Original Research

CCNB1 promotes the development of hepatocellular carcinoma by mediating DNA replication in the cell cycle

Min-Hua Rong[1,*], Jian-Di Li[1,*], Lu-Yang Zhong[1], Yu-Zhen Huang[1], Juan Chen[1], Li-Yuan Xie[1], Rong-Xing Qin[1], Xiao-Lian He[1], Zhan-Hui Zhu[1], Su-Ning Huang[2] and Xian-Guo Zhou[1]

[1]Research Department, Guangxi Medical University Cancer Hospital, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China; [2]Department of Radiotherapy, Guangxi Medical University Cancer Hospital, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China

Corresponding authors: Su-Ning Huang. Email: huangsuning@stu.gxmu.edu.cn; Xian-Guo Zhou. Email: zhouxianguo1001@163.com

*These authors contributed equally to this work.

Impact statement

CCNB1 may serve as a crucial biomarker in hepatocellular carcinoma (HCC) based on results from large sample sizes (HCC samples = 3065, normal samples = 2693). Moreover, the serum *CCNB1* could serve as a promising diagnostic biomarker due to its overexpression in the patients with early-stage HCC. *CCNB1* might contribute to the development of HCC by mediating DNA replication in the cell cycle pathway, indicating that *CCNB1* might be an important therapeutic target for exploiting a novel targeted therapy.

Abstract

In our studies, cyclin B1 (*CCNB1*) mRNA and protein were overexpressed in hepatocellular carcinoma (HCC) tissues compared with non-HCC tissues. Moreover, *CCNB1* was overexpressed in the serum of HCC patients. The expression of *CCNB1* was associated with several crucial clinicopathologic characteristics, and the HCC patients with overexpressed *CCNB1* had worse overall survival outcomes. In the screening of interactional genes, a total of 266 upregulated co-expression genes, which were positively associated with *CCNB1*, were selected from the datasets, and 67 downregulated co-expression genes, which were negatively associated with *CCNB1*, were identified. The key genes might be functionally enriched in DNA replication and the cell cycle pathways. *CDC20*, *CCNA2*, *PLK1*, and *FTCD* were selected for further research because they were highly connected in the protein-protein interaction networks. Upregulated *CDC20*, *CCNA2*, and *PLK1* and downregulated *FTCD* might result in undesirable overall survival outcomes for HCC patients. The univariate Cox analysis results showed that *CDC20* and *PLK1* might be two independent risk factors, while *FTCD* might be protective in HCC. Therefore, *CCNB1* may participate in the cell cycle of HCC by regulating DNA replication, and *CCNB1* may provide a direction for the diagnosis of early-stage HCC and targeted HCC therapy.

Keywords: Cyclin B1, hepatocellular carcinoma, DNA replication, cell cycle, prognosis, weighted gene co-expression network analysis

Experimental Biology and Medicine **2022; 247: 395–408. DOI: 10.1177/15353702211049149**

Introduction

Liver cancer remains one of the commonest digestive tumors worldwide.[1] Around 75–80% of the primary liver cancer cases are classified as hepatocellular carcinoma (HCC), which is the commonest pathological type of liver cancer.[2] However, due to the absence of clear symptoms in the early stages of HCC, patients failed to receive timely interventions, which contributes to their undesirable prognosis.[3,4] Although hepatectomy, liver transplantation, and radiofrequency ablation have been used to treat HCC, the treatment results remain unsatisfying because of long-term recurrence and drug resistance.[5,6]

B-type cyclins have attracted wide attention because their expression is upregulated in the G2/M phase to promote cell mitosis.[7] Among these B-type cyclins, cyclin B1 (*CCNB1*) is a key promoter of mitosis, and its expression varies periodically throughout the cell cycle. The knockout of *CCNB1* can suppress the invasiveness and migration of SMMC-7721 cell line.[8] The complex formed from the combination of *CCNB1* and *CDK1* can promote cell cycle

ISSN 1535-3702

progression via phosphorylation of the substrates.[9] In addition, *CDK1-CCNB1* has been demonstrated to be an indispensable factor of the spindle checkpoint that ensures the accuracy of mitosis by enabling MPS1 kinetochore localization.[10,11]

Various studies have showed that the overexpression of *CCNB1* is common in many cancers, such as adrenocortical carcinoma, cervical carcinoma, stomach cancer, non-small cell lung cancer, and rhabdomyosarcoma.[12–16] A study showed that *CCNB1* could promote the proliferation of pituitary adenomas and stimulate epithelial-to-mesenchymal transition.[17] In pancreatic cancer, *CCNB1* silencing promotes cell senescence and suppresses cell proliferation by activating the p53 signaling pathway.[18] In addition, the downregulation of miR-16 also induces G0/G1 cell cycle arrest by targeting *CCNB1*. *APC11* can also cause the degradation of *CCNB1* via the ubiquitination of *UBA52*, resulting in the enhanced proliferation ability of lung cancer cells. [19]

Increasing evidences demonstrated the intimate associations between *CCNB1* and HCC. *CCNB1* is illustrated to be an immune-infiltrations-associated prognostic biomarker for HCC, suggesting that it may be an interventional target for treating HCC patients.[20] *CCNB1* is also involved in the functioning of microRNAs, to regulate the occurrence and progress of HCC.[8,21,22] *CCNB1* overexpression has been associated with higher histological grades and higher risks of vascular invasion, and HCC patients with upregulated *CCNB1* had undesirable survival condition, indicating that *CCNB1* might play a key role in the deterioration of HCC.[23] As a result of various studies, *CCNB1* is believed to be overexpressed in HCC.[21,22,24–30] However, a large sample size is needed to verify the expression patterns of *CCNB1* in HCC, and the mechanisms of *CCNB1* in HCC are still unknown. Moreover, the potential clinical implications of *CCNB1* expression in HCC patients need to be further studied.

Therefore, we focus on exploring the potential mechanisms of *CCNB1* in HCC development and the relationship between *CCNB1* and clinicopathologic features of HCC by examining *CCNB1* at the mRNA and protein levels. In addition, we focused on finding novel therapeutic targets for HCC patients.

Materials and methods

Data enrolment of public databases

HCC expression matrices were selected and downloaded from the ArrayExpress, Gene Expression Omnibus, The Cancer Genome Atlas databases, and Genotype-Tissue Expression. The studies were integrated if they came from the same platform. The following standards were developed for study inclusion: (1) the merged studies had both HCC samples and non-HCC samples that had a clear diagnosis, (2) the total sample size of the studies was $\geq$6, and (3) the subjects of the studies were Homo sapiens. Studies containing samples with various types of treatment (virus transfection, gene silencing, gene knockout, treatment, etc.) and studies without mRNA expression data were excluded. Gene sets on the serum *CCNB1* expression level of HCC patients were also considered eligible datasets and were used to evaluate the clinical value of *CCNB1* in screening early-stage HCC patients. In addition, the association between *CCNB1* mRNA levels and the clinicopathology of HCC patients was identified based on the TCGA database.

Internal immunohistochemistry

The tissue samples were provided by the CCNB1 with 315 HCC and 195 non-HCC liver tissues. The specimens were fixed with formalin and stained using immunohistochemistry (IHC). IHC was performed with CCNB1 antibody. The clinicopathologic data of the patients were extracted and recorded, and data were collected in compliance with ethical policies. Our study was approved by the ethics committee of Guangxi Medical University Cancer Hospital (NO. 2021-KY-Guoji-099). Each participating patient signed an informed consent form, and all operations followed the manufacturer's regulations and requirements.

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) was performed to explore the conventional pathways and biological processes related to *CCNB1* in HCC. Based on the median expression value of *CCNB1* in the TCGA dataset, HCC patients were classified into two groups, and the expression level of *CCNB1* mRNA was regarded as a phenotypic label. Finally, we investigated the *CCNB1*-mediated molecular functions in HCC by using GSEA v4.0.1 software, where h.all.v6.0.symbols.gmt was exploited as a reference gene set. The false discovery rate threshold was set at <0.01.

Filtration of interactional genes of *CCNB1*

Co-expressed genes and differentially expressed genes were selected from the datasets of the aforementioned databases. Weighted Gene Co-expression Network Analysis (WGCNA) was conducted to identify the clinically significant modules of *CCNB1* from the aforementioned datasets of the TCGA database. All procedures were conducted using R v3.6.3.

The Pearson's Correlation Coefficients (PCCs) between *CCNB1* and other alternative genes in each mRNA expression matrix were calculated. The identification criteria of significant co-expression genes of *CCNB1* were (a) PCCs ≥ 0.3, $P < 0.05$ or (b) PCCs ≤ -0.3, $P < 0.05$.

"Limma-Voom" R package was used to preliminarily identify differential expression genes. The differential expression genes of *CCNB1* were selected based on the following identification criteria: (a) $|\log_2 FC| > 1$ and (b) adjusted $P < 0.05$. Standard mean differences (SMDs) were calculated to identify the final differential expression genes.

A WGCNA was conducted to determine the genes that were most related to *CCNB1* using the "WGCNA" and "limma" R packages. The number of genes was >50 to

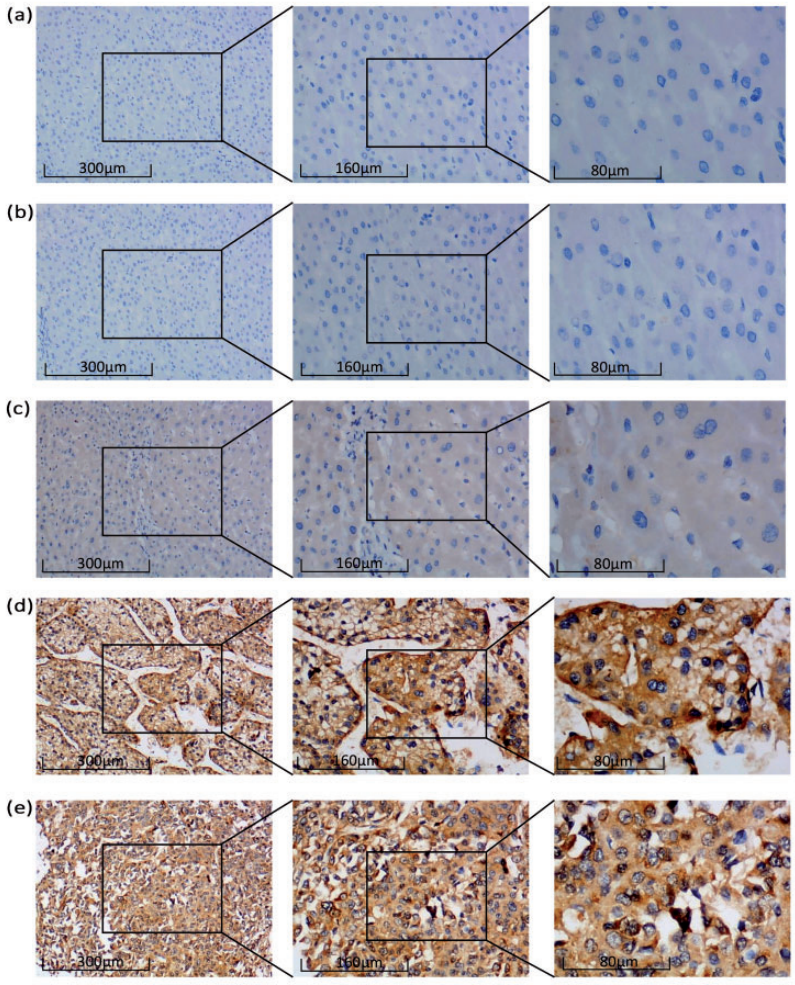


Figure 1. Protein expression of CCNB1 in HCC through immunohistochemistry. (a–c) normal tissue. (d, e) HCC tissue. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal.)

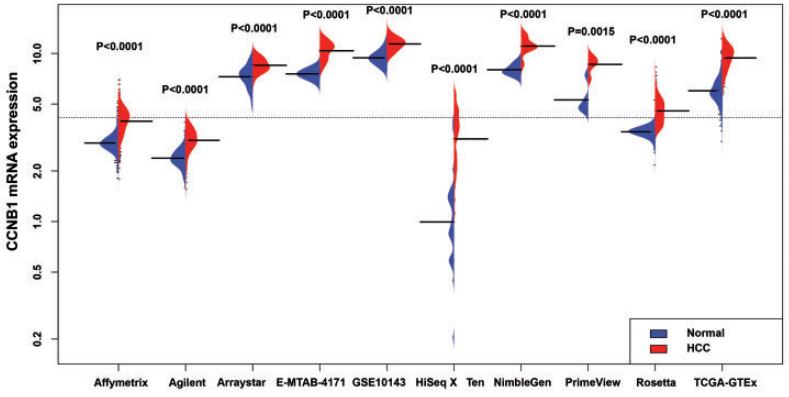


Figure 2. *CCNB1* mRNA expression in HCC tissues according to various databases. The scatter plots indicate increased *CCNB1* expression levels in HCC compared with normal liver tissue. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal.)

ensure a desirable dependability. The soft threshold was set at $\beta = 11$ to construct a scale-free topology ($R^2 > 0.90$). Through a WGCNA of the TCGA dataset, we determined the co-expression module related to HCC that contained *CCNB1*, and then the genes of this module were selected for further research.

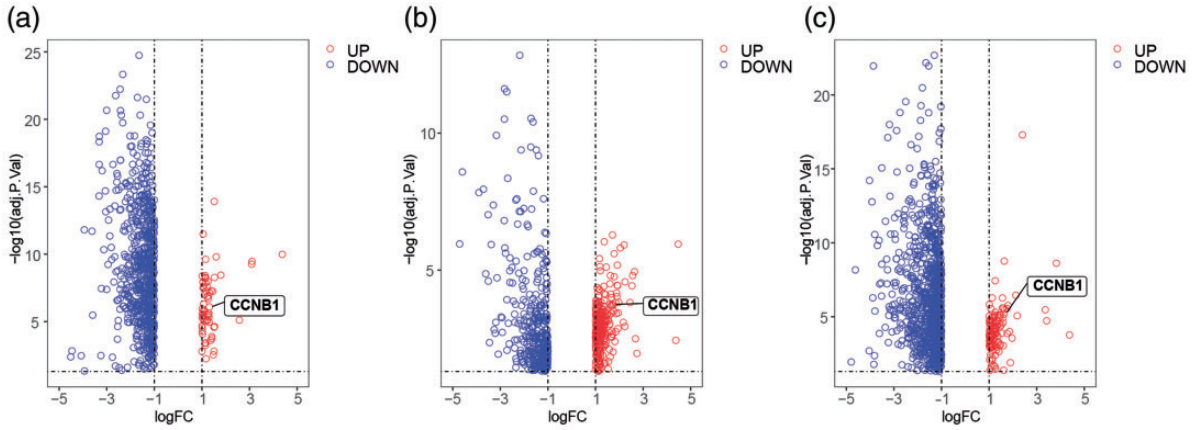


Figure 3. The expression of *CCNB1* mRNA in the serum of HCC patients. (a) HCC compared with non-HCC. (b) HCC compared with normal liver. (c) HCC compared with chronic hepatitis. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal.)

Enrichment analyses

The gene ontology (GO) terms (i.e., cellular component, molecular function, and biological process) were enriched to predict the prospective molecular functions of *CCNB1* by using DAVID v6.8. Kyoto Encyclopedia of Genes and Genomes (KEGG) and PANTHER functional annotations were conducted to identify the signaling pathways enriched by the intersected genes of the co-expression genes and the differential expression genes recovered from the above procedures. The disease ontology (DO) analysis was used to reveal the diseases that were enriched by the genes obtained from the above procedures; results were visualized via R v3.6.3. The protein–protein interaction (PPI) analyses for the two groups of genes obtained from the above procedures were based on STRING v11.0 and were performed using Cytoscape v3.8.0.

Statistical analyses

To detect the differences in *CCNB1* expression between the HCC tissues and the non-HCC tissues, an independent sample *t*-test was performed. To evaluate the diagnostic efficacy of the indicators, we assessed the ability of *CCNB1* to distinguish between HCC patients and noncancerous patients by analyzing bean plots and the receiver operating characteristic (ROC) curves with the area under curve (AUC). The summary standard mean difference (SMD) was calculated to evaluate the expression level of *CCNB1* in HCC patients. Heterogeneity and publication bias were then tested. The specificity and sensitivity of *CCNB1* in these studies were measured using Stata v12.0. For survival analyses, the Kaplan-Meier curves were plotted to further understand the prognostic value of *CCNB1* in HCC patients. All Kaplan-Meier curves were plotted by using R v3.6.3. Univariate and multivariate Cox regression analyses were performed to calculate the hazard ratios in HCC patients by using the mRNA expression levels of key genes and survival data provided by the TCGA datasets.

Results

Inclusion of expression data and collection of clinical information

Through the aforementioned databases, a total of 77 studies were obtained based on the inclusion and exclusion criteria. Ten integrated datasets of *CCNB1* mRNA expression in HCC were obtained by merging studies from the same platform and removing their batches, which covered 48 of the 77 aforementioned studies (Supplemental Table 1). GSE114564 was selected for the identification of the serum mRNA expression status of *CCNB1* in HCC patients. The protein expression values of CCNB1 in HCC was obtained using IHC. In the studies included, GSE76427, GSE10143, and TCGA were used to probe the relationship between *CCNB1* expression level and the survival condition of HCC patients.

Increased expression levels of *CCNB1* in HCC

The design of our study is shown in Supplemental Figure 1. Based on the in-house IHC staining, increased CCNB1 protein expression was more evident in HCC tissue samples in comparison to normal tissue samples (Figure 1 and Supplemental Figure 2(a)). Moreover, increased expression of CCNB1 protein was detected in the groups characterized by stages T2–T4 compared to those characterized by the T1 stage (Supplemental Figure 2(b)). As the bean plots in Figure 2 show, the expression of *CCNB1* was significantly increased in HCC tissues when compared with that in the non-HCC tissues (all with *P* values less than 0.05). Moreover, the serum mRNA expression level of *CCNB1* was elevated in the HCC group compared with the non-HCC group (Figure 3(a)). Interestingly, elevated serum *CCNB1* expression was found in the HCC patients compared with that in the normal liver and in chronic hepatitis patients (Figure 3(b) and (c), respectively). The AUC of these 11 datasets indicated that *CCNB1* might have a high ability to significantly differentiate between HCC group and non-HCC group (Supplemental Figure 3). The integrated SMD was 1.88 (95% CI = 1.62−2.15), indicating that *CCNB1* was overexpressed in HCC group compared to

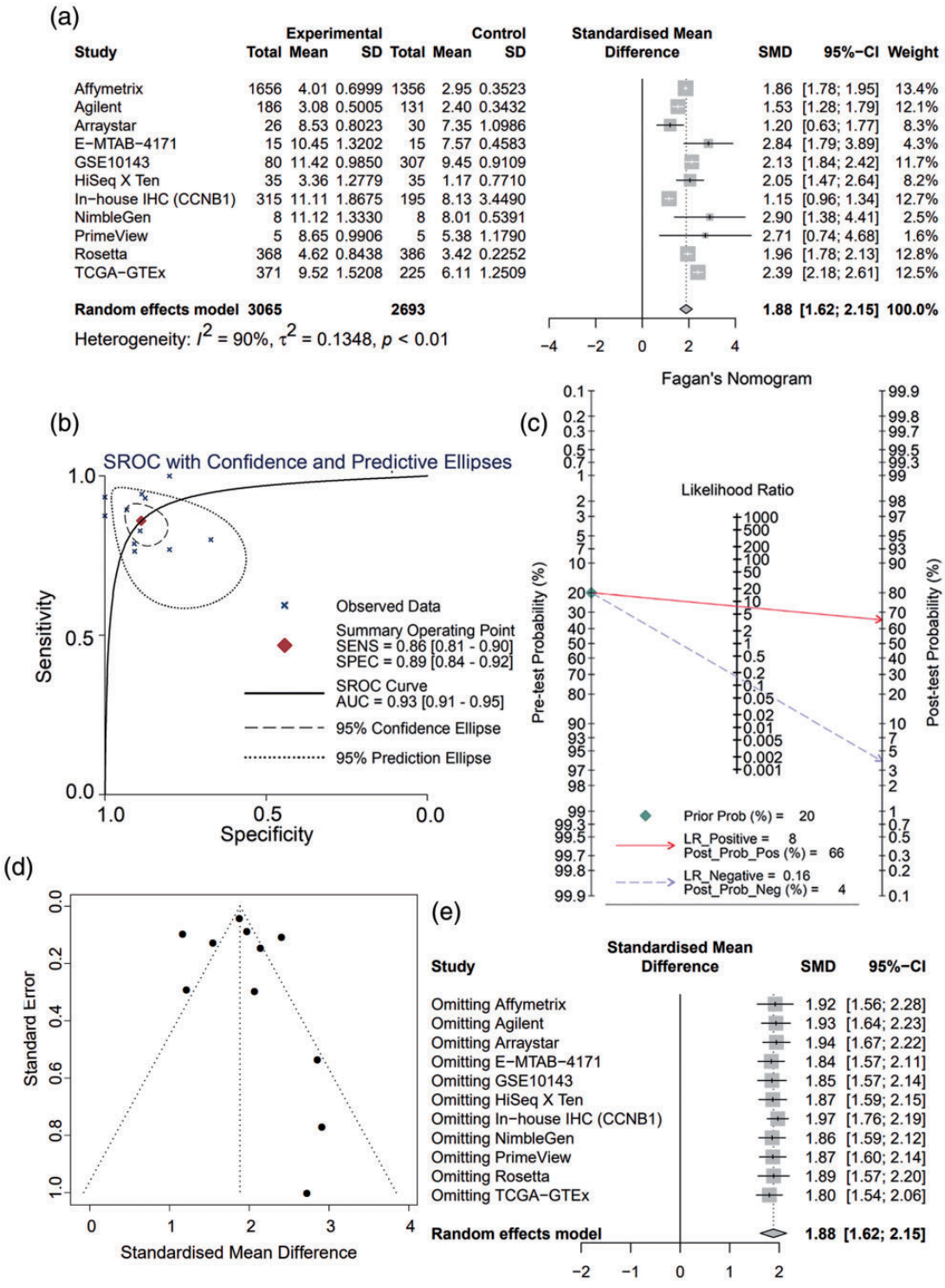


Figure 4. The expression level and differentiating capacity of *CCNB1* in hepatocellular carcinoma. SMD, standardized mean difference. (A color version of this figure is available in the online journal.)

non-HCC group (Figure 4(a)). The summarized ROC curve demonstrated that *CCNB1* had a high discriminatory capability for HCC (AUC = 93%, Figure 4(b)), and this was confirmed using the sensitivity (Figure 5(a)) and specificity results (Figure 5(b)), the positive and negative likelihood ratio (Figure 5(c) and (d)). In the Fagan plot, the probability before the test was 20%, and the probability of using *CCNB1* to detect the positive results of HCC was 66%, suggesting that *CCNB1* may be helpful for screening early-staged HCC patients (Figure 4(c)). No significant publication bias was detected (Figure 4(d)). The results showed significant heterogeneity; thus, the random effects model

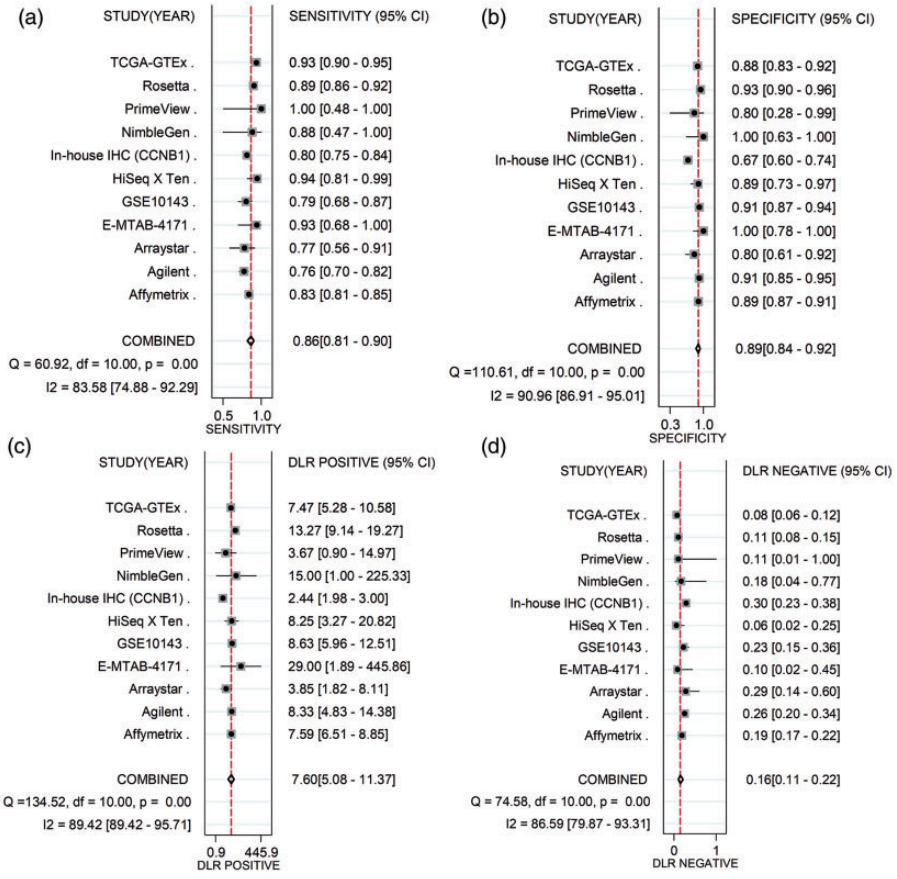


Figure 5. The discriminatory ability of *CCNB1* in hepatocellular carcinoma. (a) Sensitivity analysis. (b) Forest plot of specificity. (c and d) Forest plot of positive likelihood ratio and negative likelihood ratio, respectively. (A color version of this figure is available in the online journal.)

was used ($I^2 = 90\%$, $P < 0.05$). No significant difference existed according to the forest plot of sensitivity analysis (Figure 4(e)).

The relationship between *CCNB1* expression and clinical significance in HCC

As shown in Table 1, there were significant differences in *CCNB1* mRNA expression based on tissue type ($P < 0.0005$), race ($P = 0.0260$), neoplasm histologic grade I ($P < 0.0005$), neoplasm histologic grade II ($P = 0.0270$), and pathologic stage I ($P < 0.0005$). The differences in age ($P = 0.3810$), gender ($P = 0.0560$), and Child-Pugh classification grade ($P = 0.2780$) were insignificant. The overexpression of *CCNB1* suggested an unsatisfactory overall survival (OS) in HCC patients based on a survival analysis (Figures 6(a) to (c)).

GSEA

"DNA repair," "E2F targets," "MTORC1 signaling," and "MYC targeting" were most enriched in the *CCNB1* overexpression phenotype based on the TCGA HCC cohort ($P < 0.0005$), suggesting that *CCNB1* may be involved in HCC development through multiple signaling pathways (Figure 7).

Interactional genes of *CCNB1* in HCC

A total of 667 positive co-expression genes, which appeared in at least eight mRNA expression matrices, were screened, and 617 negative co-expression genes, which appeared in at least seven mRNA expression matrices, were selected.

Using the R package "Limma-Voom," we obtained 3736 upregulated differential expression genes and 1663 downregulated differential expression genes.

Through the WGCNA, 17 modules were obtained. We found that the module "turquoise" contained *CCNB1*. Therefore, the genes from "turquoise" were collected for subsequent screening for potential target genes of *CCNB1* (Supplemental Figure 4). Finally, 1709 genes that had a high degree of co-expression were chosen. The blue color in the normal group and the red color in the tumor group show that the genes from the module "turquoise" are mainly overexpressed in HCC (correlation coefficient = 0.56, $P < 0.0001$, Supplemental Figure 4(c)).

Functional enrichment and co-expression network analysis

By intersecting positively co-expressed genes, upregulated differential expression genes, and WGCNA *CCNB1*-related modules, we obtained 266 upregulated and HCC-associated co-expression genes, which were positively

Table 1. Clinical pathological parameters of 361 patients diagnosed with HCC from TCGA database.

Clinical parameters	*N*	Mean ± standard deviation	*T* or *F*	*P*-value
Tissue				
Normal	50 (12.2%)	6.1313 ± 0.8544	−15.359	$P < 0.0005$
Tumor	361 (87.8%)	7.1313 ± 1.8544		
Ages				
≤60	167 (46.8%)	9.6409 ± 1.5917	1.422	0.3810
>60	190 (53.2%)	9.4099 ± 1.4776		
Gender				
Female	117 (32.4%)	9.6459 ± 1.3599	1.057	0.0560
Male	244 (67.6%)	9.4644 ± 1.6014		
Race				
White	176 (50.4%)	9.3378 ± 1.5467	$F = 3.669$	0.0260
Asian	156 (44.7%)	9.7866 ± 1.4783		
Black or African American	17 (4.9%)	9.3859 ± 1.7321		
Neoplasm histologic grade-I				
G1	53 (14.9%)	8.6218 ± 1.5561	$F = 15.977$	$P < 0.0005$
G2	171 (48.0%)	9.3294 ± 1.4779		
G3	121 (34.0%)	10.143 ± 1.3557		
G4	11 (3.1%)	10.0598 ± 1.0119		
Neoplasm histologic grade-II				
G1–G2	224 (62.9%)	9.162 ± 1.5234	−6.105	0.0270
G3–G4	132 (37.1%)	10.136 ± 1.3275		
Pathologic stage-I				
Stage I	167 (49.6%)	9.1667 ± 1.4104	7.626	<0.0005
Stage II	82 (24.3%)	9.8037 ± 1.5262		
Stage III	84 (24.9%)	10.0344 ± 1.5637		
Stage IV	4 (1.2%)	9.2856 ± 0.9102		
Pathologic stage-II				
Stage I–II	249 (73.9%)	9.3765 ± 1.4773	−3.365	0.6490
Stage III–IV	88 (26.1%)	10.0004 ± 1.5447		
Child Pugh classification grade				
A	212 (90.6%)	9.3358 ± 1.4484	$F = 1.288$	0.2780
B	21 (9.0%)	9.3586 ± 1.4394		
C	1 (0.4%)	7.0112		

HCC: hepatocellular carcinoma; TCGA: The Cancer Genome Atlas; *T*: unpaired independent sample *t*-test; *F*: *F*-test or analysis of variance.

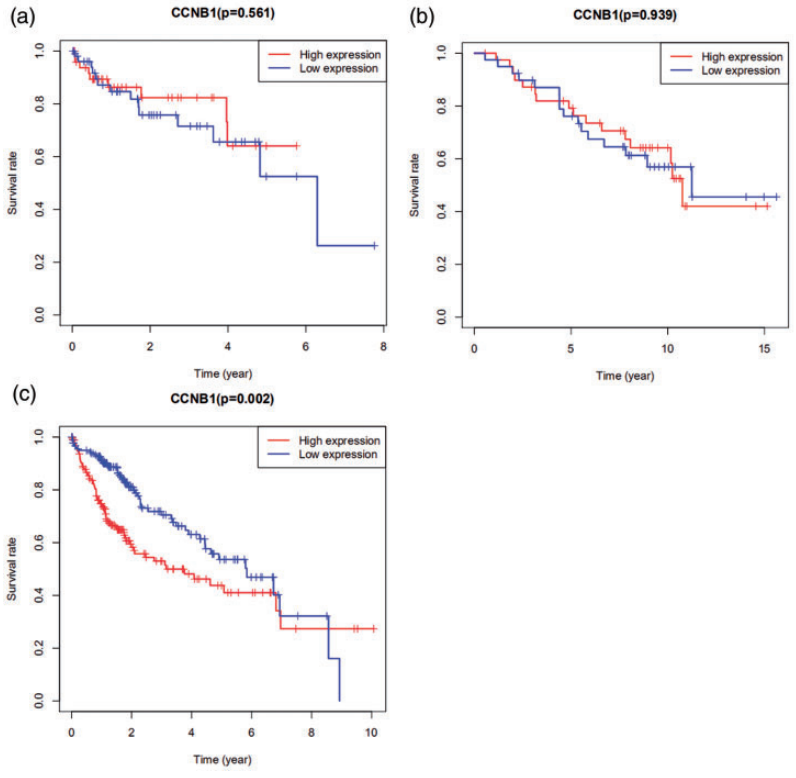


Figure 6. Evaluation of the prognostic value of *CCNB1* in hepatocellular carcinoma. (a) GSE76427. (b) GSE10143. (c) The Cancer Genome Atlas. (A color version of this figure is available in the online journal.)

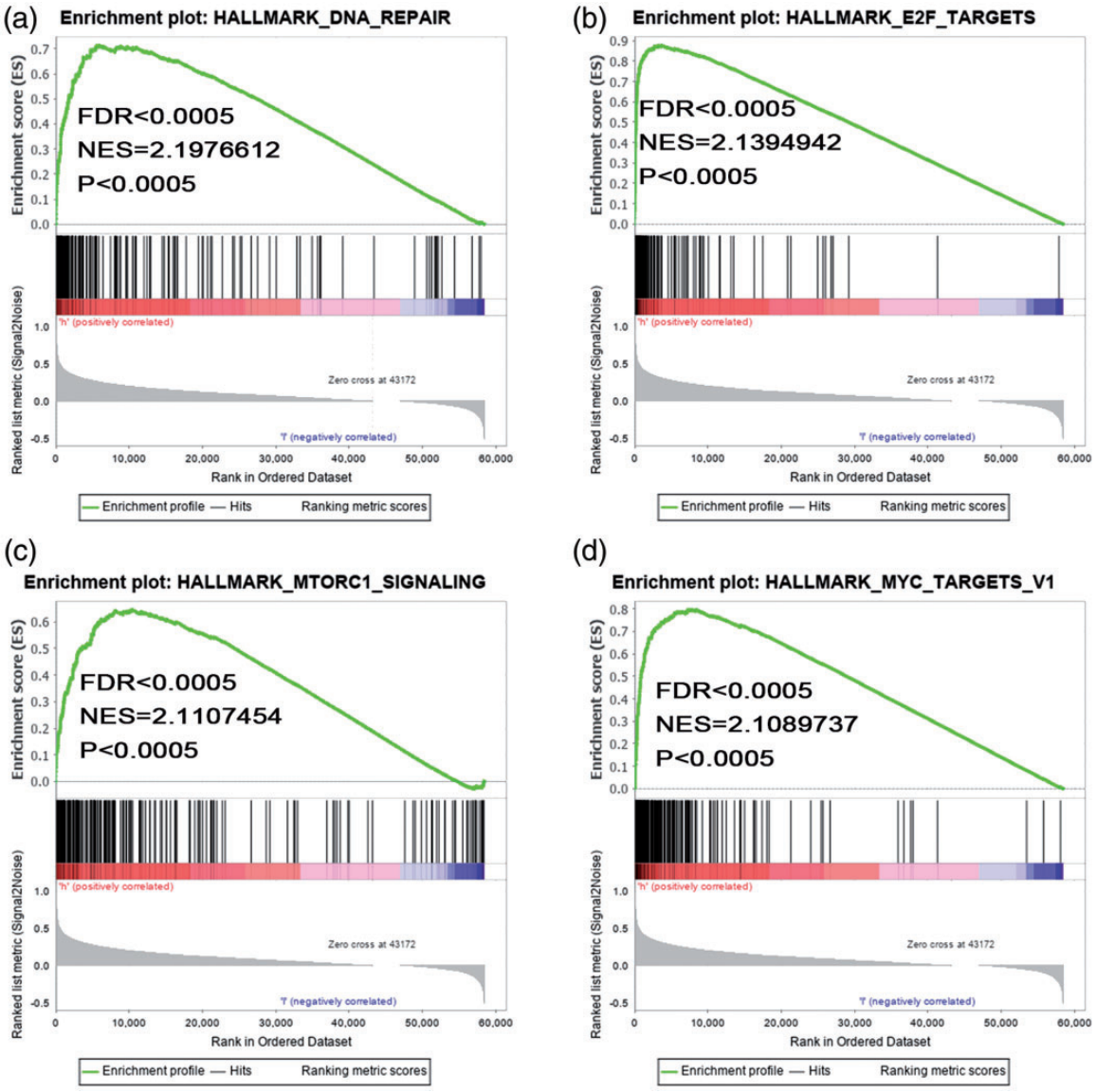


Figure 7. Gene Set Enrichment Analysis of *CCNB1* in hepatocellular carcinoma based on the Cancer Genome Atlas database. (a) DNA repair. (b) E2F targets. (c) MTORC1 signaling. (d) MYC targets. (A color version of this figure is available in the online journal.)

related to *CCNB1* (Supplemental Figure 5(a)). In GO enrichment analyses, the most notably enriched functional terms were "DNA replication," "cell division" in biological process, "nucleoplasm," and "cytosol" in cellular component, and "protein binding" and "ATP binding" in molecular function ($P < 0.001$, Figure 8(b) to (d)). KEGG pathway analyses showed that "cell cycle" and "DNA replication" played important roles in HCC ($P < 0.001$, Figure 8(e)). Interestingly, the top pathway in the PANTHER analysis was "DNA replication," followed by "cell cycle" (Figure 8 (f)). A PPI network analysis identified that three genes (*CDC20*, *CCNA2*, and *PLK1*) might play an indispensable role in *CCNB1*-mediated pathways in HCC (Figure 8(a)). The DO analysis result showed that these overlapped genes might be related to some diseases, such as ataxia telangiectasia and sensory system cancer (Figure 10(a)).

By overlapping negatively co-expressed genes, downregulated differential expression genes, and WGCNA *CCNB1*-related modules, we obtained 67 downregulated and HCC-associated co-expression genes, which were negatively related to *CCNB1* (Supplemental Figure 5(b)). In GO enrichment analyses, the most notably enriched functional terms were "oxidation-reduction process" in biological process, "extracellular exosome" in cellular component, and "monooxygenase activity" in molecular function ($P < 0.001$, Figure 9(b) to (d)). These overlapped genes were enriched in "metabolic pathways," "drug metabolism-cytochrome P450," and "metabolism of xenobiotics by cytochrome P450" (Figure 9(e) and (f)). PPI network analyses showed that *FTCD* could be a key gene in HCC (Figure 9(a)). These overlapped genes may have a close relationship with some diseases, such as coronary artery disease and myocardial infarction (Figure 10(b)).

CDC20, *CCNA2*, *PLK1*, and *FTCD* were selected to investigate the relationship between key genes and the clinicopathologic features as well as prognoses of HCC patients.

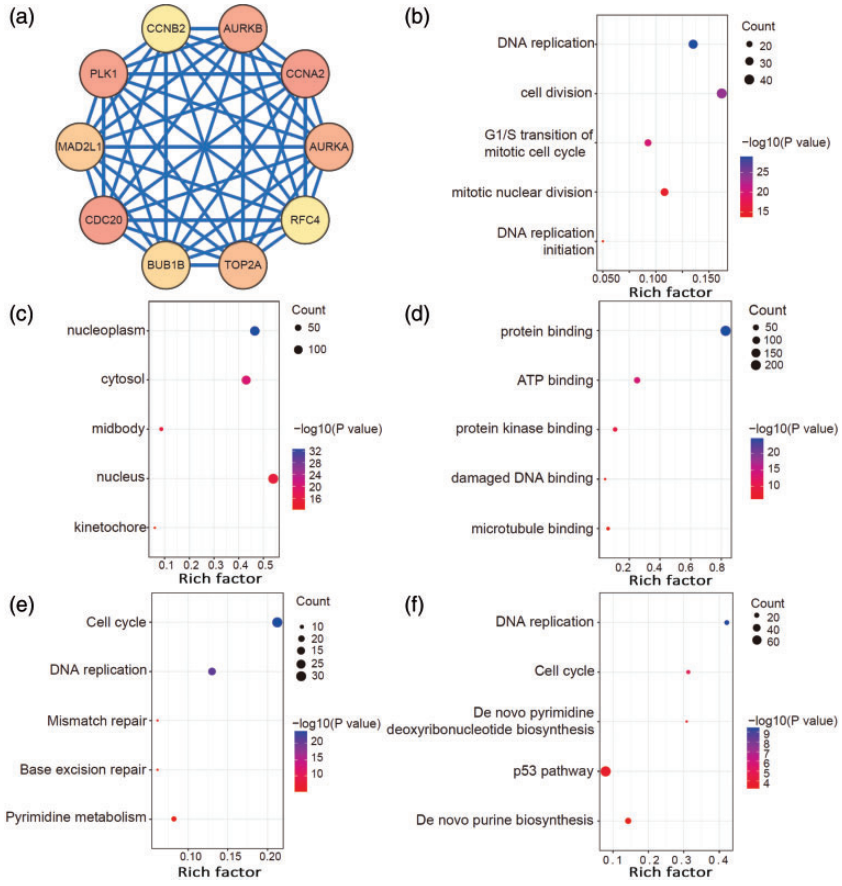


Figure 8. Enrichment analysis of 266 upregulated and hepatocellular carcinoma-associated co-expression genes, which were positively related with *CCNB1*. (a) Top

The relationship between key genes of *CCNB1* and clinicopathologic features

Based on the TCGA and GTEx databases, we found that *CDC20*, *CCNA2*, and *PLK1* were overexpressed in HCC tissues, whereas the expression of *FTCD* was downregulated in HCC tissues (Supplemental Figure 6). The group with a high expression of *CDC20*, *CCNA2*, and *PLK1* showed a worse OS rate than the low expression group ($P < 0.01$, Figure 11(a) to (c)). The survival analyses indicated that the lower *FTCD* expression predicted worse OS rates ($P = 0.009$, Figure 11(d)). The result of univariate Cox regression analysis implied that the whole clinical stages, T stage, M stage, *PLK1*, and *CDC20* expression levels might be five independent risk factors of HCC, while *FTCD* might be a protective factor of HCC ($P < 0.05$, Table 2). However, the result of multivariate Cox analysis was insignificant.

Discussion

The occurrence and progression of liver cancer is a complicated biological process. Due to recurrence, metastasis, and other factors, the prognosis of HCC patients has not been effectively improved. Therefore, to treat HCC patients, it is of great significance to search for potential specific molecular biological targets and elucidate the potential molecular mechanisms of HCC. In this study, *CCNB1* was upregulated in HCC in a large dataset containing 3065 HCC samples and 2693 normal samples. We also revealed that the serum *CCNB1* mRNA expression level was increased in HCC patients, suggesting that *CCNB1* may be a crucial indicator for the early screening of HCC patients.

A total of 266 upregulated HCC-associated co-expression genes, which were positively associated with *CCNB1*, and 67 downregulated HCC-associated co-expression genes, which were negatively related with *CCNB1*, were selected from the dataset. According to the KEGG and PANTHER results, the upregulated co-expression genes that positively associated with *CCNB1* were aggregated in the DNA replication and the cell cycle pathways. The downregulated co-expression genes, which were negatively associated with *CCNB1*, were mainly involved in some metabolic pathways. GSEA result implied that DNA repair was related to HCC. With the development of carcinogenesis in HCC, the DNA repair pathway is more enhanced in high-grade HCC compared to low-grade HCC.[31] Based on the survival analysis of *CCNB1*, its poor prognosis was related to its high expression. However, the results of the survival analysis of GSE76427 and GSE10143 were not ideal. It is likely that the sample sizes of those two datasets were not large enough to account for the different OS between two HCC groups (i.e., high *CCNB1* expression group versus low *CCNB1* expression group). Given the

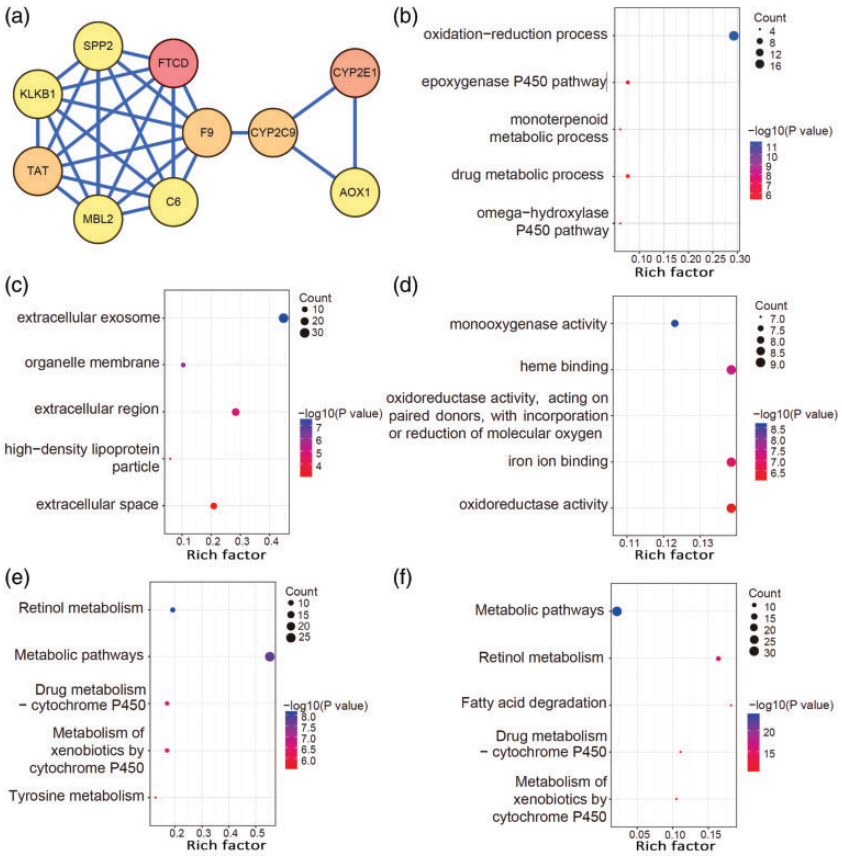
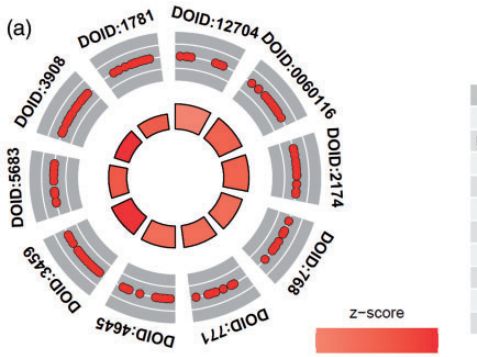
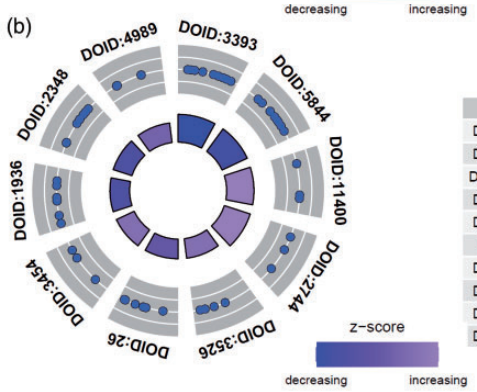


Figure 9. Enrichment analysis of 67 downregulated and hepatocellular carcinoma-associated co-expression genes, which were negatively related with *CCNB1*.



ID	Description
DOID:12704	ataxia telangiectasia
DOID:0060116	sensory system cancer
DOID:2174	ocular cancer
DOID:768	retinoblastoma
DOID:771	retinal cell cancer
DOID:4645	retinal cancer
DOID:3459	breast carcinoma
DOID:5683	hereditary breast ovarian cancer
DOID:3908	non−small cell lung carcinoma
DOID:1781	thyroid cancer



ID	Description
DOID:3393	coronary artery disease
DOID:5844	myocardial infarction
DOID:11400	pyelonephritis
DOID:2744	pyelitis
DOID:3526	cerebral infarction
DOID:26	pancreas disease
DOID:3454	brain infarction
DOID:1936	atherosclerosis
DOID:2348	arteriosclerotic cardiovascular disease
DOID:4989	pancreatitis

Figure 10. The disease ontology analyses of *CCNB1* in HCC. (a) Upregulated and HCC-associated co-expression genes, which were positively related with *CCNB1*. (b) Downregulated and HCC-associated co-expression genes, which were negatively related with *CCNB1*. HCC: hepatocellular carcinoma. (A color version of this figure is available in the online journal.)

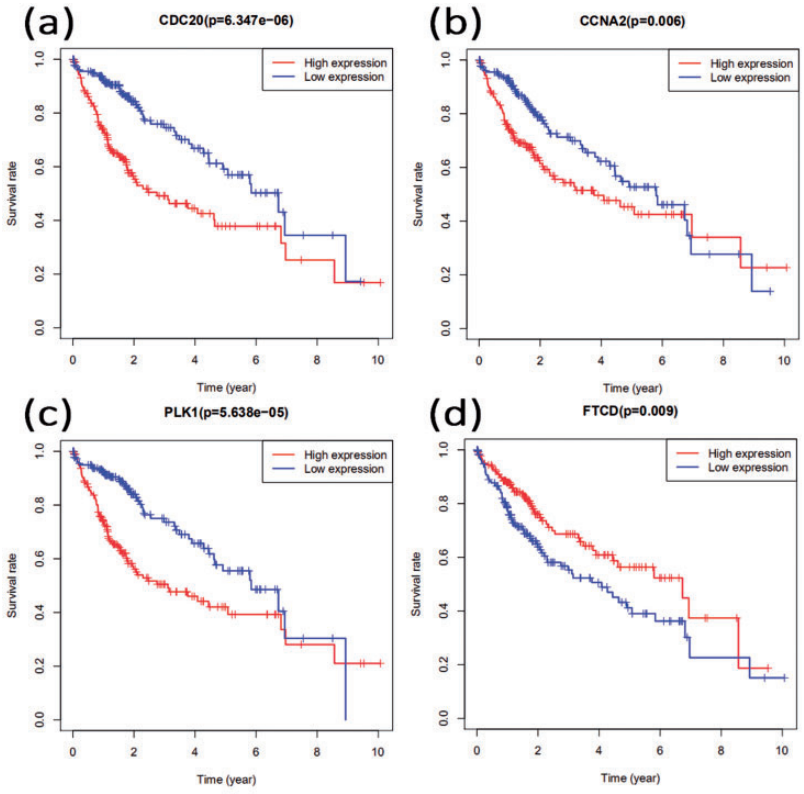


Figure 11. Survival analysis of hepatocellular carcinoma patients based on (a) *CDC20*, (b) *CCNA2*, (c) *PLK1*, and (d) *FTCD*. (A color version of this figure is available in the online journal.)

Table 2. Univariate and multiple Cox analysis of key genes of *CCNB1* in HCC.

	Univariate Cox analysis				Univariate Cox analysis			
Characteristics	**HR**	**HR.95L**	**HR.95H**	***P* value**	**HR**	**HR.95L**	**HR.95H**	***P* value**
Age	1.005006	0.986857	1.02349	0.591219				
Gender	0.780125	0.487182	1.249215	0.301298				
Grade	1.017173	0.745926	1.387055	0.914313				
Stage	1.86469	1.455816	2.388397	<0.0005	1.184675	0.491352	2.856313	0.705863
T	1.804388	1.43414	2.270223	<0.0005	1.386427	0.621025	3.095171	0.425236
M	3.849834	1.206809	12.28133	<0.0005	1.724869	0.467319	6.36647	0.413246
N	2.021833	0.493927	8.276134	0.327563				
PLK1	1.120903	1.069089	1.175227	<0.0005	1.051893	0.949687	1.165099	0.332001
CCNA2	1.016156	0.997696	1.034959	0.08665				
CDC20	1.023539	1.013634	1.033541	<0.0005	1.008825	0.988736	1.029322	0.391911
FTCD	0.994437	0.990238	0.998653	0.009761	0.998473	0.994212	1.002751	0.483545

HCC: hepatocellular carcinoma; HR: Hazard Ratio; 95L: 95% lower limit of confidence interval; 95H: 95% higher limit of confidence interval.

key promoter roles of *CCNB1* in mitosis and its overexpression in early HCC, we considered another reason for the insignificance of *CCNB1* prognosis to be that *CCNB1* might affect only early HCC and have no or an unknown effect in HCC development. Thus, *CCNB1* could not influence the OS time of patients with advanced HCC. These results provide clues on the mechanisms of *CCNB1* in HCC. The Kaplan-Meier analysis showed that *CDC20*, *CCNA2*, *PLK1*, and *FTCD* had effective prognostic value for HCC patients, and based on univariate Cox regression analyses, *CDC20*, *PLK1*, and *FTCD* were independent prognostic factors. The multivariate Cox regression analyses may have been unable to detect a statistical significance on such genes due to the small sizes of the HCC samples.

CDC20, a cell cycle regulator, is believed to perform an essential role in HCC formation.[32] It is greatly involved in the progression of HCC by managing *PHD3*.[33] Furthermore, the overexpression of *CDC20* in HCC has been shown to contribute to unsatisfactory OS and disease-free survival outcomes.[23]

Intriguingly, researchers found that viral insertion and structural rearrangements that activated *CCNA2* and *CCNE1* could be used to define a subtype of invasive

HCC.[34] A report showed that *CSN1* could enhance the proliferative and migrated abilities of MHCC-LM3 cells by upregulating *CCNA2* expression.[35] Moreover, *CCNA2* is active in the cytoplasm to activate *PLK1* during the S/G2 transition.[36]

PLK1, a crucial cell cycle regulator, is frequently upregulated in various cancers. *PLK1* has been shown to enhance carcinogenesis by causing chromosomal instability and eliminating cell cycle checkpoints.[37] *PLK1*-induced phosphorylation of *PARP10* at the T601 site can inhibit the single ADP ribosyltransferase activity of *PLK1*, sequentially promoting the transcriptional activity of NF-B and accelerating the occurrence of HCC.[38]

FTCD has been correlated with autoimmune hepatitis, and HCC patients with lower *FTCD* expression have a worse prognosis than those with higher ones.[39] During mitotic Golgi reassembly, p97 and p47 can influence membrane tethering by binding to *FTCD*.[40]

CCNB1 has been reported to have a close relation with miRNA or lncRNA in HCC. *CCNB1* expression was remarkably elevated in UPK1A-AS1-overexpressed HCC cells, which enhanced HCC deterioration by promoting cell cycle progression via interactions with EZH2.[26] In addition, *CCNB1* might be a binding target of miR-6884-3p.[22] LINC00346 regulates invasiveness, apoptosis, and the cell cycle in HCC cells by influencing *CDK1*/*CCNB1* expression.[21] Moreover, *CCNB1* was a direct target gene of miR-144 that could later inhibit HCC cell growth, migration, and invasion.[8]

The closed connection with miRNA or lncRNA implied that *CCNB1* may be a potential interventional target for HCC patients. There are several studies showing that *CCNB1* serves as a prospective interventional target in cancers.[28,41] Some studies demonstrated that the inhibition of *CCNB1* expression could enhance the apoptotic rate of HCC cells, suppress cell invasion and proliferation, and halt HCC cell lines in the G0/G1 phase.[8,21,28] Moreover, some reports have regarded *CCNB1* as an interacting gene in other potential targeted HCC therapies.[21,42,43] Taken together, this evidence suggests that *CCNB1* may serve as a possible interventional target for treating HCC. However, more in-depth experimental validations must be conducted in the future.

This study provides a novel and effective exploration method, and it suggests that *CCNB1* may be a promising target for preventing and treating HCC and is a promising prognostic gene biomarker for HCC. Despite the continuous improvements in current diagnosis and treatment methods, the mortality rate of HCC patients remains high. In this setting, more effective, sensitive, and specific diagnostic and prognostic indicators as well as targeted therapy methods are urgently needed. Currently, alpha fetoprotein (AFP) is regarded as the most commonly used biomarker for the early screening of HCC, but its value is limited: 30 percent of HCC cases do not show an increase in AFP. This study also addressed the relevance between *CCNB1* expression and clinicopathological parameters: *CCNB1* was associated with OS, pathological stages, T stage, races, and age. *CCNB1* predicted worse survival conditions of HCC patients, which could be supported by a previous research in which microRNA-144 was found to attenuate the proliferation and invasion abilities of HCC cells after *CCNB1* was knocked out.[8] In the present study, the differential genes predominantly participated in the cell cycle and the DNA replication pathways. As is known, the development of tumor partially attributes to the disturbance of the normal cell cycle. Many researchers have found that *CCNB1* expression is upregulated in tumor tissues. A study demonstrated that *CCNB1* mRNA was directly targeted by miR-718 in lung cancer and the decreased expression of *CCNB1* could inhibit the growth and migration of cancer cells.[13] Some researchers found that the expression of ectopic hnRNPR increased the expression of *CCNB1*, while the inhibition of hnRNPR decreased the expression of *CCNB1*. Silencing *CCNB1* partially reversed the effect of hnRNPR in GC cells.[12]

The merit of our study is that we found the expression of *CCNB1* to increase in HCC based on results from a large sample size and illuminated the relationship between *CCNB1* expression and clinicopathologic features. With the utilization of WGCNA, the interactional genes of *CCNB1* that we found could be more accurate. The "DNA replication" pathway acting in HCC was validated by both KEGG and PANTHER analyses, which have high reliability. Four key genes we selected (*CDC20*, *CCNA2*, *PLK1*, and *FTCD*) are involved in the progress of HCC. The expression of the four key genes differed significantly in the HCC tissues compared with the normal tissues. The four genes are also important prognostic factors in HCC. Hence, *CCNB1* is mainly involved in DNA replication to regulate the cell cycle process in HCC, and this may direct a novel avenue for the targeted therapy of HCC.

Nevertheless, this study has its limitations. First, in the IHC analysis, we only selected tissue samples from one hospital, and the sample size should be expanded. To overcome this issue, we integrated gene chips and RNA sequencing datasets to obtain more comprehensive and convincing results. Second, since this was a multi-center study, there was obvious heterogeneity in the study, which may have caused unavoidable differences between our results and those of various studies. However, several strategies were taken to minimize the adverse effects of high heterogeneity. We not only removed the inter-study batch effects, but we also utilized a random effect model when calculating the SMD result. Future studies should provide more evidence to support our findings using in vivo, in vitro, or even clinical trials.

In conclusion, the results strongly suggest that *CCNB1* may participate in the cell cycle pathway in HCC by regulating DNA replication, and the serum *CCNB1* may be a potential indictor for the screening of early-stage HCC.

AUTHORS' CONTRIBUTIONS

MHR, SNH, and XGZ designed the study; JDL and ZHZ performed the IHC; JDL, LYZ, YZH, JC, LYX, RXQ, and XLH conducted the data collection, GSEA, filtration of interactional genes, enrichment analyses, and statistical analyses; JDL, LYZ, YZH, JC, LYX, RXQ, XLH, and ZHZ drafted and edited the

manuscript; MHR, SNH, and XGZ revised the manuscript. All authors confirmed the current version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the National Natural Science Foundation of China (NSFC81860596), the Natural Science Foundation of Guangxi Province of China (2020GXNSFAA259022), the Promoting Project of Basic Capacity for Young and Middle-aged University Teachers in Guangxi (2019KY0154), the Promoting Project of Basic Capacity for Young and Middle-aged University Teachers in Guangxi (2020KY03038), the "Future Academic Star" Project of Guangxi Medical University (WLXSZX20088), and the Guangxi Medical University 2020 College Student Innovation and Entrepreneurship Training Program Project (202010598002).

ORCID iD

Xianguo Zhou https://orcid.org/0000-0001-6726-886X

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 practice guidance by the American Association for the Study of Liver Diseases. *Hepatology (Baltimore, Md)* 2018;**68**:723–50
2. 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**:394–424
3. Chu YW, Chien CH, Sung MI, Chen CW, Chen YT. dBMHCC: a comprehensive hepatocellular carcinoma (HCC) biomarker database provides a reliable prediction system for novel HCC phosphorylated biomarkers. *PloS One* 2020;**15**:e0234084
4. Chidambaranathan-Reghupaty S, Fisher PB, Sarkar D. Hepatocellular carcinoma (HCC): epidemiology, etiology and molecular classification. *Adv Cancer Res* 2021;**149**:1–61
5. Wang CH, Wey KC, Mo LR, Chang KK, Lin RC, Kuo JJ. Current trends and recent advances in diagnosis, therapy, and prevention of hepatocellular carcinoma. *Asian Pac J Cancer Prev* 2015;**16**:3595–604
6. Abdel-Rahman O, Elsayed Z. Yttrium-90 microsphere radioembolisation for unresectable hepatocellular carcinoma. *The Cochrane Database of Systematic Reviews* 2020;**1**:Cd011313
7. Vishnoi N, Yao J. Single-cell, single-mRNA analysis of Ccnb1 promoter regulation. *Sci Rep* 2017;**7**:2065
8. Gu J, Liu X, Li J, He Y. MicroRNA-144 inhibits cell proliferation, migration and invasion in human hepatocellular carcinoma by targeting CCNB1. *Cancer Cell Int* 2019;**19**:15
9. Fang L, Du WW, Awan FM, Dong J, Yang BB. The circular RNA circ-Ccnb1 dissociates Ccnb1/Cdk1 complex suppressing cell invasion and tumorigenesis. *Cancer Lett* 2019;**459**:216–26
10. Alfonso-Pérez T, Hayward D, Holder J, Gruneberg U, Barr FA. MAD1-dependent recruitment of CDK1-CCNB1 to kinetochores promotes spindle checkpoint signaling. *J Cell Biol* 2019;**218**:1108–17
11. Hayward D, Alfonso-Pérez T, Cundell MJ, Hopkins M, Holder J, Bancroft J, Hutter LH, Novak B, Barr FA, Gruneberg U. CDK1-CCNB1 creates a spindle checkpoint-permissive state by enabling MPS1 kinetochore localization. *J Cell Biol* 2019;**218**:1182–99
12. Chen EB, Qin X, Peng K, Li Q, Tang C, Wei YC, Yu S, Gan L, Liu TS. HnRNPR-CCNB1/CENPF axis contributes to gastric cancer proliferation and metastasis. *Aging (Albany NY)* 2019;**11**:7473–91
13. Wang S, Sun H, Zhan X, Wang Q. MicroRNA-718 serves a tumor-suppressive role in non-small cell lung cancer by directly targeting CCNB1. *International Journal of Molecular Medicine* 2020;**45**:33–44
14. Guo J, Gu Y, Ma X, Zhang L, Li H, Yan Z, Han Y, Xie L, Guo X. Identification of hub genes and pathways in adrenocortical carcinoma by integrated bioinformatic analysis. *J Cell Mol Med* 2020;**24**:4428–38
15. Li Q, Zhang L, Jiang J, Zhang Y, Wang X, Zhang Q, Wang Y, Liu C, Li F. CDK1 and CCNB1 as potential diagnostic markers of rhabdomyosarcoma: validation following bioinformatics analysis. *BMC Med Genomics* 2019;**12**:198
16. Li S, Liu N, Piao J, Meng F, Li Y. CCNB1 expedites the progression of cervical squamous cell carcinoma via the regulation by FOXM1. *Onco Targets Ther* 2020;**13**:12383–95
17. Li B, Zhu HB, Song GD, Cheng JH, Li CZ, Zhang YZ, Zhao P. Regulating the CCNB1 gene can affect cell proliferation and apoptosis in pituitary adenomas and activate epithelial-to-mesenchymal transition. *Oncol Lett* 2019;**18**:4651–8
18. Zhang H, Zhang X, Li X, Meng WB, Bai ZT, Rui SZ, Wang ZF, Zhou WC, Jin XD. Effect of CCNB1 silencing on cell cycle, senescence, and apoptosis through the p53 signaling pathway in pancreatic cancer. *J Cell Physiol* 2018;**234**:619–31
19. Wang F, Chen X, Yu X, Lin Q. Degradation of CCNB1 mediated by APC11 through UBA52 ubiquitination promotes cell cycle progression and proliferation of non-small cell lung cancer cells. *Am J Transl Res* 2019;**11**:7166–85
20. Zou Y, Ruan S, Jin L, Chen Z, Han H, Zhang Y, Jian Z, Lin Y, Shi N, Jin H. CDK1, CCNB1, and CCNB2 are prognostic biomarkers and correlated with immune infiltration in hepatocellular carcinoma. *Med Sci Monit* 2020;**26**:e925289
21. Jin J, Xu H, Li W, Xu X, Liu H, Wei F. LINC00346 acts as a competing endogenous RNA regulating development of hepatocellular carcinoma via modulating CDK1/CCNB1 axis. *Front Bioeng Biotechnol* 2020;**8**:54
22. Li J, Xia T, Cao J, He D, Chen Z, Liang B, Song J. RP11-295G20.2 facilitates hepatocellular carcinoma progression via the miR-6884-3p/CCNB1 pathway. *Aging (Albany NY)* 2020;**12**:14918–32
23. Zhuang L, Yang Z, Meng Z. Upregulation of BUB1B, CCNB1, CDC7, CDC20, and MCM3 in tumor tissues predicted worse overall survival and disease-free survival in hepatocellular carcinoma patients. *BioMed Research International* 2018;**2018**:7897346
24. Yue C, Ren Y, Ge H, Liang C, Xu Y, Li G, Wu J. Comprehensive analysis of potential prognostic genes for the construction of a competing endogenous RNA regulatory network in hepatocellular carcinoma. *Onco Targets Ther* 2019;**12**:561–76
25. Zhou Z, Li Y, Hao H, Wang Y, Zhou Z, Wang Z, Chu X. Screening hub genes as prognostic biomarkers of hepatocellular carcinoma by bioinformatics analysis. *Cell Transplant* 2019;**28**:76S–86S
26. Zhang DY, Sun QC, Zou XJ, Song Y, Li WW, Guo ZQ, Liu SS, Liu L, Wu DH. Long noncoding RNA UPK1A-AS1 indicates poor prognosis of hepatocellular carcinoma and promotes cell proliferation through interaction with EZH2. *J Exp Clin Cancer Res* 2020;**39**:229
27. Zhang Y, Lin Z, Lin X, Zhang X, Zhao Q, Sun Y. A gene module identification algorithm and its applications to identify gene modules and key genes of hepatocellular carcinoma. *Sci Rep* 2021;**11**:5517
28. Li M, Shang H, Wang T, Yang SQ, Li L. Huanglian decoction suppresses the growth of hepatocellular carcinoma cells by reducing CCNB1 expression. *World J Gastroenterol* 2021;**27**:939–58
29. Zhang H, Liu R, Sun L, Guo W, Ji X, Hu X. Comprehensive analysis of gene expression changes and validation in hepatocellular carcinoma. *Onco Targets Ther* 2021;**14**:1021–31

30. Li Z, Lin Y, Cheng B, Zhang Q, Cai Y. Identification and analysis of potential key genes associated with hepatocellular carcinoma based on integrated bioinformatics methods. *Front Genet* 2021;**12**:57123–1
31. Oshi M, Kim TH, Tokumaru Y, Yan L, Matsuyama R, Endo I, Cherkassky L, Takabe K. Enhanced DNA repair pathway is associated with cell proliferation and worse survival in hepatocellular carcinoma (HCC). *Cancers (Basel)* 2021;**13**:323
32. Li J, Gao JZ, Du JL, Huang ZX, Wei LX. Increased CDC20 expression is associated with development and progression of hepatocellular carcinoma. *Int J Oncol* 2014;**45**:1547–55
33. Shi M, Dai WQ, Jia RR, Zhang QH, Wei J, Wang YG, Xiang SH, Liu B, Xu L. APC(CDC20)-mediated degradation of PHD3 stabilizes HIF-1a and promotes tumorigenesis in hepatocellular carcinoma. *Cancer Lett* 2021;**496**:144–55
34. Bayard Q, Meunier L, Peneau C, Renault V, Shinde J, Nault JC, Mami I, Couchy G, Amaddeo G, Tubacher E, Bacq D, Meyer V, La Bella T, Debaillon-Vesque A, Bioulac-Sage P, Seror O, Blanc JF, Calderaro J, Deleuze JF, Imbeaud S, Zucman-Rossi J, Letouzé E. Cyclin A2/E1 activation defines a hepatocellular carcinoma subclass with a rearrangement signature of replication stress. *Nat Commun* 2018;**9**:5235
35. Fu H, Zhang Y, Chen Y, Chen J, Chen P. CSN1 facilitates proliferation and migration of hepatocellular carcinoma cells by upregulating cyclin A2 expression. *Mol Med Rep* 2021;**23**:1
36. Silva Cascales H, Burdova K, Middleton A, Kuzin V, Müllers E, Stoy H, Baranello L, Macurek L, Lindqvist A. Cyclin A2 localises in the cytoplasm at the S/G2 transition to activate PLK1. *Life Sci Alliance* 2021;**4**: e202000980
37. Gheghiani L, Wang L, Zhang Y, Moore XTR, Zhang J, Smith SC, Tian Y, Wang L, Turner K, Jackson-Cook CK, Mukhopadhyay ND, Fu Z. PLK1 induces chromosomal instability and overrides cell cycle checkpoints to drive tumorigenesis. *Cancer Res* 2021;**81**:1293–307
38. Tian L, Yao K, Liu K, Han B, Dong H, Zhao W, Jiang W, Qiu F, Qu L, Wu Z, Zhou B, Zhong M, Zhao J, Qiu X, Zhong L, Guo X, Shi T, Hong X, Lu S. PLK1/NF-κB feedforward circuit antagonizes the Mono-ADP-ribosyltransferase activity of PARP10 and facilitates HCC progression. *Oncogene* 2020;**39**:3145–62
39. Zhang S, Liu Z, Wu D, Chen L, Xie L. Single-cell RNA-Seq analysis reveals microenvironmental infiltration of plasma cells and hepatocytic prognostic markers in HCC with cirrhosis. *Front Oncol* 2020;**10**:596318
40. Kaneko Y, Shimoda K, Ayala R, Goto Y, Panico S, Zhang X, Kondo H. p97 and p47 function in membrane tethering in cooperation with FTCD during mitotic Golgi reassembly. *EMBO J* 2021;**40**:e105853
41. Zeng H, Ji J, Song X, Huang Y, Li H, Huang J, Ma X. Stemness related genes revealed by network analysis associated with tumor immune microenvironment and the clinical outcome in lung adenocarcinoma. *Front Genet* 2020;**11**:549213
42. Lin J, Hou Y, Huang S, Wang Z, Sun C, Wang Z, He X, Tam NL, Wu C, Wu L. Exportin-T promotes tumor proliferation and invasion in hepatocellular carcinoma. *Mol Carcinog* 2019;**58**:293–304
43. Zhang Q, Su R, Shan C, Gao C, Wu P. Non-SMC condensin I complex, subunit G (NCAPG) is a novel mitotic gene required for hepatocellular cancer cell proliferation and migration. *Oncol Res* 2018;**26**:269–76

(Received April 27, 2021, Accepted September 7, 2021)