

CENPF Overexpression Induced by HBV Infection Facilitates the G1/S Cell Cycle Transition of Hepatocellular Carcinoma Cells via MYC Pathway

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Background: Centromere protein F (CENPF), a mitosis-related protein, is overexpressed in hepatocellular carcinoma (HCC) and has emerged as a promising biomarker for early HCC. However, the role of hepatitis B virus (HBV) infection on CENPF overexpression in HCC remains unknown. Moreover, with ultra-large molecular weight of 358kDa of CENPF, no study has directly explored its carcinogenicity with an overexpression model.

Materials and Methods: The relationship among HBV infection, CENPF amplification and CENPF overexpression was investigated in HCC tissues. HBV X protein (HBx) transient overexpression cell models was constructed to explore its effect on CENPF expression. CENPF was upregulated and downregulated to analyze its functions in vitro and in vivo. Specifically, a CRISPR/dCas9 system was applied to construct the CENPF overexpression model.

Results: A high frequency of CENPF amplification (36.21%, 21/58) was identified in HCC tissues, predominantly in HBV-associated cases (90.48%, 19/21), and CENPF amplification correlated with CENPF overexpression. The HBx enhanced CENPF expression in HBx transfected HCC cells. In addition, CENPF knockdown cell models showed inhibition of HCC proliferation both in vitro and in vivo. Notably, as a cell cycle protein with high constitutive expression in G2/M phase, CENPF overexpression cell models also showed inhibitory effects, probably due to the toxic effect of excessive CENPF expression on G2/M transition. However, in both CENPF downregulation and overexpression models, cell cycle assays showed CENPF promoted G1/S transition in HCC cells. RNA-seq showed that CENPF overexpression activated the MYC pathway, thereby promoting G1/S transition. Rescue experiment indicated that the MYC pathway inhibitor 10058-F4 counteracted the G1/S transition induced by CENPF overexpression in HCC cells.

Conclusion: HBV infection was associated with upregulated CENPF expression in HCC and CENPF overexpression might facilitate G1/S transition of HCC cells via the MYC pathway.

Keywords: hepatocellular carcinoma, HBV infection, CENPF amplification, CENPF overexpression, G1/S cell cycle transition

Introduction

Liver cancer is the sixth most common cancer and the third leading cause of cancer-related death.¹ Hepatocellular carcinoma (HCC) is the predominant histopathological subtype of liver cancer, and one of the main risk factors is the infection of hepatitis

B virus (HBV). Despite great advances in basic and clinical research of HCC, the molecular mechanisms underlying the occurrence and development of HCC remain to be further explored.

Centromere protein F (CENPF), the largest member of the centromere-kinetochore complex, is involved in chromosome segregation during cell division and is expressed in a cell cycle-dependent pattern.^{2–4} At G1/S phase, only low levels of CENPF can be detected in the nucleus, whereas it reaches peak levels at G2/M phase and degrades rapidly after mitosis is completed. Current studies have shown that CENPF is significantly upregulated in various tumors including HCC,^{5–8} and CENPF may regulate the G2/M phase cell cycle and enhances the progression of cancers.^{5,6} CENPF overexpression is closely related to HCC oncogenesis and is correlated with poor prognosis,^{9,10} and serum CENPF autoantibody caused by CENPF overexpression might be a promising biomarker for early HCC.^{11,12} However, with the molecular weight of CENPF reaching up to 358kDa, CENPF knockdown but not overexpression models have been applied to explore the carcinogenicity of CENPF in HCC. Whether CENPF overexpression is a cause or an effect in HCC remains to be clarified.

HBV contributes to HCC tumorigenesis via direct and indirect mechanisms including DNA integrations, the expression of the HBV X protein (HBx) and immune imbalance.¹³ Genomic instability caused by HBV integration, such as amplification of oncogenes and deletion or mutation of tumor suppressor genes, plays an important role in the development and progression of HCC.^{14,15} Gene amplification may lead to overexpression, and *CENPF* gene has been reported frequently amplified in HCC,¹⁶ but the relationship between HBV infection, *CENPF* gene amplification, and CENPF overexpression in HCC remains unknown.

In the present study, we assessed whether HBV contributed to CENPF overexpression and the underlying mechanisms, and explored the role of CENPF overexpression in the development of HCC by utilizing our well-established CENPF overexpression models. Those studies are expected to provide a target for the diagnosis and treatment of HBV-related HCC in the future.

Materials and Methods

Patients

A total of 126 HCC patients treated at Beijing Friendship Hospital and Beijing You-An Hospital were enrolled in this study. A total of 100 formalin-fixed, paraffin-embedded (FFPE) HCC pathological tissue sections were available. Among these 100 HCC pathological tissue sections, 32 had matched HCC frozen tissue samples. Another 26 frozen HCC tissues not prepared as FFPE sections were also included in the study. Clinicopathological characteristics of participants are summarized in [Table S1](#). The research was approved by the Clinical Research Ethics Committee of the Beijing Friendship Hospital, Capital Medical University (NO.2015-P2-019-01) and Beijing You-an Hospital, Capital Medical University (NO.ChiCTR-ONRC-10001064), in accordance with the Declaration of Helsinki and Istanbul. Written informed consent was obtained from all patients whose tissue and clinical data were used.

Cell Lines and Cell Culture

Human HCC cell line HCCLM3 was purchased from China Centre for Type Cultures Collection. Huh-7, PLC/PRF/5, Hep3B, SNU-387, Li-7, SNU-182 and HEK293T were provided by Cell Bank, Chinese Academy of Sciences. HCCLM3, Huh-7 and HEK293T were cultured in DMEM (C11995500BT, Gibco, USA) containing 10% fetal bovine serum (16000–044, Gibco, USA). PLC/PRF/5 and Hep3B were maintained in MEM (C11095500BT, Gibco, USA) with 10% fetal bovine serum. SNU-387, Li-7 and SNU-182 were cultured in 1640 (C11875500BT, Gibco, USA) containing 10% fetal bovine serum. HBV DNA was integrated into the genome of the HCCLM3 cell line with high metastasis potential, however, HBsAg expression was negative in this cell line (China Centre for Type Cultures Collection). The HBV state of Huh-7 with high differentiation and Li-7 was negative (Cell Bank, Chinese Academy of Sciences). The cell lines of PLC/PRF/5 and Hep3B were HBV DNA integration-positive and secreted HBsAg. Southern blotting identified that SNU-387 and SNU-182 harbored integrated-HBV DNA, yet there were no detectable expression of HBV RNA.

HBx Expression Plasmid and Transfection

HBx expression plasmid was a gift from Professor Jie Wang from the Department of Microbiology and Infectious Disease Center, School of Basic Medical Science, Peking University Health Science Center. Transfection of HBx expression plasmid in Human HCC cell lines HCCLM3 and Hep3B was performed using X-treme GENE HP DNA Transfection Reagent (Roche,

Switzerland), following the manufacturer's instructions. The culture medium was changed at 8 h after transfection. Cells were harvested for total RNA extraction at 24 h after transfection and for total protein extraction at 48 h after transfection.

Construction of CENPF Short Hairpin RNA (shRNA) and CRISPR Transcriptional Activation Plasmids

We silenced CENPF expression using shRNA cloned into the pLKO.1 plasmid (#10878; Addgene, USA). The CENPF overexpression cell model was constructed as described in our previous study.¹⁷ The CRISPR/dCas9 SAM system with two vectors, LentiSAMv2 (#89308; Addgene) and LentiMPH v2 (#75112; Addgene), was used to specifically increase endogenous CENPF expression. The LentiSAMv2 empty vector encodes dCas9-VP64, MS2 loops at tetraloop and stemloop 2, and blasticidin resistance marker. The LentiMPHv2 plasmid contains the MS2-P65-HSF1 activator helper complex and hygromycin resistance marker. Single guide RNA (sgRNA) targeting the transcriptional start site of *CENPF* was designed and cloned into LentiSAMv2 vector. The shRNA and sgRNA sequences are provided in [Table S2](#).

Lentivirus Production and Transduction

HEK293T cell lines at 80–90% confluency (seeded in 100 mm TC-treated culture dishes) were transfected with 10 µg plasmid containing the vectors of interest, psPAX2 (#12260; Addgene) and pMD2.G (#12259; Addgene) in the proportion of 4:3:1. After 12 h transfection, the medium was changed. Virus supernatant was harvested at 48 h and 72 h post-transfection, filtered with a 0.45 µm PES filter (SLHPR33RS; Millipore, USA), and condensed using lentivirus concentration solution (BG20101L; BioGeek, China) according to the manufacturer's instructions. Virus to silence CENPF expression was added to infect Huh-7 and Hep3B cells overnight. After 72 h infection, cells were selected with puromycin for 3 days.

For the CRISPR transcriptional activation, Huh-7 and HCCLM3 cell lines were transduced with LentiMPHv2, treated with hygromycin for 14 days, and then transduced with LentiSAMv2 carrying a specific sgRNA for *CENPF*, followed by blasticidin selection for 14 days.¹⁸

Cell Proliferation, Migration and Invasion Assay

Cell proliferation was measured by CCK-8 assay according to the manufacturer's directions (CK04, Dojindo, Japan). Cell migration was tested using the culture-insert 2 well (80209, IBIDI, Germany). The invasion ability of the cells was investigated by transwell chambers (3422, Corning, USA) pre-coated with Matrigel matrix (356234, Corning, USA).

Cell Cycle Assay

The cell samples were fixed with 75% ethanol at −20°C overnight. After washing cells once with pre-cooled PBS, flow cytometry samples each containing approximately 1×10^6 cells were incubated with 0.5 mL FxCycle™ PI/RNase Staining Solution (F10797, Thermo Fisher Scientific, USA) for 20 min at room temperature in the dark. Cell cycle distribution was detected by the Attune NxT flow cytometer, and data were analyzed using FlowJo software.

In vivo Tumor Growth Assay

Hep3B (shCENPF or shNC) or HCCLM3 (CENPF or control) cells were subcutaneously injected into BALB/c nude mice (5-week-old, male, $n = 3/\text{group}$ for Hep3B, $n = 6/\text{group}$ for HCCLM3), and tumor growth was monitored regularly. All mice were killed on day 19 after inoculation, and tumors were excised for weight measurement and immunohistochemistry (IHC) analysis. The procedures were approved by the Laboratory Animal Ethics Committee of Beijing Friendship Hospital, Capital Medical University (NO.22–2023). All animal experimental procedures in this study were conducted in strict accordance with the core guiding principles outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition, 2011).

Quantitative Real-Time PCR (qRT-PCR) for CENPF Amplification

Genomic DNA was extracted from HCC tissues using TIANamp Genomic DNA kits (DP304, TIANGEN, China). The *CENPF* gene amplification in HCC tissue samples was detected by qRT-PCR with TaqManTM Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, USA). Briefly, each reaction mixture contained 2X TaqManTM Genotyping Master Mix (4371353, Applied Biosystems, USA), 20X TaqManTM Copy Number Reference Assay reagent (RNase P, 4403326, Applied Biosystems, USA), 20X TaqManTM Copy Number Assay reagent (*CENPF*, Hs03027722_cn, Applied Biosystems, USA) and template DNA. PCR were performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). PCR program was as follows: 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. Data files was analyzed by CopyCallerTM Software (Applied Biosystems, USA).

qRT-PCR

Total RNA was extracted from cell lines or tissues using ultrapure RNA Kit (CW0581, CWBIO, China). Then, RNA was reverse transcribed into cDNA using the Reverse Transcription Kit (RR036A, Takara, China), and qRT-PCR were performed with PowerUpTM SYBRTM Green Master Mix (A25742, Applied Biosystems, USA) in triplicate. The data were normalized to ACTB expression. The gene relative expression were analyzed using the $2^{-\Delta\Delta CT}$ method. All primers were listed in [Table S2](#).

Western Blotting

Protein was isolated using RIPA buffer (20–188, Millipore, USA) containing protease inhibitor (04693124001, Roche, Switzerland) and phosphatase inhibitor (04906845001, Roche, Switzerland). 25µg of protein per lane were electrophoresed on SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes. After blocked with 5% nonfat milk in TBST, the membranes were incubated with primary antibodies, CENPF antibody (ab5, abcam, UK) or β-actin Antibody (30102ES60, Yeasen, China). The membranes were washed with TBST, followed by the incubation with HRP-conjugated secondary antibodies for 1h at room temperature. Proteins were detected using an ECL kit (36208ES76, Yeasen, China).

IHC Analysis

HCC tissues of 4 µm in thickness were sliced from the FFPE blocks. After deparaffinization with xylene and rehydration with alcohol, antigen retrieval was conducted by boiling sections in EDTA buffer (pH 9.0) for 6 min. Endogenous peroxidase activity was blocked with commercial kits (PV-6000; ZSGB-bio, China), followed by incubation with 10% donkey serum in phosphate-buffered saline (PBS) for 20 min at room temperature. Subsequently, sections were incubated with CENPF primary antibody (ab223847; Abcam, Cambridge, UK) diluted in PBS overnight at 4°C. After washing with PBS, immunoreactive spots were detected by incubation with HRP-conjugated secondary antibody (PV-6000; ZSGB-bio), then stained with 3,3'-diaminobenzidine (DAB; ZLI-9018; ZSGB-bio). Sections were counterstained with hematoxylin to visualize tissue structures. Esophageal carcinoma tissue sections known to exhibit high CENPF expression were used as positive controls. Negative controls were established by replacing the primary antibodies with non-immune IgG from the same species. The CENPF score was obtained as the proportion of cells with positive staining to the total number of cells. Five high-power views were used per slice, and 100 cells in each view were counted for analysis. The final value was determined by averaging the scores from two independent observers blinded to the clinical data. Receiver operating characteristic curve was employed to assess the performance of the CENPF-positive rate for differentiating poorly differentiated HCC from well-to-moderately differentiated cases performed by the MedCalc software. High and low expression of CENPF was defined at a cutoff value calculated by the Youden index. The morphologic information of HCC tissues used in this research was collected from the medical record of the corresponding HCC patients, which was defined by the senior pathologists.

RNA-Sequencing and Bioinformatics Analysis

Total RNA was isolated from the CENPF knockdown Huh-7 cells and the CENPF-overexpressing HCCLM3 and Huh-7 cells using an Ultrapure RNA Kit (CW0581; CWBIO, China), then RNA-sequencing was performed by Annoroad Gene Tech.Co.,

Ltd. (Beijing, China). Hallmark gene sets from the Molecular Signature Database were used for GSEA by R studio based on the RNA-seq data.¹⁹ The pan-cancer *CENPF* gene expression analysis was performed by the online website UALCAN.²⁰ Furthermore, the GEPIA database was utilized to compare the mRNA expression of *CENPF* between HCC and normal liver tissues from TCGA and GTEx datasets.²¹ The prognostic value of *CENPF* mRNA expression in HCC was evaluated using two online databases, Kaplan–Meier Plotter and GEPIA.^{21,22} cBioPortal was utilized to analyze the alterations in *CENPF* in HCC, including parameters such as mutations, amplification, and deep deletion.²³

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software. The normality of the data distribution was tested by Shapiro–Wilk test. Continuous variables were described as mean with standard deviation or median with interquartile range, and frequency was used for categorical data. Differences in continuous data between two groups were analyzed using the Mann–Whitney *U*-test or Student's *t* test. Kruskal–Wallis test or one-way ANOVA, with multiple comparison corrections, were employed for multiple groups data. The chi-squared test was performed to compare the differences in frequency between two groups. The correlation between *CENPF* copy number and mRNA levels in tissue samples was analyzed using Spearman's rank correlation test. $P < 0.05$ was considered statistically significant.

Results

CENPF Expression Was Significantly Higher in HBV-Related HCC Tissues, and CENPF Overexpression Correlated with Poor Cell Differentiation in HCC Cases

To analyze *CENPF* expression in HCC, 100 FFPE tissues and 27 pairs of frozen tissue available for RNA analysis were used for IHC and qRT-PCR analysis respectively. The protein and mRNA expression levels of *CENPF* were significantly higher in HCC tissues than that in para-HCC tissues (Figure 1A and B), consistent with that observed in UALCAN and the GEPIA database (Figure S1A and B). The higher level of *CENPF* mRNA expression was correlated with the poorer prognosis of HCC patients (Figure S1C and D). Moreover, significantly higher *CENPF* expression levels were identified in poorly differentiated HCC tissues compared with well or moderately differentiated cases (Figure 1C and Table 1), and the *CENPF*-positive rate examined by IHC had the AUC of 0.893 to discriminate poor differentiation HCC from moderate or well differentiated cases at a cutoff value of 11% (Figure S2).

Specifically, IHC and qRT-PCR analyses showed that the *CENPF* expression was higher in HBV-related tissues than that in HBV-negative tissues both at the mRNA and protein level (Figure 1D and E), and *CENPF* expression was significantly correlated HBV infection ($P = 0.0075$) of HCC (Table 1).

High Frequency of CENPF Amplification Identified in HCC Was Associated with HBV Infection, and HBx Promoted the Expression of CENPF in HCC Cells

Genomic instability such as oncogene amplification caused by HBV DNA integrations plays a significant role in the tumorigenesis of HCC.¹⁵ To explore the mechanism of *CENPF* overexpression in HBV-related HCC, we firstly searched for information about the genetic alterations of *CENPF* in TCGA LIHC database using cBioPortal. As shown in Figure S3, amplification but not mutation was the most frequent alteration of *CENPF* in HCC. By TaqMan Copy Number Assay, we identified *CENPF* amplification in 21 of the 58 frozen HCC tissues (36.21%, Figure 2A and B), consistent with previous findings.¹⁶

Notably, a significantly higher prevalence of *CENPF* amplification was identified in HBV-related HCC cases (19/39, 48.72%) than that without HBV infection (2/19, 10.53%) (Figure 2A and B), with the *CENPF* amplification predominantly identified in HBV-associated cases (90.48%, 19/21). Moreover, through the analysis of 39 HCC tissues available for the detection of *CENPF* mRNA levels, we found that *CENPF* copy number was positively correlated with *CENPF* mRNA expression (Figure 2C), suggesting that *CENPF* amplification might be a mechanism for *CENPF* overexpression in HBV-related HCC.

To explore whether HBx has the direct effect on *CENPF* overexpression in HBV-related HCC, HBx transient overexpression cell models was constructed. The results showed that HBx increased the expression of *CENPF* in both

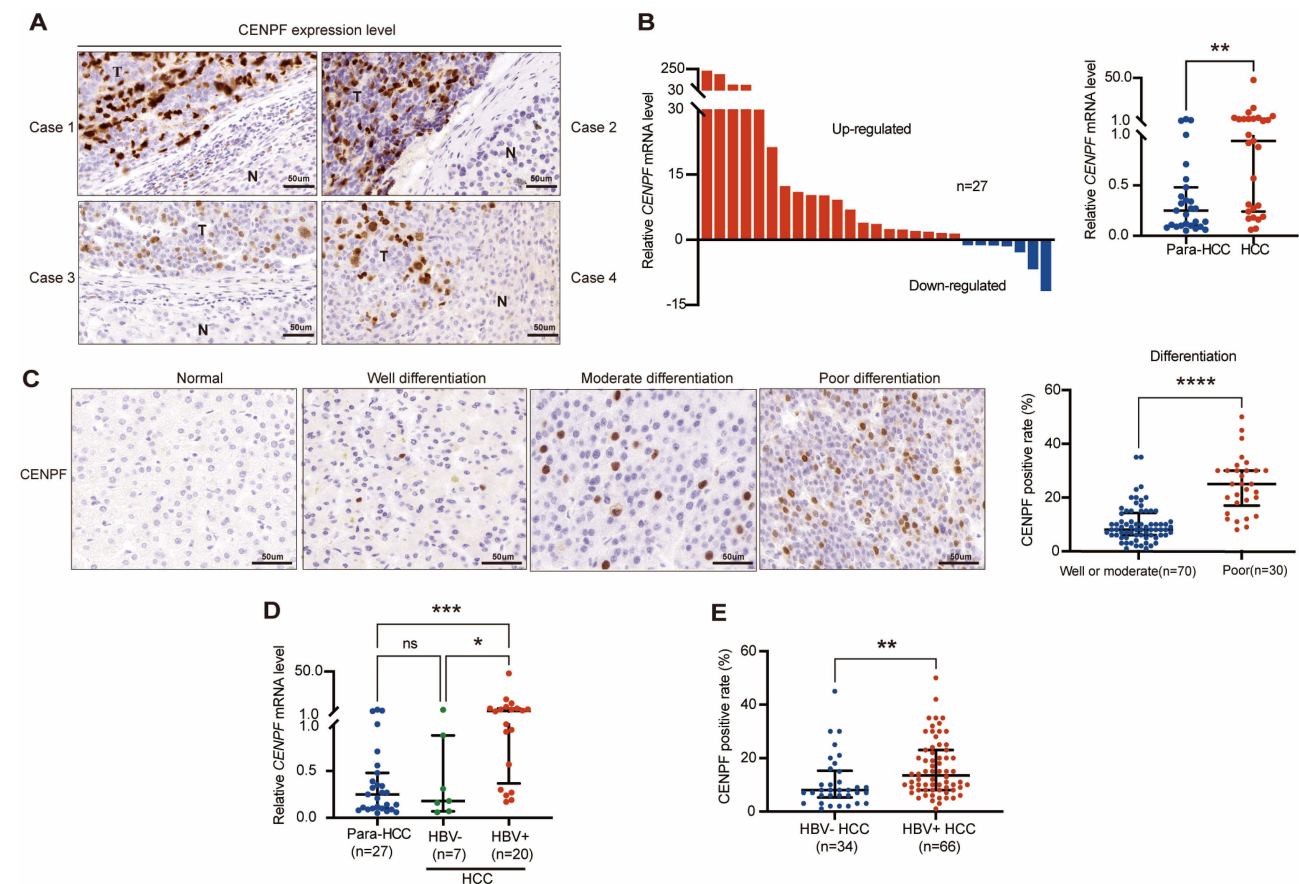


Figure 1 The expression of CENPF was higher in HCC tissues with HBV infection. **(A)** Representative immunohistochemical images of CENPF expression in HCC and adjacent tissues (n=100). **(B)** CENPF mRNA expression in HCC and adjacent tissues was examined by real-time PCR (n=27 pairs). **(C)** Immunohistochemical analysis showed that the positive rate of CENPF in poorly differentiated HCC tissues was higher than that in well-differentiated or moderately differentiated HCC tissues (n=100). **(D)** and **(E)** CENPF expression in HBV-negative and -positive HCC tissues was measured by qRT-PCR (n=27 pairs) and immunohistochemical analysis (n=100). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Abbreviations: N, Para-HCC; T, HCC; ns, no significance.

mRNA and protein level in HCCLM3 and Hep3B cells (Figure 2D and E). The results suggested that HBV infection might also contribute to CENPF overexpression via HBx.

In vitro and in vivo Models of Both CENPF Knockdown and CENPF Overexpression Showed Inhibitory Effects on the Tumor Biological Behavior of HCC Cells

To examine the role of CENPF in HCC development, we detected basic CENPF expression levels in seven HCC cell lines, HCCLM3, Huh-7, PLC/PRF/5, Hep3B, SNU-387, Li-7, and SNU-182, to select appropriate cell models for knockdown or overexpression assays (Figure 3A). Taking into consideration the proliferation characteristics of cells and the constitutive expression levels of CENPF, Huh-7 and Hep3B cells were selected to construct the knockdown model, whereas HCCLM3 and Huh-7 cells with low constitutive CENPF expression were selected to construct the overexpression model (Figure 3B and C). Specially, the CENPF-overexpressing model of HCCLM3 and Huh-7 cells was constructed using the CRISPR/dCas9 synergistic activation mediator (SAM) system according to our previous study.¹⁷

CCK-8 assays showed that the proliferation of the CENPF knockdown cell models was significantly inhibited (Figure 3D). Wound healing experiments demonstrated that the migration of Huh-7 and Hep3B cells was suppressed after CENPF expression was decreased (Figure S4A). Downregulated CENPF expression inhibited the invasive ability of Hep3B cells but had no effect on that of Huh-7 cells (Figure S4B).

Table 1 Correlation Between CENPF Expression and Clinicopathological Characteristics of 100 HCC Patients Used for Immunohistochemical Analysis

Clinical Parameter	Number	CENPF Expression		P-value
		Low	High	
Gender				
Female	19	12	7	0.4271
Male	81	43	38	
Age (years)				
<50	18	11	7	0.5649
≥50	82	44	38	
HBV infection				
Positive	66	30	36	0.0075
Negative	34	25	9	
Stage				
I+II	65	36	29	0.9161
III+IV	35	19	16	
Differentiation				
Well or moderate	70	52	18	<0.0001
Poor	30	3	27	

However, the proliferation rates of CENPF-upregulated HCCLM3 and Huh-7 cells were also overtly lower compared with that of the control cells (Figure 3E). Correspondingly, CENPF knockdown and CENPF overexpression both inhibited the tumorigenicity of HCC cell lines in a subcutaneous xenograft tumor model (Figure 3F–I).

CENPF Overexpression Promotes the Transition from G1 to S Phase in HCC Cells

Cell cycle analyses by flow cytometry indicated that the proportion of G0/G1 cells was significantly higher and the S phase cell proportion was significantly lower in CENPF knockdown Huh-7 cells and there was a consistent tendency for Hep3B cells with higher constitutive expression of CENPF (Figure 4A and B). Moreover, the proportion of G0/G1 cells was significantly lower and the S phase cell proportion was significantly higher in CENPF-overexpressing HCCLM3 cells with lower constitutive CENPF expression (Figure 4C). All the above results implied that CENPF promoted the transition from G1 to S phase of the cell cycle in HCC. Whereas there was no significant effect of CENPF overexpression on the cell cycle G1/S transition in Huh-7 cells (Figure 4D), and this difference might be caused by the higher constitutive expression of CENPF in Huh-7 cells than that in HCCLM3 cells.

CENPF Overexpression Activated the MYC Pathway and Thus Promoted G1/S Cell Cycle Transition

RNA-seq analysis showed that downregulated CENPF mainly suppressed cell proliferation-related hallmark gene sets (MYC targets v1, MYC targets v2, E2F targets and G2M checkpoint) in CENPF-silenced Huh-7 cells (Figure 5A and B, Table S3). Consistently, RNA-seq analysis of HCCLM3 cells with endogenous CENPF overexpression indicated the hallmark collections of MYC targets v1 and the MYC targets v2 were activated, which might be responsible for promoting the cell cycle transition from G1 to S phase (Figure 5C and D, Table S4). Whereas the two proliferation-related hallmark gene sets of G2M checkpoint and mitotic spindle were suppressed.

However, gene set enrichment analysis (GSEA) analysis of CENPF-upregulated Huh-7 cells showed that no proliferation-related pathways were enriched (Figure S5), suggesting differential effects of HCC cells with different basal expression of CENPF on the MYC pathway.

Real-time PCR analyses demonstrated that *CDK4*, *CDK6*, *CCNB1*, *CDK1* and *MYC* genes, which play a role in promoting cell cycle progression, were significantly downregulated in the CENPF knockdown Huh-7 cells (Figure 5E). The cell cycle

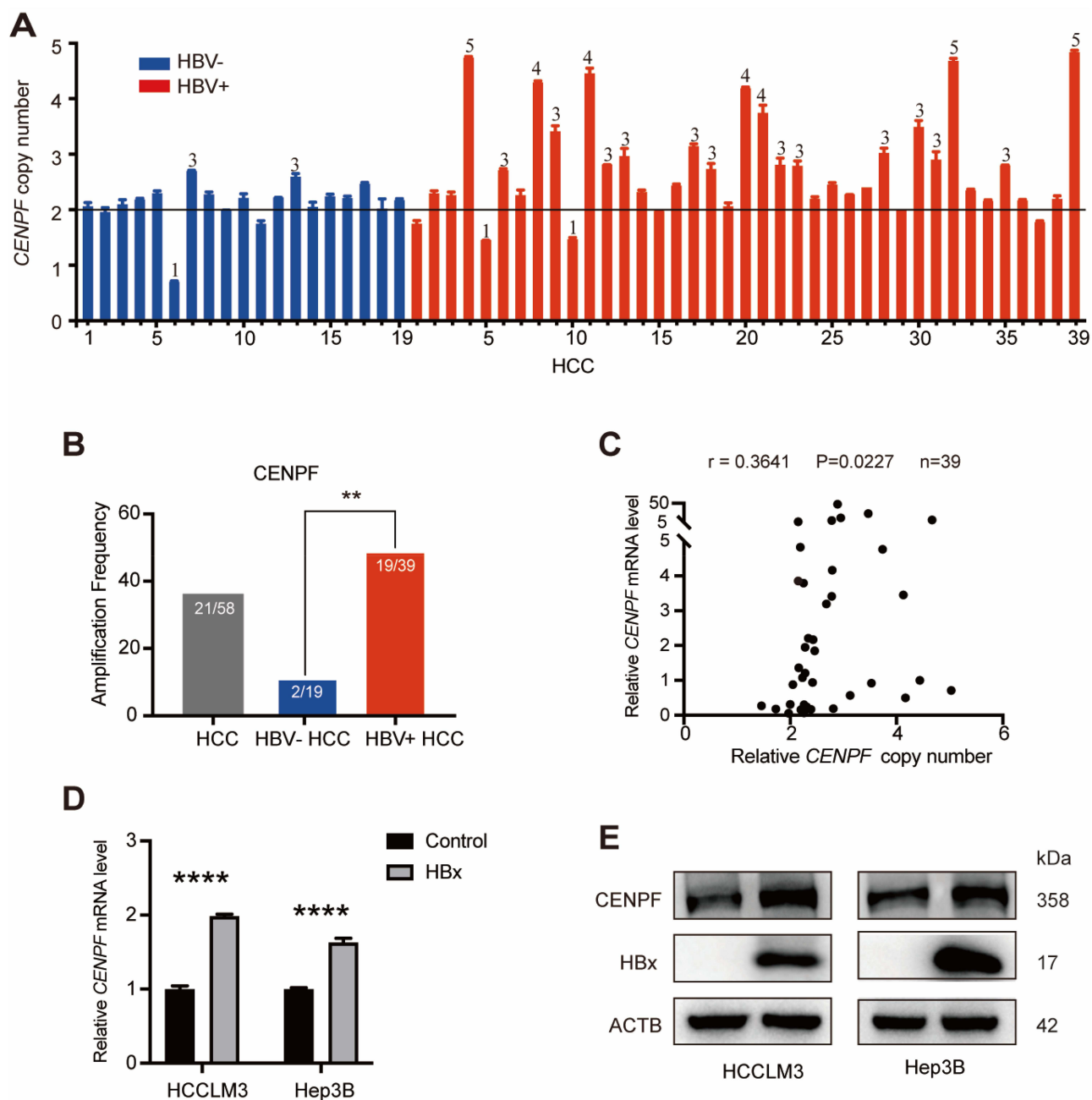


Figure 2 The frequency of *CENPF* amplification was higher in HBV-related HCC and HBx enhanced the expression of *CENPF* in HCC. (**A** and **B**) The amplification frequency of *CENPF* in HBV-positive HCC tissues ($n=39$) was higher than that in HBV-negative HCC tissues ($n=19$). (**C**) *CENPF* copy number was positively correlated with *CENPF* mRNA expression levels ($n=39$). (**D** and **E**) The effects of transient HBx overexpression in HCCLM3 and Hep3B cell lines on the mRNA and protein expression levels of *CENPF*. Data are mean $\pm$ SD of three independent experiments. (** $p < 0.01$, **** $p < 0.0001$).

suppressor genes *CDKN1A* and *CDKN1B* were upregulated, while the expression changes of *CCND1* were not statistically significant. Consistently, the expression of *MYC* and *CCND1* genes was significantly increased in the *CENPF*-overexpressing HCCLM3 cells (Figure 5F). We further treated *CENPF*-overexpressing HCCLM3 cells with the MYC pathway inhibitor 10058-F4 (60 μ M) for 24 h, followed by flow cytometric of the cell cycle analysis. 10058-F4 is a MYC inhibitor that specifically blocks the transcriptional activation of *MYC* target gene expression. Cell cycle analysis revealed that, compared to the *CENPF* overexpression group, 10058-F4 treatment reversed the *CENPF* overexpression-promoted G1/S transition in HCCLM3 cells. The proportion of cells in the G0/G1 phase significantly increased, while the proportion of cells in the S phase markedly

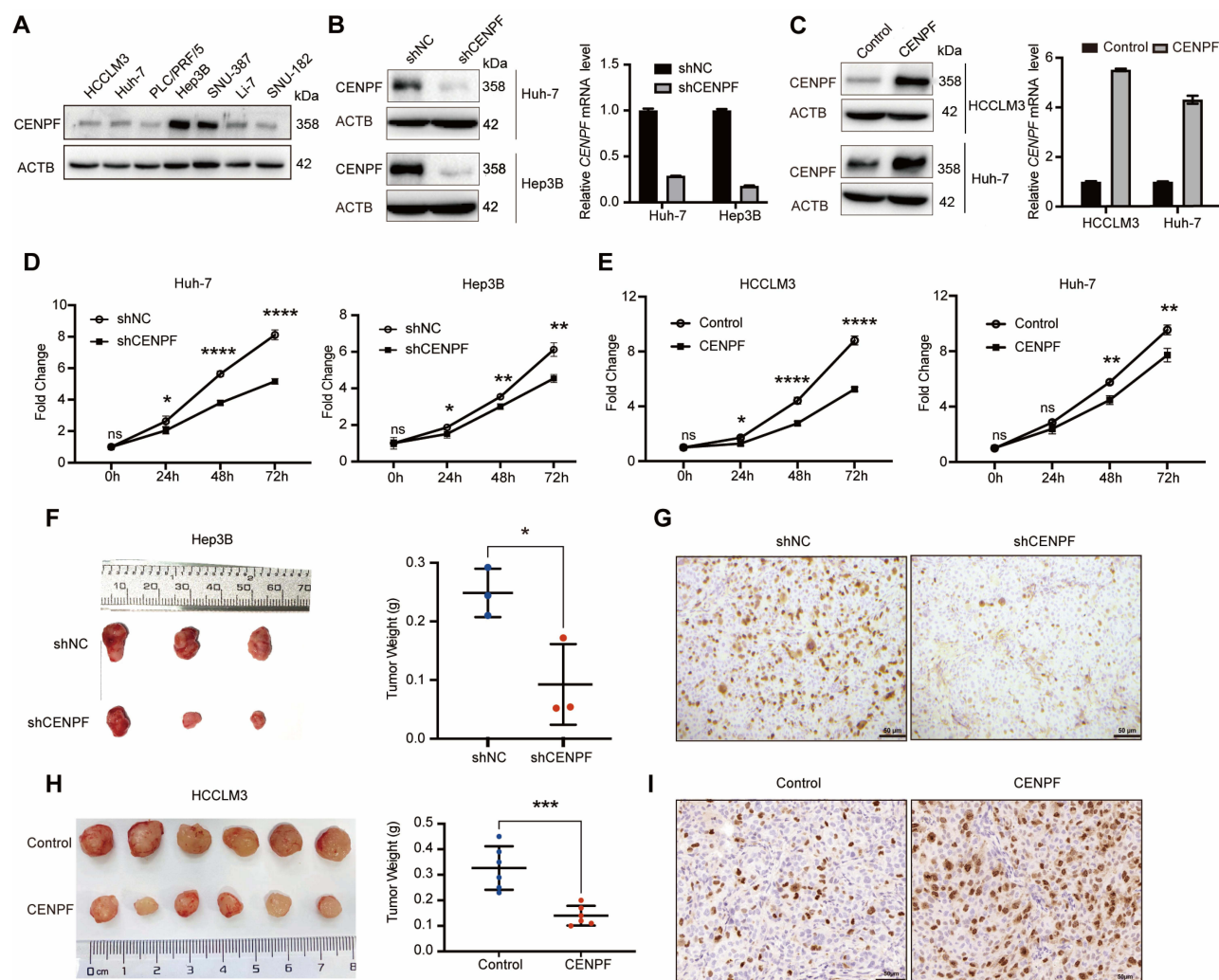
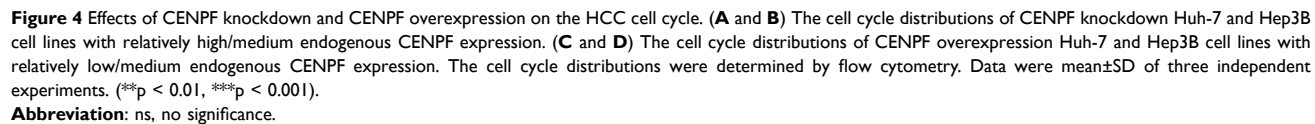


Figure 3 Effects of CENPF knockdown and CENPF overexpression on HCC cell proliferation. **(A)** Western blotting analysis of CENPF expression in HCC cell lines. **(B)** CENPF knockdown was confirmed by Western blotting and qRT-PCR. Data were mean $\pm$ SD of three independent experiments. **(C)** CENPF overexpression cell models were successfully constructed using the CRISPR/dCas9 SAM system. The SAM system relies on sgRNA to target specific sequences. Upon binding to the target sequence, the transcriptional activation system recruits the transcription initiation complex to activate the expression of the target gene. Data were mean $\pm$ SD of three independent experiments. **(D and E)** The proliferation rates of HCC cell lines with CENPF knockdown or CENPF overexpression were analyzed by CCK-8. Data were mean $\pm$ SD of four replicate wells. **(F and H)** Photos and weight analyses of xenografts excised from nude mice inoculated with CENPF-knockdown ($n = 3/\text{group}$) or CENPF-overexpressing ($n = 6/\text{group}$) HCC cell lines. **(G and I)** Representative images of CENPF IHC staining in nude mice tumor tissues. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **Abbreviation:** ns, no significance.

decreased (Figure 5G). Collectively, these results suggested that CENPF overexpression might activate MYC activity and thus promote G1/S transition in HCC development.

Discussion

Current studies have shown that CENPF is overexpressed in HCC and that CENPF expression is closely associated with the tumorigenesis of HCC.^{8,10,24} However, the cause of CENPF overexpression, and specifically, as one of the main cause of HCC, the role of HBV infection on CENPF overexpression remains unknown. In the present study, we revealed for the first time that the HBV infection might be associated with CENPF expression in HCC via the *CENPF* amplification induced by HBV integrations and the expression of HBx. In addition, combining the CENPF knockdown, and the overexpression models constructed by our well established CRISPR/dCas9 SAM system, we reported that CENPF overexpression might promote HCC proliferation by facilitating G1/S transition of HCC cells via the MYC pathway (Figure 6).



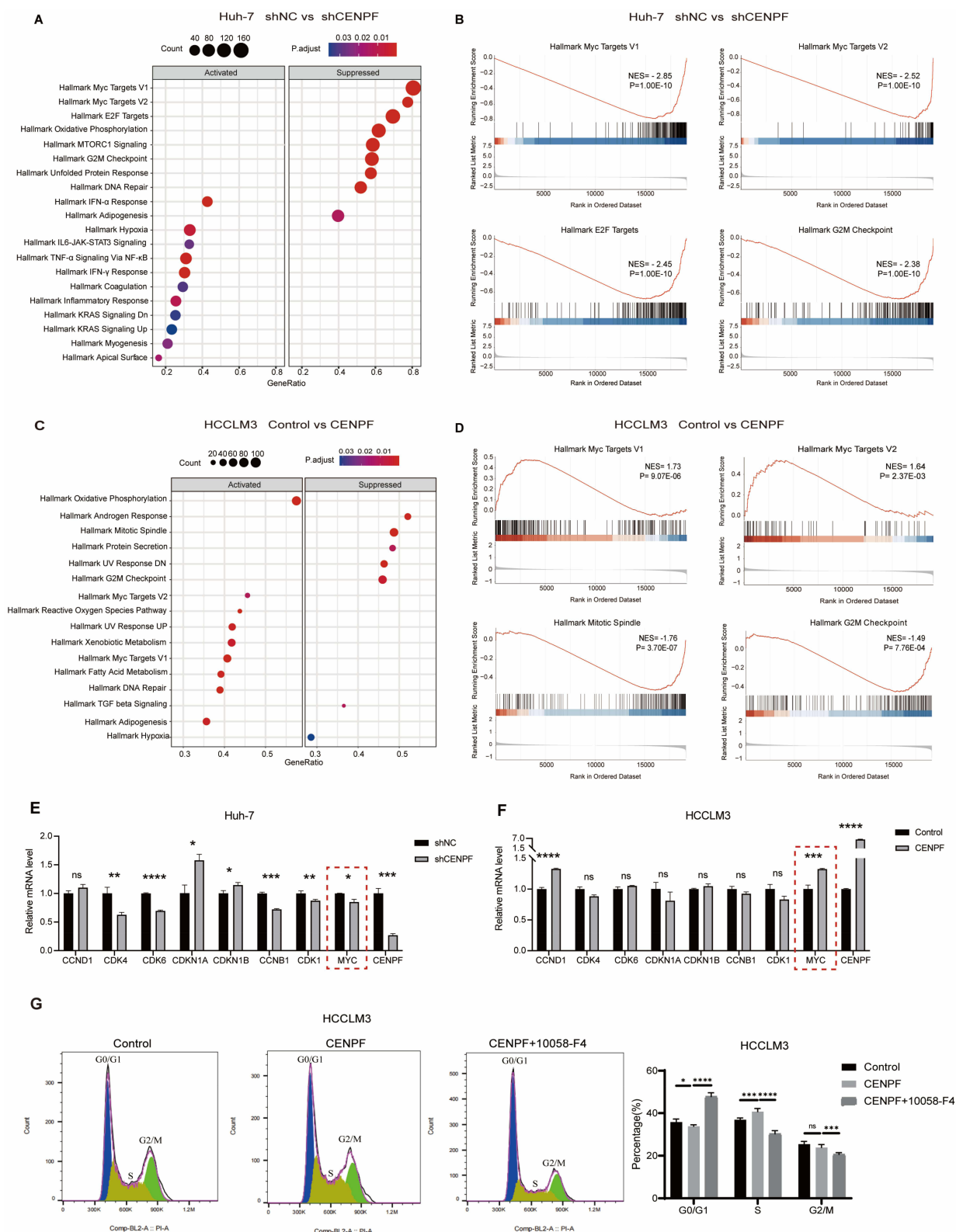


Figure 5 Transcriptome analysis of HCC cells with CENPF knockdown and CENPF overexpression respectively. (A and C) Significantly enriched hallmark gene sets analyzed by GSEA based on the RNA-seq data. (B and D) GSEA plots showed significantly enriched proliferation-related hallmarks. (E and F) Validation of the RNA-seq analysis by qRT-PCR. The red colored dotted rectangular boxes highlighted the expressions of the MYC gene as detected by real-time PCR in the CENPF knockdown Huh-7 cell line and the CENPF overexpression HCCLM3 cell line. (G) Flow cytometry was used to analyze the effect of the MYC pathway inhibitor 10058-F4 on the cell cycle of CENPF-overexpressing HCCLM3 cells. Data were mean $\pm$ SD of three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). **Abbreviation:** ns, no significance.

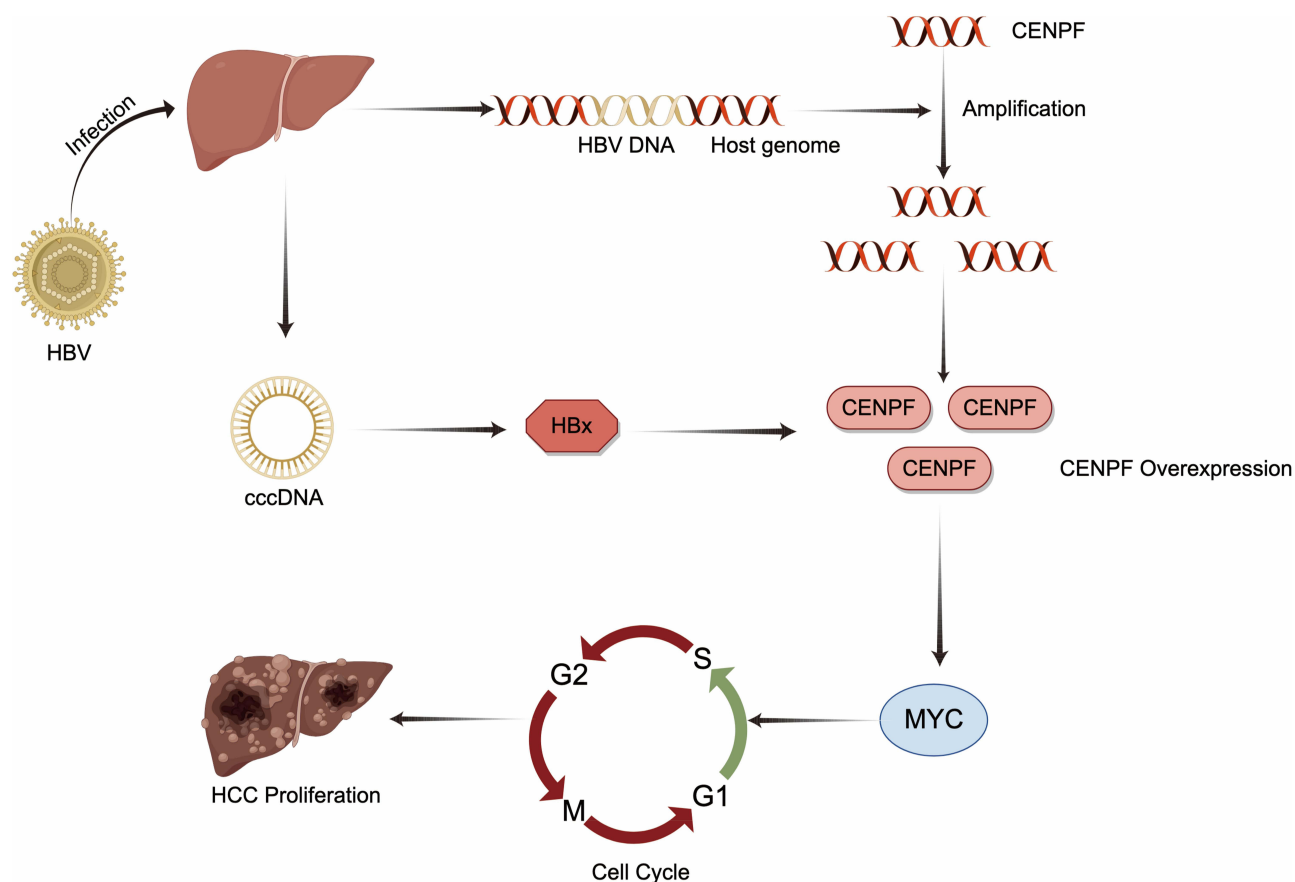


Figure 6 A schematic model illustrating the procedure of HBV induced CENPF overexpression in the proliferation of HCC cells. HBV integrates into human genome, lead to genomic instability and the *CENPF* gene amplification, resulted in the upregulated CENPF expression in HCC. The CENPF overexpression promotes HCC proliferation by facilitating G1/S transition via the MYC pathway.

As the largest member of the centromere-kinetochore complex, CENPF plays a vital role in chromosome segregation.^{25,26} In this study, we reported that CENPF was significantly overexpressed in HCC, and that CENPF expression was positively associated with HBV infection and the differentiation stage of HCC, and that higher CENPF expression indicated a poorer prognosis. However, the mechanisms of CENPF overexpression remain unknown. Kim et al reported that *CENPF* is frequently amplified in HCC.¹⁶ Analysis of genetic alterations of *CENPF* in TCGA LIHC database using cBioPortal showed that amplification but not mutation was the most frequent alteration of *CENPF* in HCC. Therefore, *CENPF* amplification might cause CENPF overexpression in HCC.

Furthermore, the effects of both CENPF knockdown and CENPF overexpression on HCC cell proliferation were evaluated in the present study. In CENPF-silenced cells, the results demonstrated that the proliferation of HCC cell lines was suppressed, which is consistent with previous studies.⁸ However, the endogenous CENPF overexpression also significantly suppressed the proliferation of HCC cells. It is well known that the overexpression of some proteins may impair cellular proliferation because of pathway modulation, promiscuous interactions, stoichiometric imbalance, and resource overload.^{27–29} Based on the high constitutive expression level of CENPF in G2/M-phase cells, the sustained CENPF overexpression model might overload the cells in this phase, resulting in disordered G2/M transition and aberrant cell division, thereby inhibiting cell proliferation. That is to say, the expression level of CENPF in our overexpression system might exceeded the physiological requirements of G2/M-phase cells, implying that the physiological upregulation of CENPF might promote HCC cells progression, but supra-physiological levels were toxic.

However, the constitutive expression level of CENPF in G1/S-phase of cells is relatively low, so the CENPF overexpression caused by CRISPR/dCas9 SAM system may not lead to cytotoxic effects in the G1/S-phase of cells as observed in our results. That is why in the CENPF-overexpressing HCCLM3 cells, cycle analyses showed that CENPF

promoted the progression of the cell cycle during G1 to S phase, which is consistent with the observations in the CENPF-silenced cells. Anyway, the in depth mechanism needs deeper exploration in the future study.

The mechanisms of HBV-mediated HCC development included the HBV DNA integrations reshaping genomic structures and the prolonged expression of HBx.¹³ Genomic instability induced by HBV integrations, such as tumor associated genes amplification, plays a significant role in the development of HCC.^{14,15} Studies have reported that CENPF expression is associated with genomic instability.^{5,30,31} Integration of HBV into the intron of CENPF gene was also report in a previous study.³² In the present study, we found for the first time that the amplification rate of *CENPF* was significantly higher in HCC tissues with HBV infection than that in HBV-negative HCC tissues, and the copy number of *CENPF* was positively associated with *CENPF* mRNA expression. These data suggested that *CENPF* gene amplification might be the underlying mechanism for CENPF upregulation in HBV-related HCC. However, it's worth noting that the results obtained from HCC tissues may not be totally relevant for the individual HCC patient because of intratumoral heterogeneity. In addition, future research would be helpful to perform high-throughput sequencing-based analysis of HBV integration sites in tumors with CENPF amplification, aiming to meticulously characterize whether HBV integration events occur at the CENPF genomic locus to provide definitive molecular evidence for a causal relationship between HBV integration and CENPF amplification.

In addition, accumulating studies have demonstrated that HBx is a transcriptional cofactor that enhances the transcription of hepatoma-related genes.³³ We found that HBx had a significant effect on CENPF overexpression in HCC in this study. Overall, the overexpressed CENPF in HBV-related HCC might be caused by both the CENPF amplification associated with the genomic instability induced by HBV integrations, and the direct effect of HBx.

Notably, cell cycle analyses showed that CENPF promoted the progression of the cell cycle during G1 to S phase in the CENPF-overexpressing HCCLM3 cell, which consistent with the observations in the CENPF-silenced cells. Based on the RNA-seq data, GSEA analysis revealed that the two proliferation-related hallmark gene sets of MYC targets v1 and MYC targets v2 were activated in the CENPF-overexpressing HCCLM3 cells and inhibited in CENPF knockdown Huh-7 cells. MYC functions in cell cycle progression by facilitating G1/S transition.^{34,35} Those results demonstrated that the overexpressed CENPF might activate MYC activity and thus promoted G1/S transition in HCC development. In addition, in the CENPF overexpression model of Huh-7 cell line, we observed that CENPF overexpression had no significant effect on the cell cycle and correspondingly GSEA analysis of the hallmark gene sets demonstrated that no proliferation-related pathways were enriched. The difference might be caused by the different basal expression levels of CENPF or the heterogeneity of the genetic background of the different cell lines. Actually, when analyzing molecular functions using the overexpression models, the results are always interfered by the endogenous expression of the molecule in different cell lines with different heredity background, as observed in the present study. Anyway, the discrepancy in different cells needs deeper exploration in the future study.

In addition, the interaction between PANoptosis and cell cycle has been reported,³⁶ future study would be helpful to explore whether CENPF regulates the tumor microenvironment through PANoptosis of HCC. Meanwhile, existing studies have pointed out the biomarker gap in HCC immune rechallenge and that lymphatic vessel density is independently associated with postoperative recurrence of HBV-HCC,^{37,38} so it would be helpful to explore the role CENPF and serum CENPF autoantibodies in the diagnosis and prognosis to improve clinical translation.

There were also limitations in this study. First, given the potential cytotoxic effects on the G2/M transition of our established CENPF sustained high expression model in HCC, the present study was limited to evaluate only its promotion on the G1/S transition when the constitutive CENPF expression was relative low, suggesting the current overexpression model's limitation in simulating physiological upregulation of CENPF. Further study is needed to develop a controlled CENPF expression system. Second, the detailed relationship between HBV infection and *CENPF* amplification and the underling mechanisms need to be further explored in the future study, such as using HBV transgenic mice model.

Conclusions

HBV infection was associated with the upregulated CENPF expression in HCC via the CENPF amplification induced by HBV integrations and the expression of HBx. Furthermore, CENPF overexpression may facilitate the G1/S cell cycle transition of HCC cells via the MYC pathway, thereby promoting HCC development. These findings may supplement the current understanding of HCC progression, and provide basis for the CENPF target diagnosis and therapy in HBV-related HCC.

Data Sharing Statement

The data supporting the findings of this study are available from the corresponding author, Jian Huang, upon reasonable request. The RNA-sequencing data have been uploaded to the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) (Accession Number: GSE319803).

Ethics Approval

The use of clinical specimens was approved by the Clinical Research Ethics Committee of Beijing Friendship Hospital, Capital Medical University. Animal experiments were approved by the Laboratory Animal Ethics Committee of Beijing Friendship Hospital, Capital Medical University.

Acknowledgments

We would like to thank Professor Jie Wang from the Department of Microbiology and Infectious Disease Center, School of Basic Medical Science, Peking University Health Science Center for supplying the HBx plasmid.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was funded by National Natural Science Foundation of China (81071973 and 81602032), National Major Science and Technology Projects of China (2017ZX10201201007002) and High-level Traditional Chinese Medicine Hospital SM Project (DGMG-GLZH-23001).

Disclosure

The authors declare no competing interests.

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