Knockdown of CENPW Inhibits Hepatocellular Carcinoma Progression by Inactivating E2F Signaling

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Abstract

Aim: This study aimed to evaluate the effects of centromere protein W (CENPW, also known as CUG2) in hepatocellular carcinoma (HCC). **Methods:** CENPW expression in HCC tissues and cells was detected by RT-qPCR assay. CCK-8 and colony formation assay were used to assess cell proliferation. Wound healing and Transwell assay was used to detect cell migration and invasion, respectively. The flow cytometry was used to analyze the cell cycle distribution and apoptosis. **Results:** CENPW expression was upregulated in HCC tissues and cells. Knockdown of CENPW inhibited cell proliferation, migration, and invasion and induced the G0/G1 phase arrest and cell apoptosis in HCC cells, which might involve the E2F signaling regulation. **Conclusion:** CENPW acted as an oncogenic role in HCC progression via activation E2F signaling. Our findings may provide new insights into the studying mechanisms of HCC.

Keywords

hepatocellular carcinoma, CENPW (also known as CUG2), E2F signaling, cell proliferation, cell cycle

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths around the world.[1] The main risk factors for HCC occurrence include the changes in life-styles,[2] chronic hepatitis B virus infection,[3] cirrhosis,[4] meta-bolic disorders.[5] Patients with HCC are usually diagnosed at late stages, although the early diagnosis and treatment for HCC patients have been significantly improved, the clinical efficacy of HCC is still not optimistic.[6,7] Metastasis and relapse may contribute to the severe mortality of HCC.[8] Therefore, studying the molecular pathogenesis of HCC may be helpful for early screening, timely treatment and more efficient therapies, so as to improve the clinical efficacy of patients.

DNA microarrays have shown that the expression of cancer upregulated gene 2 (CUG2) are usually increased in various tumor tissues, such as ovarian, liver, colon and lung.[9] Hori *et al*[10] have found that CUG2 is a component of CCAN (Consti-tutive Centromere Associated Network), which can form a complex with CENP-T, so it is also called CENPW. Human CENPW is located on chromosome 6q22.32 and have an open reading frame of 267 bp, its total length of mRNA is about 600 bp and the size of cDNA is about 531 bp.[11] It has been reported that CUG2 is a new centromere component that is required for

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kinetochore functioning during cell division.[12] In addition, Lee et al[9] also showed that CUG2 exerted an oncogenic effect in an NIH3T3 cell transplantation model.

E2F family of transcription factors play important roles in various biological process, including cell proliferation, differentiation, DNA replication, and apoptosis.[13] It can also regulate the cell cycle transition from G1 to S phase through the interaction with pRB and other cell cycle dependent proteins and kinases.[14,15] So far, 8 different members of E2F transcription factor family have been found, namely E2F1-E2F8.[16] Among them, E2F1 is the first discovered and most thoroughly studied member of the E2F transcription factors family in human malignancies.[17] E2F1 can act as an oncogene or a tumor suppressor to regulate tumorigenesis according to different cell environment.[18,19] However, the interaction between CENPW and E2F signaling is not clear in HCC.

In this study, we investigated the mRNA expression of CENPW in HCC tissues and cells. Moreover, we also studied the effects of CENPW on HCC cell proliferation, cycle distribution, migration, invasion, and apoptosis and explored the underlying molecular mechanism of CENPW in HCC. Our findings might uncover a novel mechanism for the oncogenic role of CENPW in HCC.

Materials and Methods

Patients and Tissue Samples

In this study, a total of 73 patients with pathologically confirmed HCC were recruited in our hospital from 2017 to 2019. The paired HCC and adjacent normal tissues were obtained from each patient through surgical resection. All patients did not receive any preoperative treatments (chemotherapy, radiotherapy, immunotherapy, and so on) before surgery. The surgical specimens were immediately frozen in liquid nitrogen and stored at -80°C until further analyses. Every patient signed the written informed content, and the study was approved by the Ethics Committee of our hospital.

Cell Culture

Human hepatoma cell lines (MHCC97 H, Bel-7402, Huh-7, and HepG2) and human normal liver cells (LO2) were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, NY, USA) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin at 37°C and 5% CO_2.

Cell Transfection

HepG2 and Bel-7402 cells were divided into control (without treatment), si-NC (cells transfected with siRNA negative control), si1-CENPW (cells transfected with siRNA1-CENPW) and si2-CENPW group (cells transfected with siRNA2-CENPW). In addition, HepG2 cells of si-NC and si1-CENPW group transfected with empty vector (pcDNA3.1),

which was called si-NC+Vector and si1-CENPW+Vector group respectively. HepG2 cells of si1-CENPW group transfected with pcDNA3.1-E2F1, which was called si1-CENPW+E2F1 group. CENPW siRNA, pcDNA3.1-E2F1 and their negative control were designed and synthesized by Genepharma (Shanghai, China). Transfection was carried out by Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-qPCR or Western blot analysis was used to assess the transfection efficiency after transfection for 48 h.

RT-qPCR Assay

Total RNA was isolated using the Trizol reagent (Invitrogen) following the manufacturer's guidelines. Next, the extracted RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen). The real-time PCR experiment was performed on ABI 7500 StepOne RT-qPCR system (Applied Biosystems, USA) by SYBR Green PCR Kit (Takara, Japan). The following primers for CENPW and GAPDH (as an internal standard) were used in this study: CENPW (Forward: 5'-AGTGGTGACTTATTGGTCCATCTG-3', Reverse: 5'-AAGCGTTTGTCCTGGACTCTTC-3') and GAPDH (Forward: 5'-CAATGACCCCTTCATTGACC-3', Reverse: 5'-TGGAAGATGGTGATGGGATT-3'). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression.

Western Blot Analysis

Total proteins were extracted from cells by ice-cold RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), and then protein concentration was measured by BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal quantities of protein were separated by the 10% SDS-PAGE, and then transferred onto PVDF membranes (Millipore, Boston, MA, USA). After being blocked in $1\times$ PBS containing 5% skimmed milk, the membranes were incubated with the primary antibodies (Bax, 5023; caspase-3, 14220; Bcl-2, 3498; E2F1, 3742; CDK2, 18048; p-Rb, 8516; Rb, 9313; GAPDH, 5174. 1: 1000 dilution. Cell Signaling Technology, USA) at 4°C overnight. Next, the membranes were incubated with secondary antibodies conjugated with anti-rabbit horseradish peroxidase (1:2000 dilution; Cell Signaling Technology) at room temperature for 1 h. GAPDH was used as a loading control. Finally, the bands of protein were detected by the ECL detection system (Thermo Scientific, USA).

Cell Viability Assay

Cell viability was detected by Cell Counting Kit-8 (CCK-8, Sigma-Aldrich). Briefly, the transfected cells (1×10^3 cells/ well) were seeded into a 96-well plate. CCK-8 reagent (10 μL) was added to each well after incubation for 0, 24, 48, and 72 h. The optical density (OD) at 450 nm wavelength was detected by a microplate reader (Thermo Scientific).

Colony Formation Assay

The transfected cells (400 cells/well) were seeded into a 6-well plate and incubated at 37°C in humidified 5% CO_2 atmosphere for 14 days. Subsequently, cells were stained with 0.1% crystal violet (Sigma-Aldrich). Finally, the number of colonies was counted in 5 randomly selected visual fields under a microscope (Olympus, Tokyo, Japan).

Cell Cycle Assay

The transfected cells (3×10^3 cells/well) were seeded into a 6-well plate and incubated at 37°C in humidified 5% CO_2 atmosphere for 48 h. Next, cells were fixed with ice-cold 70% ethanol for 4 h, and then stained with 50 μg/mL propidium iodide (PI, Sigma-Aldrich) for 30 min at 37°C in the dark. Finally, the flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze the cell cycle distribution.

Wound Healing Assay

The wound healing assay was used to assess cell migration. Briefly, the transfected cells (5×10^5 cells/well) were seeded into a 6-well plate and grown to 90% confluence. Subsequently, a P-200 pipette tip was used to scratch the monolayer cells. After incubation for 0 and 24 h, wound healing areas were calculated by phase-contrast microscopy (Olympus).

Invasion Assay

Transwell assay was carried out in a Transwell chambers with an 8.0-μm pore (Corning, USA). Briefly, the transfected cells (5×10^5 cells/well) were seeded into the upper chamber coated with Matrigel and contained serum-free medium, and the lower chambers were filled with 10% FBS medium. After incubation for 48 h, the cells on the upper compartment were removed with sterile cotton swabs. Next, the cells were fixed with 3.7% formaldehyde (Sigma-Aldrich) for 1 h and stained with 0.2% crystal violet (Sigma-Aldrich) for 15 min. Finally, the invasive cells were observed and counted under a phase-contrast microscope (Olympus).

Cell Apoptosis Assay

The cell apoptosis was detected by flow cytometry using the Annexin V-FITC apoptosis detection kit (BD, San Jose, CA, USA). In brief, the transfected cells (5×10^5 cells/well) were cultured in 6-well plates for 48 h. Next, cells were re-suspended in 1 ×binding buffer, and then incubated with Annexin V FITC (10 μL) and PI (5 μL) for 15 min at room temperature in the dark. Finally, cell apoptosis was analyzed by a flow cytometry (BD Biosciences).

Statistical Analysis

The data was displayed as mean ± SD, and GraphPad Prism 7.0 software (San Diego, CA, USA) was used for data analysis. Student's t-test and one-way ANOVA with Tukey post-test was used for comparison between 2 groups and multiple groups, respectively. A value of $P < 0.05$ was accepted as statistically significant.

Results

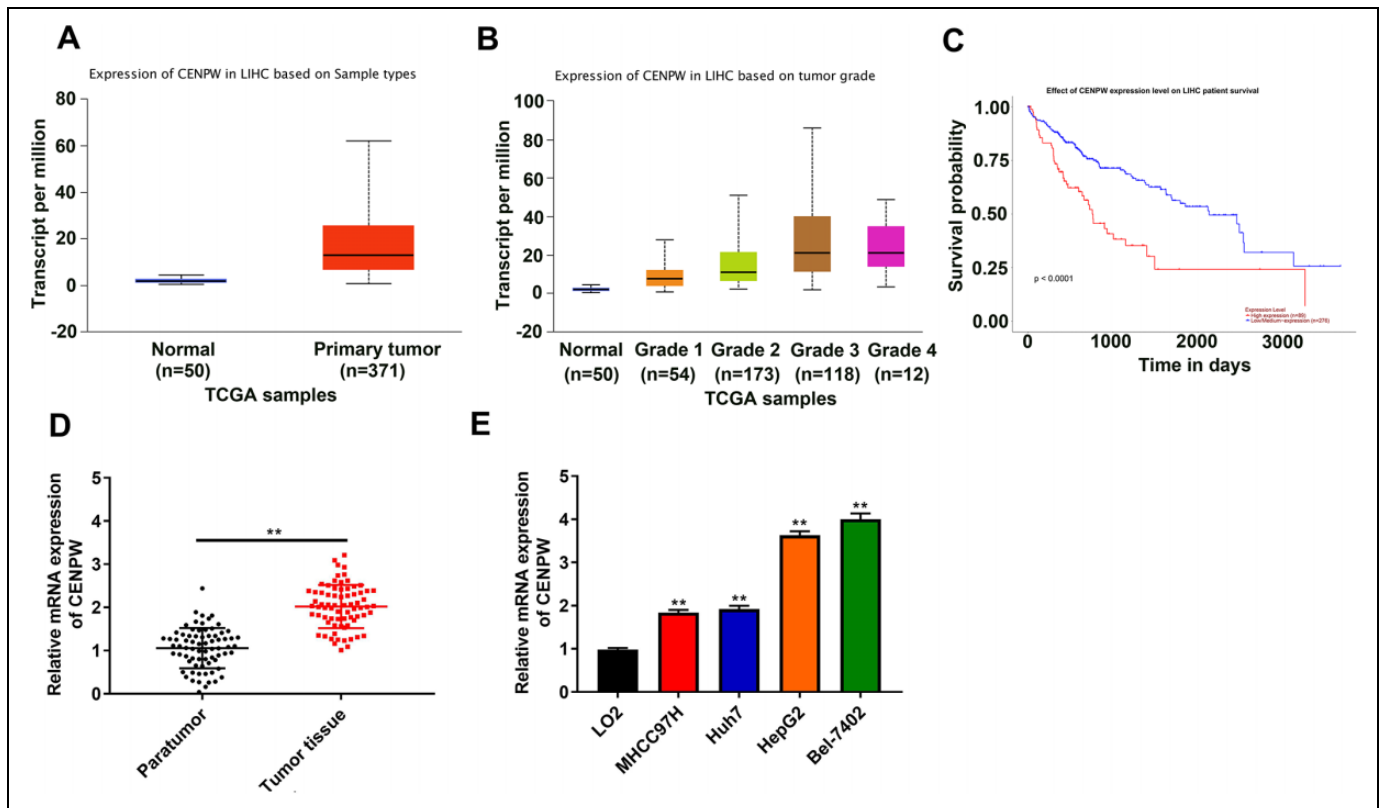## CENPW Expression Is Upregulated in HCC Tissues and Cells

To explore the role of CENPW in HCC, we first analyzed the expression difference of CENPW between HCC and normal liver tissues using TCGA database. As shown in Figure 1A, CENPW expression significantly increased in HCC tissue. Moreover, the TCGA database revealed that CENPW expression showed a step-wise increase toward higher tumor grade (Figure 1B). Of significant note, survival analysis from TCGA database showed that high CENPW expression in HCC tissues predicted poor survival ($P < 0.0001$, Figure 1C). We next assessed CENPW expression in HCC tissues and cells using RT-qPCR assay. The results showed that CENPW expression in HCC tissues and cells (MHCC97 H, Bel-7402, Huh-7, and HepG2) increased compared to adjacent normal tissues and human normal liver cells (LO2), respectively ($P < 0.01$, Figure 1D and E). CENPW expression in Bel-7402 and HepG2 cells was higher than that in MHCC97 H and Huh-7 cells, thus Bel-7402 and HepG2 cells were used in the following experiments.

Knockdown of CENPW Inhibits Cell Proliferation and Induces the G0/G1 Phase Arrest in HCC Cells

To further study the function of CENPW in HCC cells, we transfected 2 specific siRNAs against CENPW into Bel-7402 and HepG2 cells. As shown in Figure 2A, CENPW expression decreased in si1-CENPW-infected cells compared with control and si-NC group ($P < 0.01$). We then assessed the effects of si1-CENPW knockdown in Bel-7402 and HepG2 cells. CCK-8 and colony formation assay showed that knockdown of CENPW inhibited cell proliferation in both Bel-7402 and HepG2 cells ($P < 0.01$, Figure 2B and C). Next, using cell cycle assay, we found that knockdown of CENPW increased the portion of cells in the G0/G1 phase and decreased the portion of cells in S phases ($P < 0.05$, Figure 2D).

Knockdown of CENPW Inhibits Cell Migration and Invasion in HCC Cells

One of the most threatening aspects of HCC is tumor metastasis. Subsequently, the motility of Bel-7402 and HepG2 cells was also assessed through wound healing and transwell assay. As shown in Figure 3A, knockdown of CENPW reduced the wound healing rate both in Bel-7402 and HepG2 cells ($P < 0.05$). In addition, transwell assay showed that knockdown of CENPW decreased the number of invasive cells compared to control and si-NC group ($P < 0.01$, Figure 3B).

Figure 1. CENPW expression was upregulated in HCC tissues and cells. (A) CENPW expression in HCC (n = 371) and normal liver tissues (n = 50) from TCGA database. (B) CENPW expression in normal liver tissues and diffeerrent HCC grades from TCGA database. (C) Kaplan-Meyer survival curves for 2 groups of HCC patients from TCGA database. Red: high expression group; Blue: low expression group. (D) RT-qPCR assay was used to detect CENPW expression in HCC tissues and adjacent normal tissues; n = 73. (E) RT-qPCR assay was used to detect CENPW expression in HCC cells (MHCC97 H, Bel-7402, Huh-7 and HepG2) and human normal liver cells (LO2). **P < 0.01 compared with normal tissues and LO2 cells.

Knockdown of CENPW Promotes Cell Apoptosis in HCC Cells

We then detected the effect of CENPW knockdown on the apoptosis of Bel-7402 and HepG2 cells. As shown in Figure 4A, knockdown of CENPW increased the apoptotic rate in Bel-7402 and HepG2 cells compared with control and si-NC group (P < 0.01). In addition, the expression of apoptosis-regulating proteins (Bax, Bcl-2, and caspase-3) was detected using western blot assay. Our data showed that the protein expression of Bax and caspase-3 increased by knockdown of CENPW, while the protein expression of Bcl-2 decreased by knockdown of CENPW in Bel-7402 and HepG2 cells (P < 0.01, Figure 4B).

Knockdown of CENPW Inhibits E2F Signaling

To identify the mechanism of CENPW on HCC cells, we performed GSEA analysis with the Hallmark gene set (Figure 5A). We found that high expression of CENPW was significantly associated with the E2F signaling (P = 0.0018, Figure 5B). We then measured the expression of E2F signaling-related proteins by western blot assay. As shown in Figure 5C, knockdown of CENPW decreased the protein expression of E2F1, CDK2, and

p-Rb/ Rb in Bel-7402 and HepG2 cells (P < 0.01). The results suggested that CENPW played an important role in the regulation of E2F signaling in HCC.

Knockdown of CENPW Inhibits HCC Cell Proliferation and Invasion and Induces the G0/G1 Phase Arrest by Inactivating E2F Signaling

To further confirm the relationship between CENPW and E2F signaling, we transfected with siRNA1-CENPW and pcDNA3.1-E2F1 into HepG2 cells. As shown in Figure 6, pcDNA3.1-E2F1 reversed the inhibition of E2F1 expression, cell proliferation, cell cycle distribution, and invasion induced by knockdown of CENPW. Taken together, these results suggested that knockdown of CENPW inhibited HCC cell proliferation and invasion and induced the G0/G1 phase arrest by inactivating E2F signaling.

Discussion

In the past decade, despite the great progress have made in the diagnosis and treatment of HCC, the incidence rate of HCC is still high and the 5-year survival rate remains low.[20]
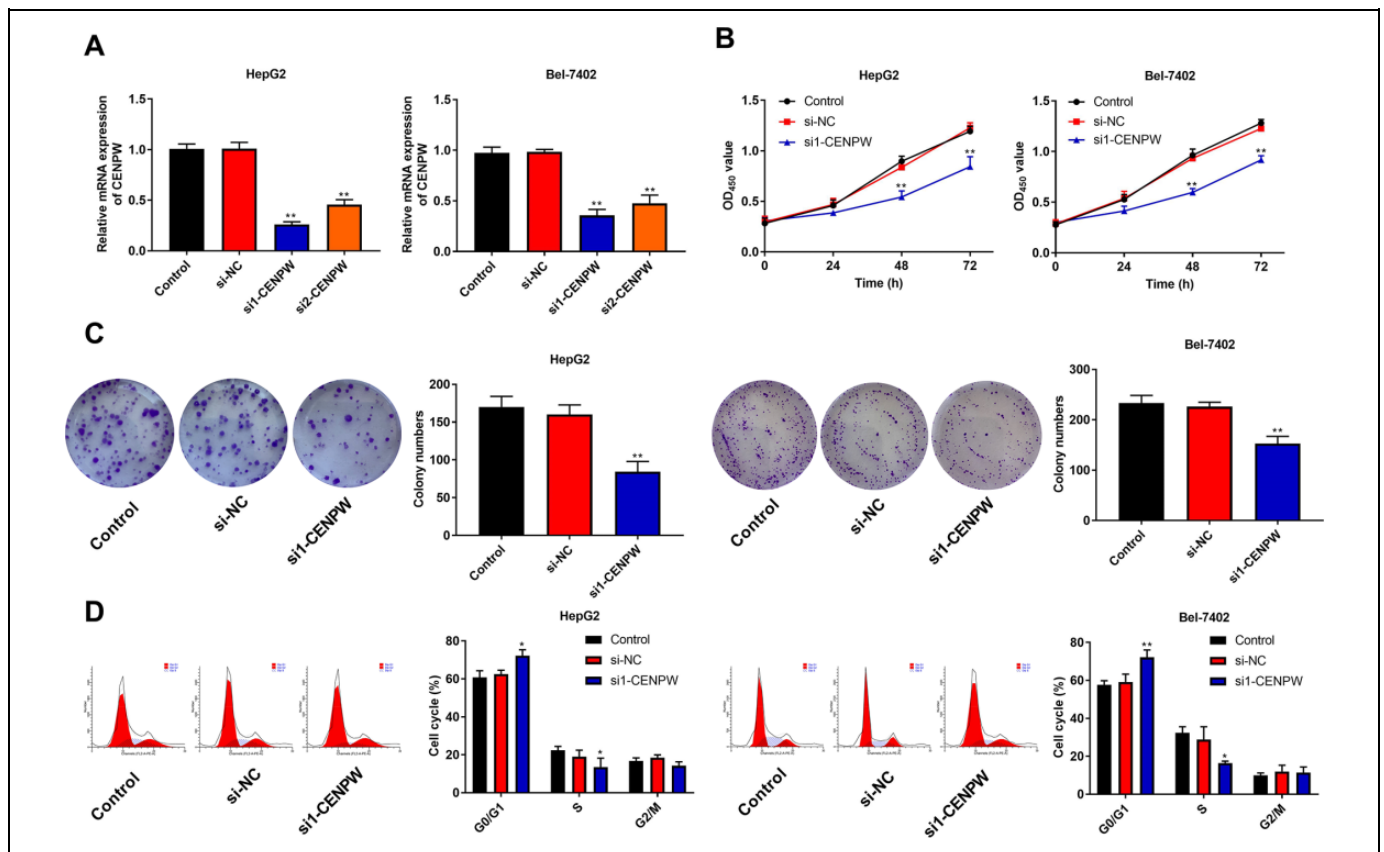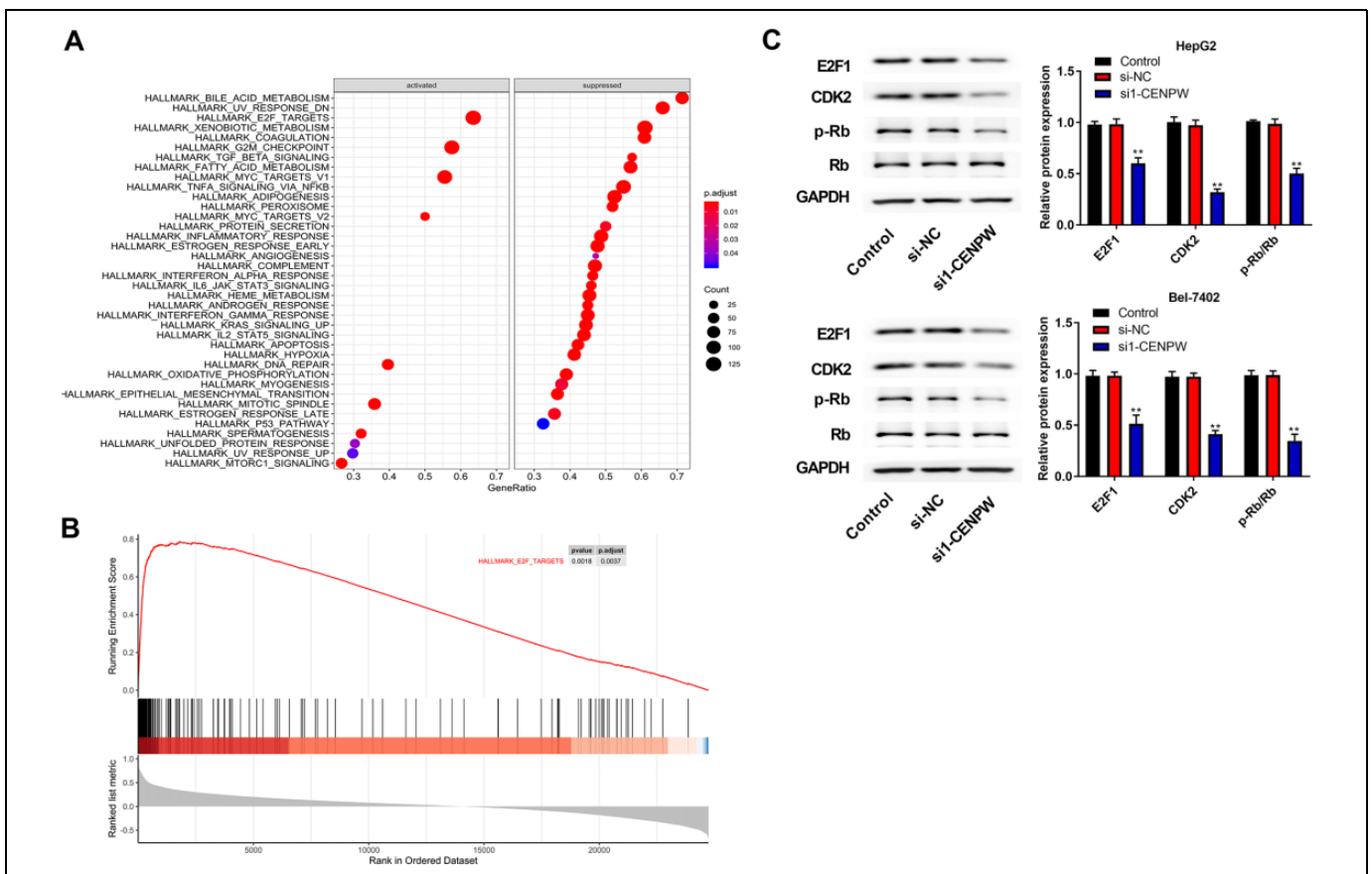
Figure 2. Knockdown of CENPW inhibited cell proliferation and induced the G0/G1 phase arrest in HCC cells. (A) RT-qPCR assay was used to detect CENPW expression in Bel-7402 and HepG2 cells. (B) CCK-8 assay was used to detect cell viability in Bel-7402 and HepG2 cells. (C) Colony formation assay was used to detect cell proliferation in Bel-7402 and HepG2 cells. (D) The flow cytometry was used to analyze the cell cycle distribution. $*P < 0.05$, $**P < 0.01$ compared with Control and si-NC group.
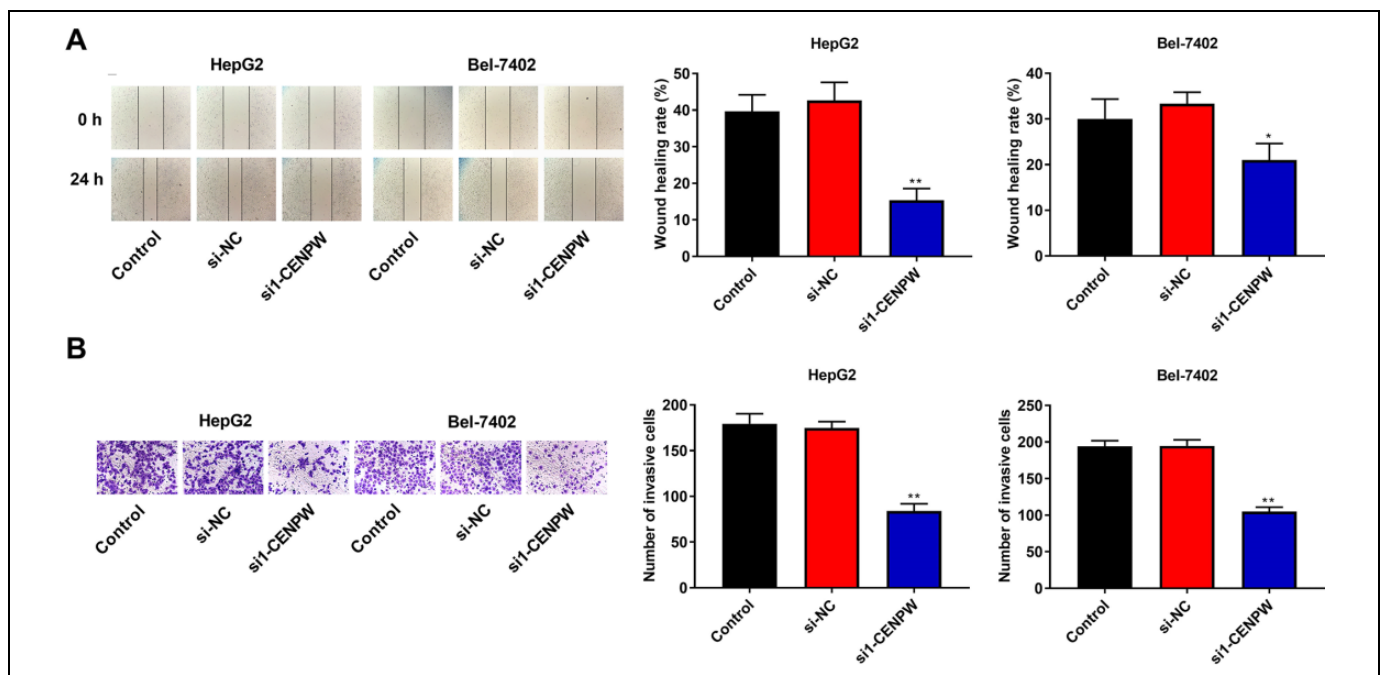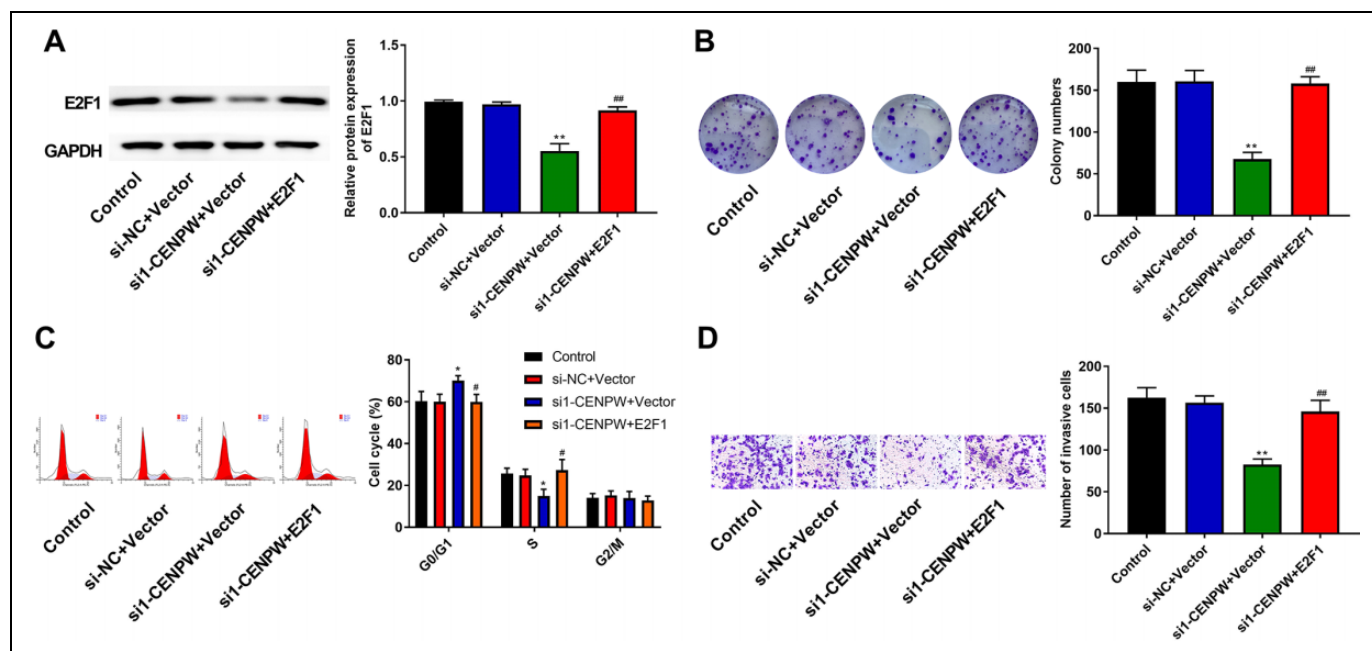

Figure 3. Knockdown of CENPW inhibited cell migration and invasion in HCC cells. (A) Wound healing assay was used to detect cell migration in Bel-7402 and HepG2 cells. (B) Transwell assay was used to detect cell invasion in Bel-7402 and HepG2 cells. $*P < 0.05$, $**P < 0.01$ compared with Control and si-NC group.
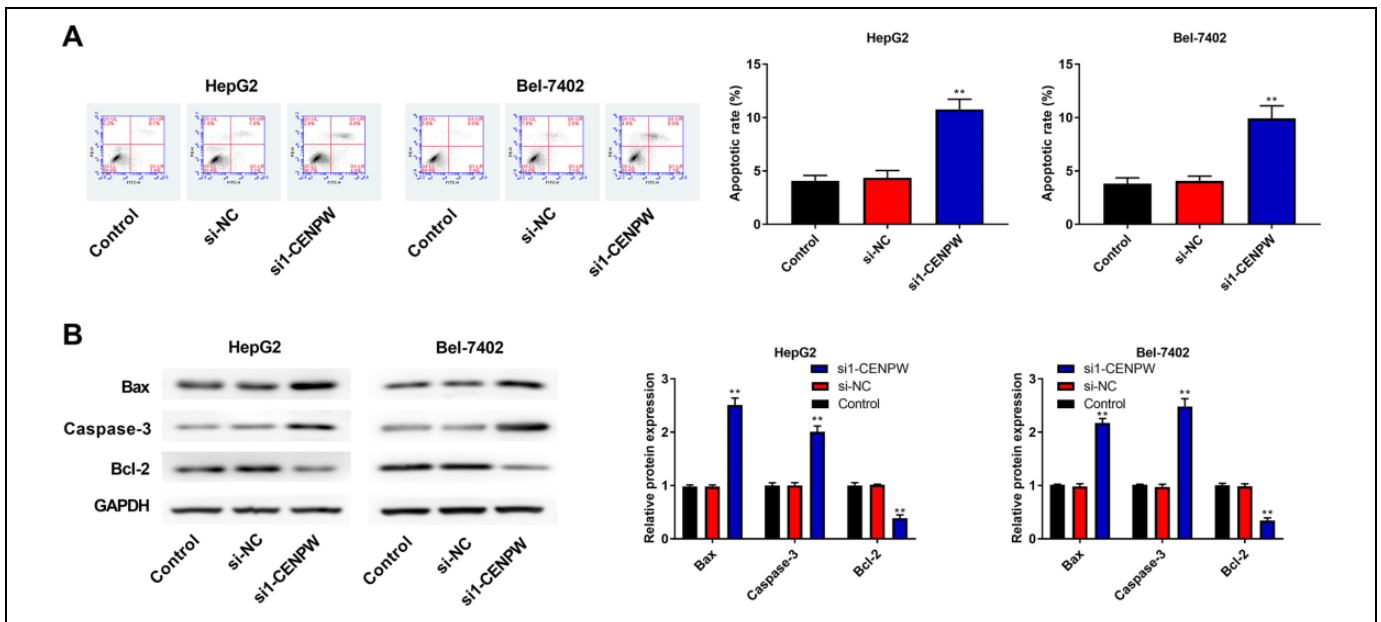
Figure 4. Knockdown of CENPW promoted cell apoptosis in HCC cells. (A) The cell apoptosis in Bel-7402 and HepG2 cells was detected by flow cytometry. (B) The expression of apoptosis-regulating proteins (Bax, Bcl-2, and Caspase-3) was detected by western blot assay. **$P < 0.01$ compared with Control and si-NC group.

Figure 5. Knockdown of CENPW inhibited E2F signaling. (A) GSEA analysis with the Hallmark gene set was performed to choose significant enriched pathways related to CENPW. (B) GSEA analysis showed that high expression of CENPW was significantly associated with the E2F signaling. (C) The expression of E2F signaling-related proteins (E2F1, CDK2, p-Rb and Rb) was detected by western blot assay. **$P < 0.01$ compared with Control and si-NC group.

Figure 6. Knockdown of CENPW inhibited HCC cell proliferation and invasion, and induced the G0/G1 phase arrest by inactivating E2F signaling. (A) The expression of E2F1 protein in HepG2 cells was detected by western blot assay. (B) Colony formation assay was used to detect cell proliferation in HepG2 cells. (C) The flow cytometry was used to analyze the cell cycle distribution in HepG2 cells. (D) Transwell assay was used to detect cell invasion in HepG2 cells. $*P < 0.05$, $**P < 0.01$ compared with Control and si-NC+Vector group, $#P < 0.05$, $##P < 0.01$ compared with si1-CENPW+Vector group.

Understanding the pathogenesis of HCC can be helpful for early diagnosis and targeted treatment, thereby improving the patient's clinical outcome.[21,22] In the present study, we elucidated that CENPW expression was upregulated in HCC tissues and cells. Knockdown of CENPW inhibited cell proliferation, migration, and invasion and induced the G0/G1 phase arrest and cell apoptosis in Bel-7402 and HepG2 cells. Moreover, E2F1 reversed the inhibition of cell proliferation, cell cycle distribution, and invasion induced by knockdown of CENPW in HepG2 cells.

Centromere proteins (CENPs) play an important role in the cell cycle, and the deregulation of CENPs is associated with carcinogenesis.[23] For instance, CENP-A expression in lung adenocarcinoma cancer[24] and colorectal cancer tissues samples[25] was upregulated. CENP-H expression in nasopharyngeal carcinoma[26] and colorectal cancers[27] also increased. In addition, CENP-F expression in breast cancer is upregulated, and it has been identified as a new biomarker associated with poor prognosis in patients with breast cancer.[28] In the present study, we showed that CENPW expression was upregulated in HCC tissues and cells (MHCC97 H, Bel-7402, Huh-7 and HepG2), which was consistent with previous studies. Moreover, we found that knockdown of CENPW increased the portion of cells in the G0/G1 phase and decreased the portion of cells in S phases in both Bel-7402 and HepG2 cells. These results suggested that CENPW might play an indispensable role in the development of HCC.

Tumor invasion and metastasis are very important for the prognosis and survival of clinical patients. Therefore, it is necessary to have a detailed understanding of CENPW and the mechanism of tumor invasion and metastasis. Kaowinn et al[29] have reported that CUG2 specifically activates and stabilizes β-catenin to promote cell migration, invasion, and enforces sphere formation in A549 cells and tumor formation in vivo. Moreover, Kaowinn et al[30] also found that CUG2 overexpression increased YAP1 expression, while knockdown of YAP1 inhibited cell migration and invasion and reduced the protein expression of N-cadherin and Vimentin in A549-CUG2 and BEAS-CUG2 cells. A study by Malilas et al[31] showed that the levels of phosphorylation STAT1 was higher in a colon cancer cell line stably expressing CUG2 (Colon26L5-CUG2), and knockdown of STAT1 expression inhibited cell migration and sensitized Colon26L5-CUG2 cells to doxorubicin-induced apoptosis. In the present study, our results showed that knockdown of CENPW not only inhibited cell proliferation, migration, and invasion, but also induced the G0/G1 phase arrest and cell apoptosis in Bel-7402 and HepG2 cells. The findings indicated that knockdown of CENPW expression could effectively inhibit many biological behaviors of HCC cells.

The basic feature of cancer is the sustaining cell division, mainly involving the activation of E2F signaling.[32] E2F transcription factor was originally discovered as an activator of adenovirus E2 promoter. It has been reported that E2F signaling played important roles in human malignancies.[19] In particular, E2F1 can control the G1/S transition of the cell cycle, which is crucial to cell proliferation and growth.[33] Huang et al[34] found that DEPDC1 promoted cell proliferation and drove G1 to S phase cell cycle transition via activation of E2F

signaling in prostate cancer. Park et al[35] showed that LncRNA EPEL promoted cell proliferation through E2F target activation in lung cancer. In addition, Chen et al[36] found that overexpression of E2F1 could increase the expansion of cancer cells and promoted tumor formation *in vivo* and *in vitro*. In this study, GSEA analysis showed that high expression of CENPW was significantly associated with the E2F signaling. Study has shown that Rb binds to E2F as a heterodimer to inhibit the transcriptional regulation of E2F.[37] In the middle and late stages of G1, Rb becomes phosphorylated, which induces the release of E2F complexes and promotes the transition of G1/S phase.[38-40] Moreover, the cyclin E-CDK2 complex promotes Rb phosphorylation.[41] Our results showed that knockdown of CENPW decreased the protein expression of E2F1, CDK2, and p-Rb/Rb in Bel-7402 and HepG2 cells. Moreover, siRNA1-CENPW and pcDNA3.1-E2F1 were co-transfected into HepG2 cells to further confirm the relationship between CENPW and E2F signaling. It was found that E2F1 reversed the inhibition of cell proliferation, cell cycle distribution, and invasion induced by knockdown of CENPW. Taken together, these results indicated that CENPW could regulate many biological behaviors by E2F signaling in HCC.

Conclusion

Overall, CENPW expression was upregulated in HCC tissues and cells. Knockdown of CENPW inhibited cell proliferation, migration, and invasion and induced the G0/G1 phase arrest and cell apoptosis in HCC cells. Moreover, we demonstrated for the first time that the role of CENPW in HCC was involved in the E2F signaling. Our findings may help for facilitating the development of new therapeutic strategies against HCC.

Authors' Note

Declaration of Conflicting Interests

Funding

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.

2. Wallace MC, Preen D, Jeffrey GP, Adams LA. The evolving epidemiology of hepatocellular carcinoma: a global perspective. Review. *Expert Rev Gastroenterol Hepatol*. 2015;9(6):765-779.

3. Yu R, Fan R, Hou J. Chronic hepatitis B virus infection: epidemiology, prevention, and treatment in China. Research Support, Non-U S Gov't Review. *Front Med*. 2014;8(2):135-144.

4. Janevska D, Chaloska-Ivanova V, Janevski V.Hepatocellular carcinoma: risk factors, diagnosis and treatment. Review. *Open Access Maced J Med Sci*. 2015;3(4):732-736.

5. Klein J, Dawson LA, Tran TH, et al. Metabolic syndrome-related hepatocellular carcinoma treated by volumetric modulated arc therapy. *Curr Oncol*. 2014;21(2):e340-344.

6. Burak KW.Prognosis in the early stages of hepatocellular carcinoma: Predicting outcomes and properly selecting patients for curative options. *Can J Gastroenterol*. 2011;25(9):482-484. doi: 10.1155/2011/302457

7. Fitzmorris P, Shoreibah M, Anand BS, Singal AK. Management of hepatocellular carcinoma. Review. *J Cancer Res Clin Oncol*. 2015;141(5):861-876.

8. Page AJ, Cosgrove DC, Philosophe B, Pawlik TM. Hepatocellular carcinoma: diagnosis, management, and prognosis. Review. *Surg Oncol Clin N Am*. 2014;23(2):289-311.

9. Lee S, Gang J, Jeon SB, et al. Molecular cloning and functional analysis of a novel oncogene, cancer-upregulated gene 2 (CUG2). Research Support, Non-U S Gov't. *Biochem Biophys Res Commun*. 2007;360(3):633-639.

10. Hori T, Amano M, Suzuki A, et al. CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. Research Support, Non-U S Gov't. *Cell*. 2008; 135(6):1039-1052.

11. Kim H, Lee S, Park B, Che L, Lee S.Sp1 and Sp3 mediate basal and serum-induced expression of human CENP-W. Research Support, Non-U S Gov't. *Mol Biol Rep*. 2010;37(7):3593-3600.

12. Kim H, Lee M, Lee S, et al. Cancer-upregulated gene 2 (CUG2), a new component of centromere complex, is required for kinetochore function. Research Support, Non-U S Gov't. *Mol Cells*. 2009;27(6):697-701.

13. Gokhman D, Livyatan I, Sailaja BS, Melcer S, Meshorer E.Multi-layered chromatin analysis reveals E2f, Smad and Zfx as transcriptional regulators of histones. Research Support, Non-U S Gov't. *Nat Struct Mol Biol*. 2013;20(1):119-126.

14. Li SZ, Zeng F, Li J, et al. Nemo-like kinase (NLK) primes colorectal cancer progression by releasing the E2F1 complex from HDAC1. Research Support, Non-U S Gov't. *Cancer Lett*. 2018; 431:43-53.

15. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Research Support, Non-U S Gov't Research Support, U S Gov't, P H S. *Mol Cell Biol*. 1995;15(8):4215-4224.

16. Shen ZG, Liu XZ, Chen CX, Lu JM. Knockdown of E2F3 inhibits proliferation, migration, and invasion and increases apoptosis in glioma cells. *Oncol Res*. 2017;25(9):1555-1566.

17. Xu TP, Wang YF, Xiong WL, et al. E2F1 induces TINCR transcriptional activity and accelerates gastric cancer progression via activation of TINCR/STAU1/CDKN2B signaling axis. Research Support, Non-U S Gov't. *Cell Death Dis*. 2017;8(6):205.

18. Tarangelo A, Lo N, Teng R, et al. Recruitment of Pontin/Reptin by E2f1 amplifies E2f transcriptional response during cancer progression. Research Support, Non-U S Gov't. *Nat Commun*. 2015; 6:10028.

19. Engelmann D, Pützer BM. The dark side of E2F1: in transit beyond apoptosis. Research Support, Non-U S Gov't Review. *Cancer Res*. 2012;72(3):571-575.

20. Golabi P, Fazel S, Otgonsuren M, Sayiner M, Locklear CT, Younossi ZM. Mortality assessment of patients with hepatocellular carcinoma according to underlying disease and treatment modalities. *Medicine*. 2017;96(9): e5904.

21. Gomes MA, Priolli DG, Tralhão JG, Botelho MF. Hepatocellular carcinoma: epidemiology, biology, diagnosis, and therapies. Review. *Rev Assoc Med Bras*. 1992;59(5):514-524.

22. Rich N, Singal AG. Hepatocellular carcinoma tumour markers: current role and expectations. Review. *Best Pract Res Clin Gastroenterol*. 2014;28(5):843-853.

23. Kaowinn S, Seo EJ, Heo W, et al. Cancer upregulated gene 2 (CUG2), a novel oncogene, promotes stemness-like properties via the NPM1-TGF-β signaling axis. Research Support, Non-U S Gov't. *Biochem Biophys Res Commun*. 2019;514(4):1278-1284.

24. Wu Q, Qian YM, Zhao XL, et al. Expression and prognostic significance of centromere protein A in human lung adenocarcinoma. Research Support, Non-U S Gov't. *Lung Cancer*. 2012; 77(2):407-414.

25. Tomonaga T, Matsushita K, Yamaguchi S, et al. Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res*. 2003;63(13):3511-3516.

26. Liao WT, Song LB, Zhang HZ, et al. Centromere protein H is a novel prognostic marker for nasopharyngeal carcinoma progression and overall patient survival. Research Support, Non-U S Gov't. *Clin Cancer Res*. 2007;13(2 Pt 1):508-514.

27. Tomonaga T, Matsushita K, Ishibashi M, et al. Centromere protein H is up-regulated in primary human colorectal cancer and its overexpression induces aneuploidy. Research Support, Non-U S Gov't. *Cancer Res*. 2005;65(11):4683-4689.

28. O'Brien SL, Fagan A, Fox EJ, et al. CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. Research Support, Non-U S Gov't. *Int J Cancer*. 2007;120(7):1434-1443.

29. Kaowinn S, Oh S, Moon J, et al. CGK062, a small chemical molecule, inhibits cancer upregulated gene 2-induced oncogenesis through NEK2 and β-catenin. *Int J Oncol*. 2019;54(4): 1295-1305.

30. Kaowinn S, Yawut N, Koh SS, Chung YH. Cancer upregulated gene (CUG)2 elevates YAP1 expression, leading to enhancement of epithelial-mesenchymal transition in human lung cancer cells. Research Support, Non-U S Gov't. *Biochem Biophys Res Commun*. 2019;511(1):122-128.

31. Malilas W, Koh SS, Kim S, et al. Cancer upregulated gene 2, a novel oncogene, enhances migration and drug resistance of colon cancer cells via STAT1 activation. Research Support, Non-U S Gov't. *Int J Oncol*. 2013;43(4):1111-1116.

32. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Research Support, N I H, Extramural Review. *Cell*. 2011; 144(5):646-674.

33. Hallstrom TC, Nevins JR. Balancing the decision of cell proliferation and cell fate. Review. *Cell Cycle*. 2009;8(4):532-535.

34. Huang L, Chen K, Cai ZP, et al. DEPDC1 promotes cell proliferation and tumor growth via activation of E2F signaling in prostate cancer. *Biochem Biophys Res Commun*. 2017;490(3): 707-712.

35. Park SM, Choi EY, Bae DH, Sohn HA, Kim SY, Kim YJ. The LncRNA EPEL promotes lung cancer cell proliferation through E2F target activation. *Cell Physiol Biochem*. 2018;45(3): 1270-1283.

36. Chen YL, Uen YH, Li CF, et al. The E2F transcription factor 1 transactives Stathmin 1 in hepatocellular carcinoma. *Research Support, Non-U S Gov't. Ann Surg Oncol*. 2013;20(12): 4041-4054.

37. Park SA, Kim MJ, Park SY, et al. EW-7197 inhibits hepatic, renal, and pulmonary fibrosis by blocking TGF-β/Smad and ROS signaling. Research Support, Non-U S Gov't. *Cell Mol Life Sci*. 2015;72(10):2023-2039.

38. Hassan M, Ghozlan H, Abdel-Kader O. Activation of RB/E2F signaling pathway is required for the modulation of hepatitis C virus core protein-induced cell growth in liver and non-liver cells. *Cell Signal*. 2004;16(12):1375-1385.

39. Slansky JE, Li Y, Kaelin WG, Farnham PJ. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Research Support, Non-U S Gov't Research Support, U S Gov't, P H S. *Mol Cell Biol*. 1993;13(3):1610-1618.

40. Ohtani K, DeGregori J, Nevins JR. Regulation of the cyclin E gene by transcription factor E2F1. Research Support, U S Gov't, P H S. *Proc Natl Acad Sci U S A*. 1995;92(26):12146-12150.

41. Li W, Kotoshiba S, Berthet C, Hilton MB, Kaldis P.Rb/Cdk2/Cdk4 triple mutant mice elicit an alternative mechanism for regulation of the G1/S transition. Research Support, N I H, Intramural Research Support, Non-U S Gov't. *Proc Natl Acad Sci U S A*. 2009;106(2):486-491.