MEDICAL SCIENCE MONITOR

DATABASE ANALYSIS

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e925289
DOI: 10.12659/MSM.925289

Received: 2020.04.21
Accepted: 2020.06.03
Available online: 2020.06.30
Published: 2020.08.31

CDK1, *CCNB1*, and *CCNB2* are Prognostic Biomarkers and Correlated with Immune Infiltration in Hepatocellular Carcinoma

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: This study was funded by the Natural Science Foundation of Guangdong (c17140500000053) and Natural Science Foundation of China (81972792)

Background: Orderly G2/M transition in the cell cycle is controlled by the cyclin-dependent kinase 1/cyclin B (CDK1/CCNB) complex. We aimed to comprehensively investigate the roles of CDK1, CCNB1, and CCNB2 via multi-omics analysis and their relationships with immune infiltration in hepatocellular carcinoma (HCC).

Material/Methods: The transcriptional data and the epigenetic and genetic alterations of *CDK1*, *CCNB1*, and *CCNB2*, as well as their impacts on prognosis in HCC patients, were identified using multiple databases. The correlations between expression of these genes and immune infiltration in HCC were then explored using the TIMER database.

Results: Overall, mRNA expression of *CDK1*, *CCNB1*, and *CCNB2* was up-regulated in various tumor tissues including HCC. Higher expression of these genes was associated with poorer prognosis in HCC patients. Lower promoter methylation of these genes might cause higher expression levels in tumor tissues of HCC. Genetic alterations and several methylated-CpG sites in these genes were significantly associated with survival. Notably, expression levels of *CDK1*, *CCNB1*, and *CCNB2* were positively correlated with infiltrating levels of $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells in HCC. In addition, significant correlations between the expression of these genes and various immune markers in HCC, such as PD-1, PDL-1, and CTLA-4, were also observed.

Conclusions: *CDK1*, *CCNB1*, and *CCNB2* are potential prognostic biomarkers and associated with immune cell infiltration in HCC. The genes may be utilized to predict the reaction of immunotherapy. Combining inhibitors of these genes with immunotherapy may improve the survival time of HCC patients.

MeSH Keywords: **Biological Markers • Carcinoma, Hepatocellular • CDC2 Protein Kinase • Tumor Escape**

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/925289

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Background

Liver cancer is one of the most common types of cancer and the third leading cause of cancer death worldwide. Hepatocellular carcinoma (HCC), which composes 75% to 85% of primary liver cancer cases, is the major pathological type [1]. HCC is often secondary to chronic liver cirrhosis, and various risk factors have been identified, such as chronic hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, autoimmune hepatitis, alcohol abuse, and several metabolic diseases [2,3]. Current therapeutic strategies for HCC, such as surgical resection, liver transplantation, and radiofrequency ablation, have been widely used and improved, but the long-term survival rate of HCC is still unsatisfactory due to the high percentage of cases that are at an advanced stage at diagnosis and the high recurrence rate after surgical resection [4,5]. Only 2 first-line therapeutic drugs, the oral multikinase inhibitors sorafenib and lenvatinib, have shown clinical benefits in patients with advanced HCC [6,7]. Therefore, in order to improve survival of HCC patients, it is critical to evaluate carcinogenesis mechanisms and explore potential drug targets.

Cyclin B1 (CCNB1) and cyclin B2 (CCNB2) can form complexes with CDK1 (cyclin-dependent kinase 1) to regulate the G2/M phases of the mammalian cell cycle, which plays an important role in the initiation of mitosis [8]. Dysregulation of CDKs is associated with the uncontrolled cell proliferation in human cancers. In addition, higher expression of cyclin B is associated with poorer outcomes for gastric, esophageal, breast, and non-small-cell lung cancer [9]. As shown in recent research, the knockdown of *CDK1*, *CCNB1*, or *CCNB2* could inhibit cell proliferation, invasion, and migration in HCC cell lines [10–12]. Recently, several bioinformatics studies have identified *CDK1*, *CCNB1*, and *CCNB2* as hub genes associated with HCC. These findings indicate that these genes play a critical role in malignancy and poorer outcomes of HCC [13–16]. Nevertheless, the precise functions of these genes and their relationship within the immune microenvironment in HCC need to be elucidated.

In the current study, we used several public databases to comprehensively analyze the expression levels of *CDK1*, *CCNB1*, and *CCNB2* in several types of carcinomas. In addition, we evaluated their correlations with prognosis in HCC patients. We also performed a comprehensive analysis of DNA methylation and genetic alterations of these genes to identify epigenetic and genetic changes. Finally, we investigated the correlations between the mRNA levels of these genes and the levels of immune infiltration cells and several immune markers in HCC.

Material and methods

The description of all databases

All bioinformatics analyses were based on sequencing or microarray data obtained from tumor tissues and corresponding normal tissues. The data in this study involved samples from The Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC), and the Gene Expression Omnibus (GEO). Figure 1E shows the number of samples from different datasets. Samples with missing data on survival information and clinical information such as tumor pathological grade were excluded from survival analysis and corresponding subgroup analyses. Using various online database analysis tools, we explored the transcription of *CDK1*, *CCNB1*, and *CCNB2* in various tumors and the prognostic values of these genes in HCC. In addition, we identified the promoter methylation and genetic alterations of *CDK1*, *CCNB1*, and *CCNB2* and their impacts of prognosis in HCC patients. Finally, we explored the correlations between expression of these genes and immune infiltration in HCC. The specific online databases and the statistical methods used are as follows.

ONCOMINE database analysis

ONCOMINE (*https://www.oncomine.org/resource/main.html*) is an online tumor data analysis platform [17]. By using the Oncomine database 4.5, we identified the mRNA expression levels of the *CDK1*, *CCNB1*, and *CCNB2* genes in various cancers.

HCCDB database analysis

HCCDB (*http://lifeome.net/database/hccdb/home.html*) is a novel database containing 3917 samples, which were obtained from 15 public datasets of gene expression in HCC [18]. The database includes 2 RNA-Seq datasets (TCGA and ICGC) and 13 microarray datasets from GEO. In our study, HCCDB was used to provide the visualization for differential expression analysis of *CDK1*, *CCNB1*, and *CCNB2* in HCC from several datasets.

UALCAN database analysis

UALCAN (*http://ualcan.path.uab.edu*) is a publicly available interactive online portal that is used to analyze the relative expression and methylation of genes in normal and tumor tissues from TCGA [19]. Subgroups based on pathological grades and individual cancer stages can be further investigated in depth. Histological grades of HCC were defined as follows: well-differentiated (I), moderately differentiated (II), poorly differentiated (III), and undifferentiated (IV). Individual cancer stages were based on the American Joint Committee on Cancer (AJCC) stage.

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GEPIA dataset analysis

GEPIA (*http://gepia.cancer-pku.cn/*) is an online database that contains expression data based on 9736 tumors and 8587 normal tissues from GTEx and TCGA [20]. We used this dataset to analyze the correlations between expression of *CDK1*, *CCNB1*, and *CCNB2* and survival, including overall survival (OS) and disease-free survival (DFS). Furthermore, gene expression correlation analyses of those 3 genes were performed based on the TCGA expression dataset.

LinkedOmics database analysis

The LinkedOmics database (*http://www.linkedomics.org/login.php*) is an easy-to-use online tool for analyzing 32 TCGA cancer-associated multidimensional datasets [21]. Using this database, we analyzed genes that were positively correlated with *CDK1*, *CCNB1*, and *CCNB2*. Heat maps were applied to show the top 50 positively correlated genes.

GO analysis and KEGG analysis

Based on the co-occurrence genes from the LinkedOmics database, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed, using the online tool DAVID (*https://david.ncifcrf.gov/*). The GO analysis contained the gene annotation results for biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The results of GO analysis and KEGG analysis were further visualized using the ggplot2 package of R software.

MethSurv database analysis

MethSurv (*https://biit.cs.ut.ee/methsurv*) is a visualization web tool used to investigate methylation biomarkers associated with survival in different types of cancer [22]. By analyzing the TCGA methylation data from the MethSurv, we obtained the prognostic value of each DNA methylation CpG site for *CDK1*, *CCNB1*, and *CCNB2* in HCC.

c-BioPortal database analysis

The c-BioPortal (*https://www.cbioportal.org*) is an online database for analyzing multidimensional cancer genomics data [23]. We used the dataset that included 360 cases of liver hepatocellular carcinoma (LIHC) from TCGA. Using the c-BioPortal, we identified mutations, copy-number alterations (CNAs), and mRNA expression of *CDK1*, *CCNB1*, and *CCNB2* in HCC. Furthermore, we assessed the relationships between the degree of methylation of the 3 genes and their mRNA expression levels.

TIMER database analysis

TIMER (*https://cistrome.shinyapps.io/timer/*) is a useful web server for analyzing the immune infiltration in different types of cancer from the TCGA database [24]. By applying a deconvolution method, TIMER determines the infiltration levels of immune cells from gene expression profiles in tumor tissues. We analyzed the correlation of *CDK1*, *CCNB1*, and *CCNB2* expression in HCC with the infiltrating levels of immune cells, respectively, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, dendritic cells (DCs), and neutrophils. In addition, we investigated the genetic markers for immune cell infiltration in tumors, including tumor-associated macrophages (TAMs), M1 macrophages, M2 macrophages, DCs, neutrophils, T-helper 1 (Th1) cells, T-helper (Th2) cells, regulatory T cells (Tregs), natural killer (NK) cells, and B cell and T cell exhaustion.

Statistical analysis

Expression levels of *CDK1*, *CCNB1*, and *CCNB2* from the HCCDB and UALCAN databases were analyzed using the Student *t* test. The Kaplan-Meier survival curves with log-rank text were plotted to compare the survival of patients in different groups. Then the log-rank *P* values and hazard ratios (HRs) were calculated. The correlation between *CDK1*, *CCNB1*, and *CCNB2* and other gene markers were evaluated by Spearman correlation analysis. The strengths of the correlations were defined by the absolute values using the following guide: weak ($0.00 < r \leq 0.30$), moderate ($0.30 < r \leq 0.60$), strong ($0.60 < r \leq 0.80$), very strong ($0.80 < r \leq 1.00$). A 2-tailed *P* value <0.05 was considered statistically significant.

Results

Aberrant expression of *CDK1*, *CCNB1*, and *CCNB2* in pan-cancer and in-depth verification in HCC

Using the ONCOMINE database, we analyzed the mRNA expression levels for *CDK1*, *CCNB1*, and *CCNB2* in different types of cancer. The results showed that compared with normal tissues, the levels were significantly higher in several types of tumor tissues, such as liver cancer, kidney cancer, breast cancer, colorectal cancer, gastric cancer, esophageal cancer, and lung cancer, among others (Figure 1A). We further used the TIMER database to analyze the RNA-seq data from the TCGA database. The result also confirmed that mRNA expression levels of *CDK1*, *CCNB1*, and *CCNB2* were significantly higher in LIHC, kidney renal clear cell carcinoma, esophageal carcinoma, colon adenocarcinoma, lung adenocarcinoma, stomach adenocarcinoma, and so forth than in the adjacent normal tissues (Figure 1B–1D). Using the HCCDB databases, we evaluated the expression levels reported in HCC studies from GEO, TCGA, and ICGC. Among these studies, 11 datasets indicated that mRNA

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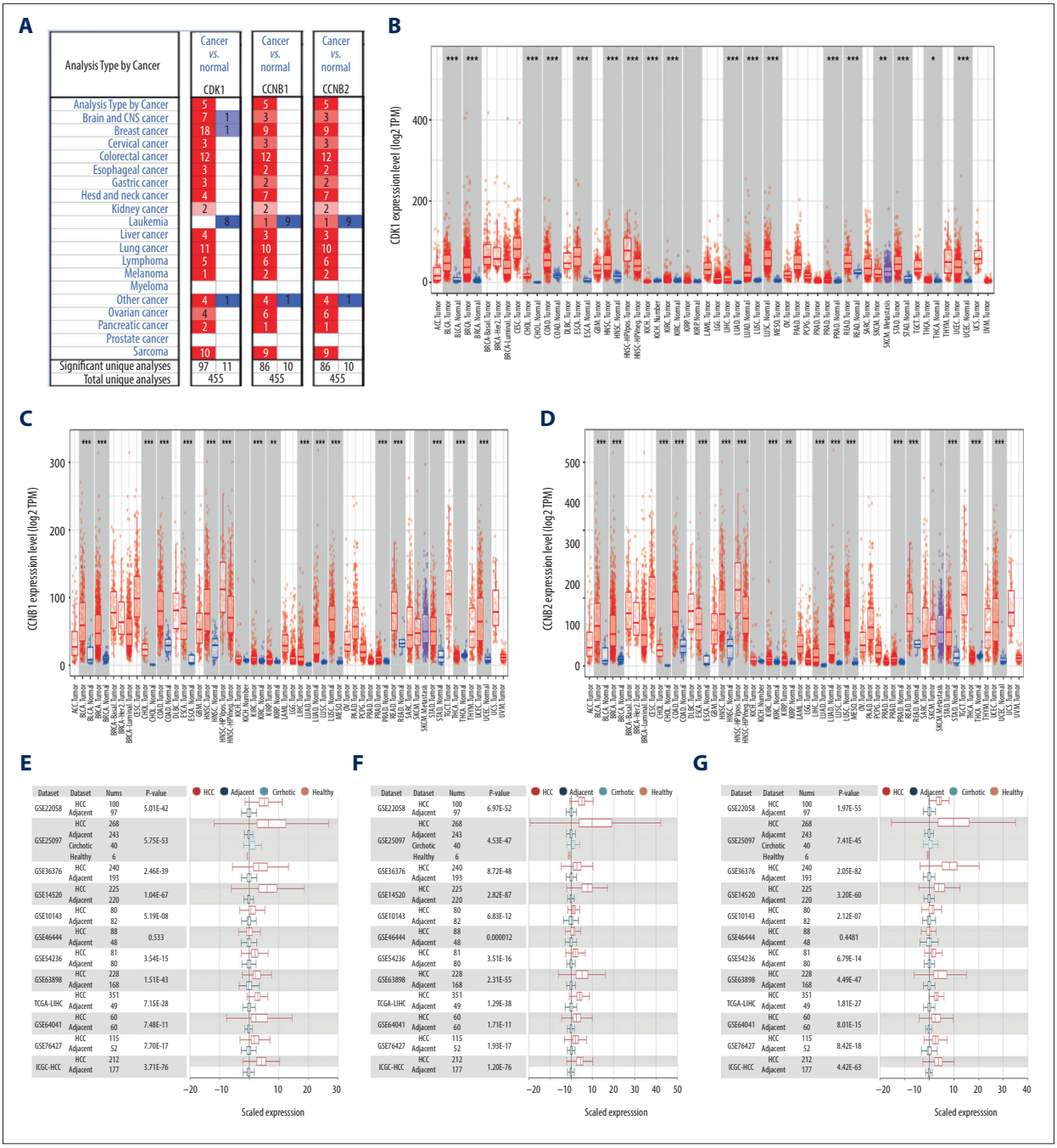

Figure 1. (**A**) Expression levels of *CDK1*, *CCNB1*, and *CCNB2* for different types of tumors in ONCOMINE. (**B–D**) The levels of *CDK1*, *CCNB1*, and *CCNB2* expression in different types of tumors from the TCGA database in TIMER. * *P*<0.05, ** *P*<0.01, *** *P*<0.001. (**E–G**) Charts and plots showing the expression of *CDK1*, *CCNB1*, and *CCNB2* in HCC tissues and the adjacent normal tissues in HCCDB.

expression levels of *CDK1* and *CCNB2* were significantly higher in the HCC tissues than in the adjacent normal tissues. In addition, 12 datasets revealed that mRNA expression levels of *CCNB1* in the HCC tissues were significantly higher than in the adjacent normal tissues (Figure 1E–1G).

Transcription in subgroups of *CDK1*, *CCNB1*, and *CCNB2* based on different pathological stages and grades in patients with HCC

We next explored the different expression levels of the 3 genes in HCC, stratified according to the AJCC stage and pathological

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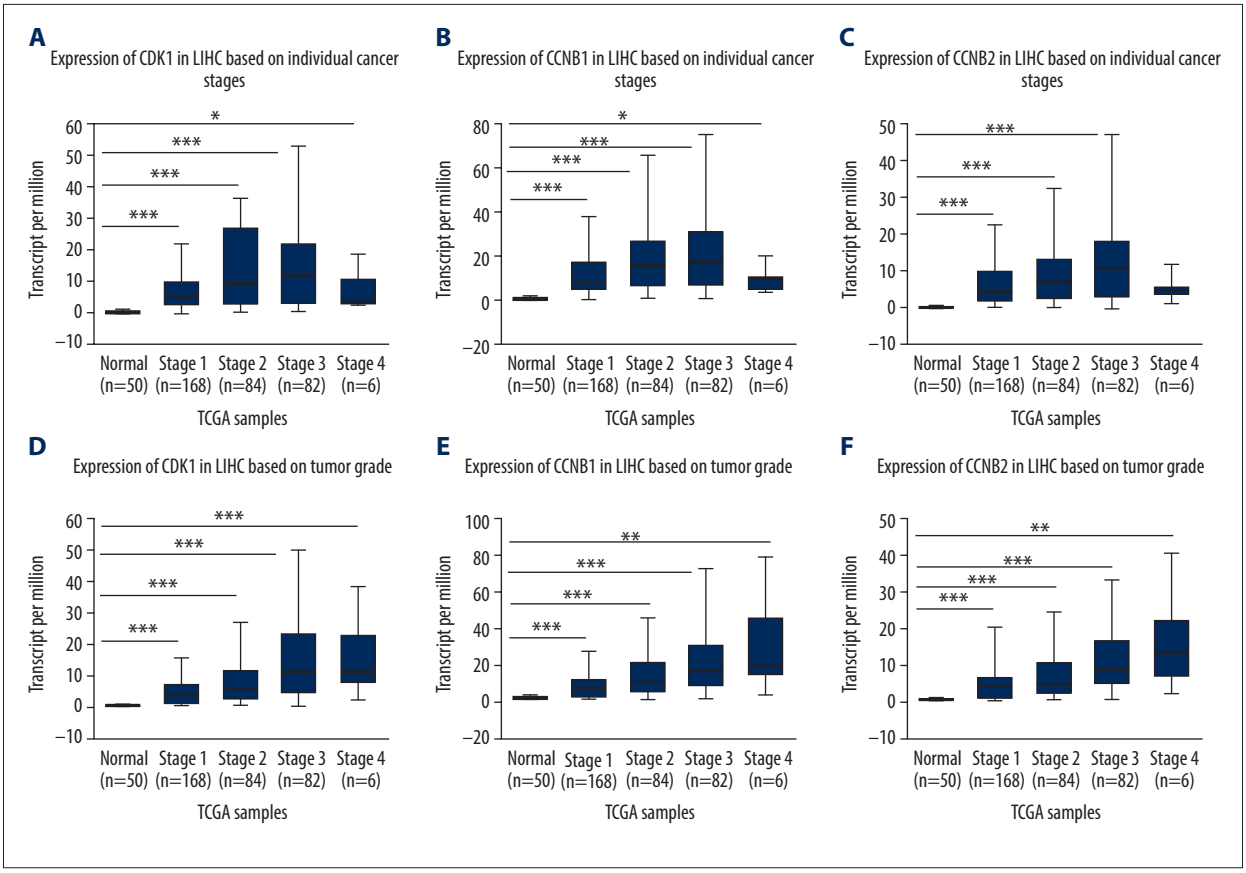

Figure 2. (**A–C**) Expression levels of *CDK1*, *CCNB1*, and *CCNB2* in normal tissues or in HCC tissues at different stages. (**D–F**) Expression levels of *CDK1*, *CCNB1*, and *CCNB2* in normal tissues or in HCC tissues with different grades. * *P*<0.05; ** *P*<0.01; *** *P*<0.001.

grade. The results demonstrated that the expression of the 3 genes was higher in HCC tissues than in normal tissues based on different pathological grades and individual cancer stages. Therefore, expression levels of *CDK1*, *CCNB1*, and *CCNB2* may serve as potential diagnostic markers in patients with HCC. Furthermore, overexpression of these genes was also related to advanced pathological grades and individual cancer stages, except stage IV (with only 6 cases) (Figure 2A–2F). Therefore, the results indicated that expression of these genes plays an important role in the tumorigenesis and progression of HCC.

The prognostic values and relationships of *CDK1*, *CCNB1*, and *CCNB2* in HCC patients

Using the data for LIHC from TCGA in the GEPIA database, we assessed the correlation between differential expression of *CDK1*, *CCNB1*, and *CCNB2* and clinical outcomes. Based on results from 364 HCC patients, poorer prognosis in terms of DFS and OS (*P*<0.05), except OS of *CCNB2* (*P*=0.052), were associated with higher mRNA expression levels for *CDK1*, *CCNB1*, and *CCNB2* (Figure 3A–3F). We further investigated the prognostic value of these genes in the ICGC dataset by using the HCCDB database. The results further confirmed that increased expression of *CDK1*, *CCNB1*, and *CCNB2* was significantly associated with poor OS in HCC (*P*<0.05) (Figure 3G–3I). Hence, higher expression levels of *CDK1*, *CCNB1*, and *CCNB2* are indicators of poor survival for patients with HCC.

Analysis of co-expressed genes in HCC

We next analyzed the correlation between expression levels of *CDK1*, *CCNB1*, and *CCNB2* in HCC using the GEPIA database. The results indicated that these 3 genes were significantly positively correlated: *CDK1* and *CCNB1* (*r*=0.91, *P*<0.001), *CDK1* and *CCNB2* (*r*=0.92, *P*<0.001), and *CCNB1* and *CCNB2* (*r*=0.93, *P*<0.001) (Figure 4A–4C). Genes positively co-expressed with *CDK1*, *CCNB1*, and *CCNB2* were analyzed in the LIHC cohort from TCGA by using the LinkedOmics database. The top 50 genes that were significantly correlated with these 3 genes are shown in the heat maps (Figure 4D–4F). This result further confirmed that *CDK1*, *CCNB1*, and *CCNB2* were strongly positively co-expressed in HCC. We combined genes that were co-expressed with these 3 genes, deleted duplicate values, and identified 62 co-expressed genes, which were then extracted for GO and KEGG analysis. We showed the top 5 GO of BPs, CCs, and MFs based on the minimum values of false discovery

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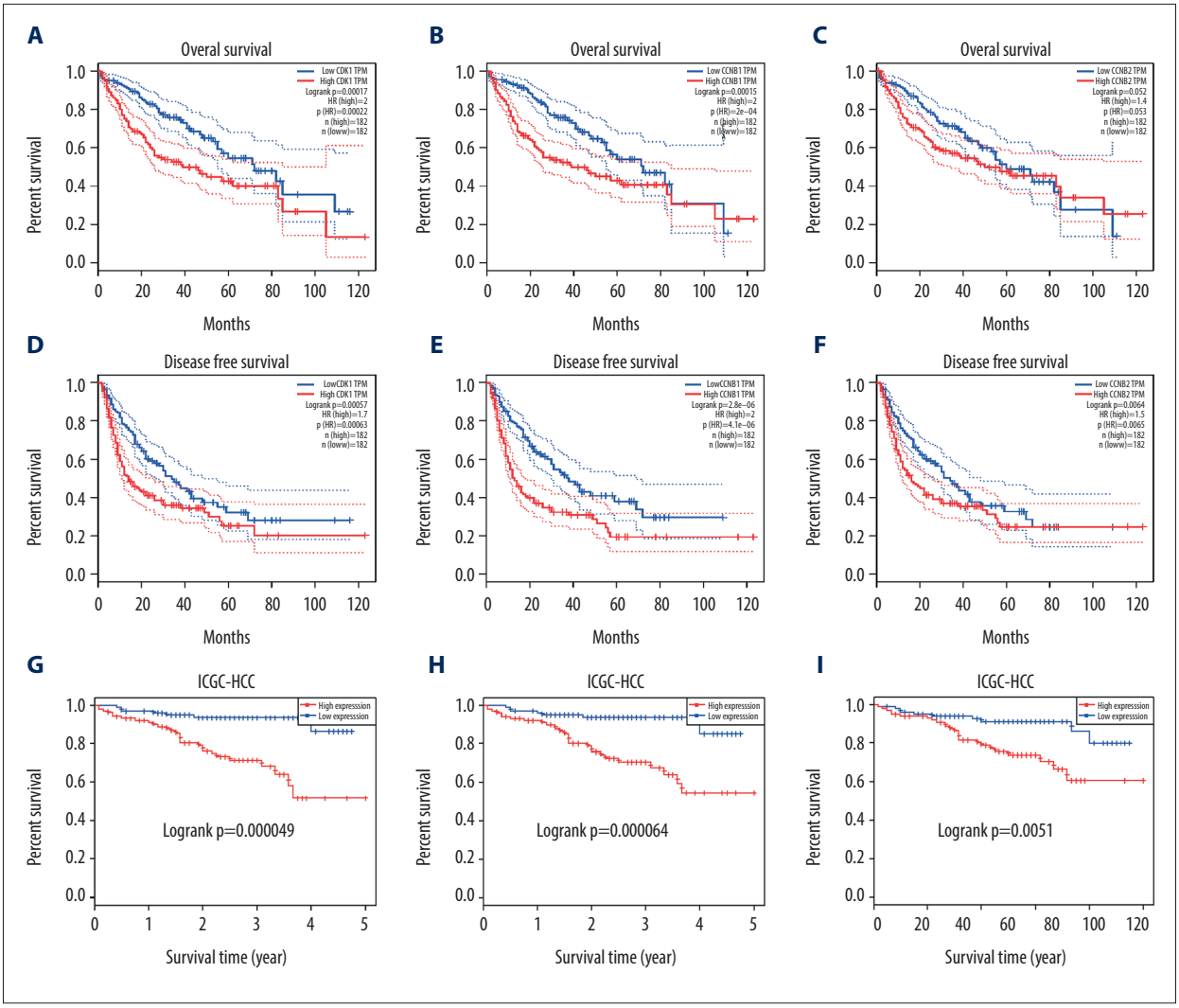

Figure 3. (**A–C**) Overall survival (OS) and differential *CDK1*, *CCNB1*, and *CCNB2* expression in the TCGA-LIHC cohort. (**D–F**) Disease-free survival (DFS) and differential *CDK1*, *CCNB1*, and *CCNB2* expression in the TCGA-LIHC cohort. (**G–I**) Overall survival (OS) and differential *CDK1*, *CCNB1*, and *CCNB2* expression in the ICGC-HCC cohort.

rate (FDR) and maximum counts of GO. The results indicated that the most significant BPs, CCs, and MFs were cell cycle, non-membrane-bound organelles, and ATP binding, respectively (Figure 4G). KEGG analysis revealed the key pathways of these genes: cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, and the p53 signaling pathway (Figure 4H).

Promoter methylation of *CDK1*, *CCNB1*, and *CCNB2* in HCC

Moreover, by analyzing the LIHC samples from TCGA in the UALCAN database, we evaluated the levels of *CDK1*, *CCNB1*, and *CCNB2* promoter methylation in HCC and normal tissues. The results suggested that the levels of methylation were lower in HCC than in normal tissues ($P<0.05$) (Figure 5A–5C). In addition, we assessed the relationships between the degree of methylation of these 3 genes and the mRNA levels using the c-BioPortal database. The results indicated significant negative correlations between the methylation levels and the mRNA expression levels of these genes in HCC ($P<0.05$) (Figure 5D–5F). Thus, the results suggest that lower levels of *CDK1*, *CCNB1*, and *CCNB2* promoter methylation might cause higher expression levels of these genes in HCC. In addition, the prognostic values associated with diverse CpG sites were also analyzed via the MethSurv database. Ultimately, the results showed that 7 CpG sites in *CDK1*, 6 CpG sites in *CCNB1*, and 2 CpG sites in *CCNB2* were significantly associated with the prognosis of patients with HCC (Table 1).

Genomic alterations of *CDK1*, *CCNB1*, and *CCNB2* in HCC

The frequency and types of genetic alterations in *CDK1*, *CCNB1*, and *CCNB2* in patients with HCC were analyzed by using the

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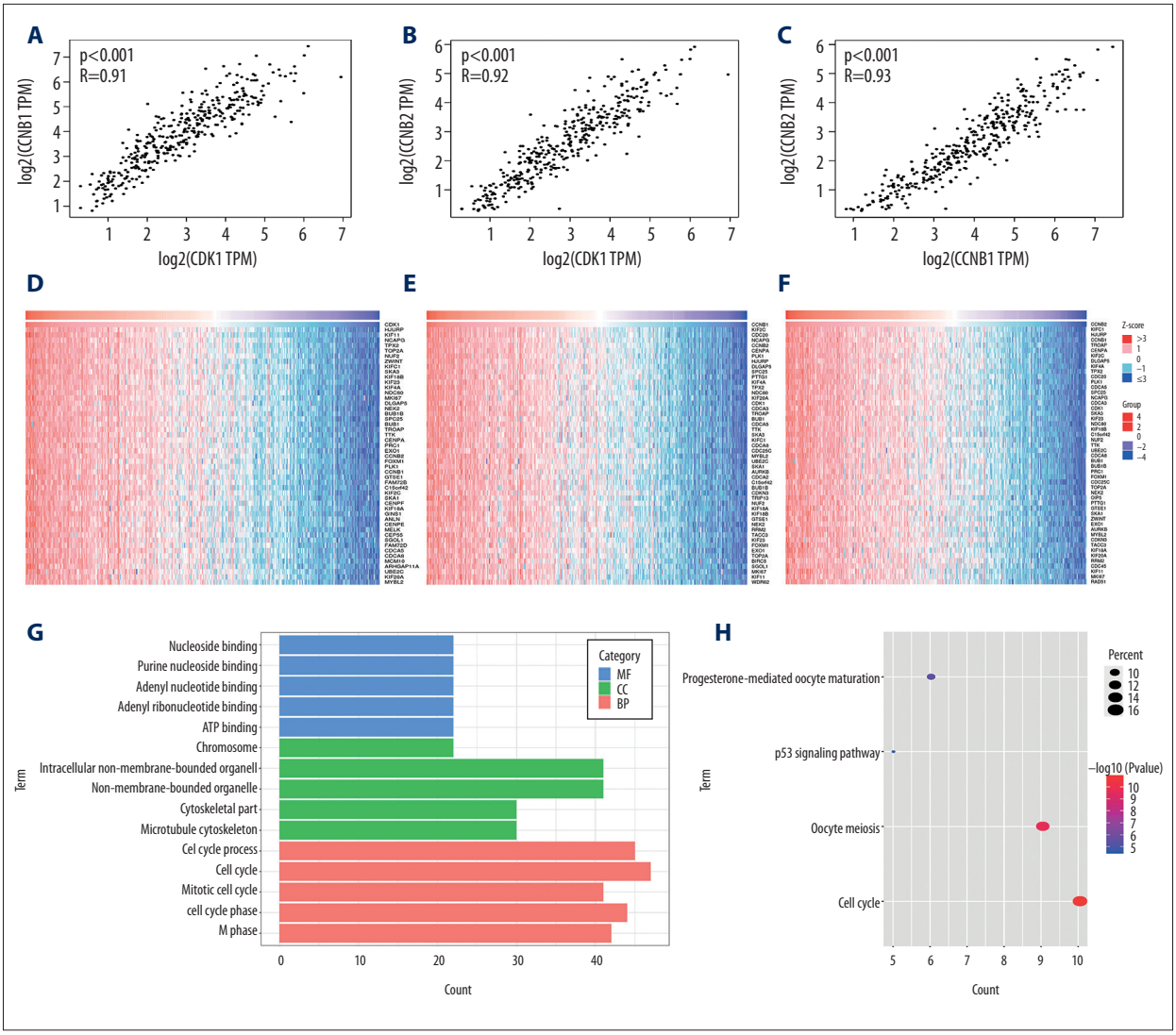

Figure 4. (**A**) The correlation between *CDK1* and *CCNB1* in HCC. (**B**) The correlation between *CDK1* and *CCNB2* in HCC. (**C**) The correlation between *CCNB1* and *CCNB2* in HCC. (**D–F**) Top 50 genes positively correlated with *CDK1*, *CCNB1*, and *CCNB2* in LIHC from the TCGA database. (**G, H**) Significantly enriched GO annotations and KEGG pathways of *CDK1*, *CCNB1*, and *CCNB2* in the LIHC cohort. GO – Gene Ontology; CC – cellular component; BP – biological process; MF – molecular function; KEGG – Kyoto Encyclopedia of Genes and Genomes.

cBioPortal database. A total of 360 LIHC cases from TCGA were explored. *CDK1*, *CCNB1*, and *CCNB2* were altered in 6%, 9%, and 9% of LIHC cases, respectively. The most frequent alteration type in these samples was mRNA upregulation (10.8%) (Figure 6A). Furthermore, the Kaplan-Meier curves for the altered and unaltered groups of these genes demonstrated significant differences in OS (P<0.001) and DFS (P=0.0368) in patients with HCC (Figure 6B, 6C). Therefore, genomic alterations of these genes were considered as poor prognosis factors in HCC patients.

The correlation between *CDK1*, *CCNB1*, and *CCNB2* expression levels and infiltration levels of immune cells in tumor microenvironment of HCC

By using the TIMER database, we explored whether the mRNA expression levels of *CDK1*, *CCNB1*, and *CCNB2* were correlated with infiltrating immune cells in HCC. The results demonstrated that overexpression of each of these genes was significantly associated with higher immune cell infiltration levels. Specifically, the *CDK1* expression level was positively correlated with infiltration levels of $CD8^+$ T cells (r=0.316, P=2.38e-09), $CD4^+$ T cells (r=0.332, P=2.72e-10), B cells (r=0.469, P=2.97e-20), macrophages (r=0.449, P=2.60e-18), neutrophils (r=0.344,

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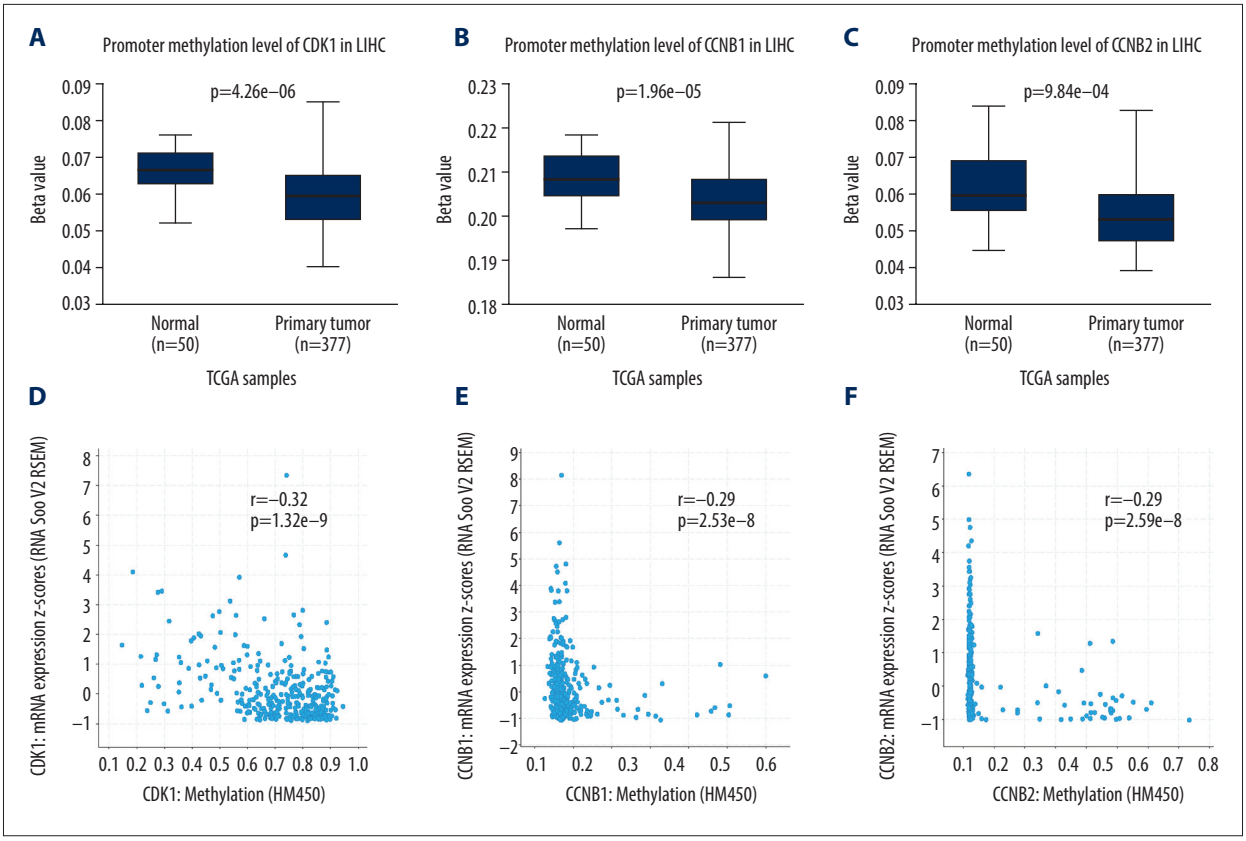

Figure 5. (**A–C**) Boxplots showing relative promoter methylation levels of *CDK1*, *CCNB1*, and *CCNB2* in normal and LIHC samples. (**D–F**) The correlation between expression levels of *CDK1*, *CCNB1*, and *CCNB2* and their methylation levels in LIHC samples.

P=4.98e-11), and DCs (*r*=0.442, *P* =1.17e-17) (Figure 7A). The *CCNB1* expression level was positively correlated with infiltration levels of CD8$^+$ T cells (*r*=0.303, *P*=1.12e-08), CD4$^+$ T cells (*r*=0.283, *P*=9.33e-08), B cells (*r*=0.469, *P* =2.9e-20), macrophages (*r*=0.42, *P*=5.42e-16), neutrophils (*r*=0.342, *P*=6.81e-11), and DCs (*r*=0.429, *P* =1.15e-16) (Figure 7B). Similarly, the *CCNB2* expression level was also positively correlated with infiltration levels of CD8$^+$ T cells (*r*=0.35, *P*=2.78e-11), CD4$^+$ T cells (*r*=0.305, *P*=7.86e-09), B cells (*r*=0.487, *P*=6.57e-22), macrophages (*r*=0.444, *P*=6.27e-18), neutrophils (*r*=0.318, *P*=1.49e-09), and DCs (*r*=0.457, *P*=6.57e-19) (Figure 7C). The results provided strong evidence that these genes play crucial roles for various immune infiltration cells, including CD4$^+$ T cells, CD8$^+$ T cells, B cells, neutrophils, macrophages, and DCs.

Moreover, the relationships between somatic copy number alterations (SCNA) of the 3 genes and tumor infiltration levels among HCC were investigated. Interestingly, the results showed that the CNA of *CDK1* had significant correlations with the infiltration levels of CD4$^+$ T cells and B cells; the CNA of *CCNB1* had significant correlations with CD4$^+$ T cells, neutrophils, and macrophages; and the CNA of *CCNB2* had a significant correlation with CD4$^+$ T cells (Figure 7D).

Correlation analysis between *CDK1*, *CCNB1*, and *CCNB2* expression levels and immune markers

To further explore the relationships between *CDK1*, *CCNB1*, and *CCNB2* expression and various immune infiltrating cells, we explored the correlations between these genes and immune markers for different subsets of immune cells in HCC. The immune markers analyzed in our study were used to characterize immune cells, including TAMs, monocytes, M1 and M2 macrophages, neutrophils, DCs, NK cells, and B cells. We also investigated diverse functional T cells, such as Th1, Th2, Tregs, and exhausted T cells. After the correlation was adjusted by tumor purity, the expression levels of *CDK1*, *CCNB1*, and *CCNB2* were found to be significantly correlated with diverse immune markers in different immune cells in HCC (Table 2).

Notably, the expression levels of most immune markers for TAMs, monocytes, and M2 macrophages had moderate correlations with *CDK1*, *CCNB1*, and *CCNB2* expression levels. However, the markers for M1 macrophages did not show significant correlations with *CDK1*, *CCNB1*, and *CCNB2* expression levels. These results implied that *CDK1*, *CCNB1*, and *CCNB2* might participate in regulating the polarization of macrophages in HCC.

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Table 1. The prognostic values of CpG in *CDK1*, *CCNB1*, and *CCNB2* genes by MethSurv.

Gene-CpG	HR	Log-rank test P value
CDK1-5'UTR-Island-cg02401235	0.429	<0.001*
CDK1-5'UTR-Island-cg25793692	0.778	0.15
CDK1-TSS200; 5'UTR; 1stExon-Island-cg04271103	0.866	0.41
CDK1-TSS200; 5'UTR; 1stExon-Island-cg06793798	1.534	0.034*
CDK1-TSS200; 5'UTR; 1stExon-Island-cg13227273	1.678	0.0038*
CDK1-TSS200; 5'UTR; 1stExon-Island-cg13954297	0.663	0.024*
CDK1-TSS200; 5'UTR; 1stExon-Island-cg14922279	0.631	0.0094*
CDK1-5'UTR; 1stExon; TSS200-Island-cg13554667	0.866	0.41
CDK1-5'UTR; 1stExon; TSS200-Island-cg25228510	2.349	<0.001*
CDK1-TSS200; TSS1500-Island-cg15172601	0.834	0.3
CDK1-TSS200; TSS1500-Island-cg27457323	0.766	0.18
CDK1-5'UTR; 1stExon; Island-cg18827378	0.674	0.043
CCNB1-TSS1500-N_Shore-cg00290373	1.387	0.062
CCNB1-TSS1500-N_Shore-cg13849825	1.766	0.0016*
CCNB1-Body-S_Shore-cg01276222	0.571	0.0016*
CCNB1-Body-Island-cg06452669	1.895	<0.001*
CCNB1-TSS1500-Island-cg06979550	1.136	0.47
CCNB1-TSS1500-Island-cg20440575	1.907	0.0025*
CCNB1-TSS200-Island-cg09999250	0.758	0.12
CCNB1-TSS200-Island-cg24088685	1.502	0.032*
CCNB-1stExon; 5'UTR-Island-cg10556830	0.816	0.25
CCNB-1stExon; 5'UTR-Island-cg23935746	0.826	0.3
CCNB1-3'UTR-Open_Sea-cg13647309	2.688	<0.001*
CCNB1-Body-S_Shelf-cg17668562	1.418	0.072
CCNB2-TSS1500-N_Shore-cg01738168	1.149	0.43
CCNB2-TSS1500-N_Shore-cg08366813	1.765	0.0018*
CCNB2-TSS200-N_Shore-cg03950590	1.279	0.16
CCNB2-Body-Island-cg01763821	0.903	0.56
CCNB2-TSS200-Island-cg17236576	1.075	0.72
CCNB2-TSS200-Island-cg17260725	1.184	0.41
CCNB2-Body-S_Shore-cg13581437	1.919	<0.001*

HR – hazard ratio; * $P<0.05$.

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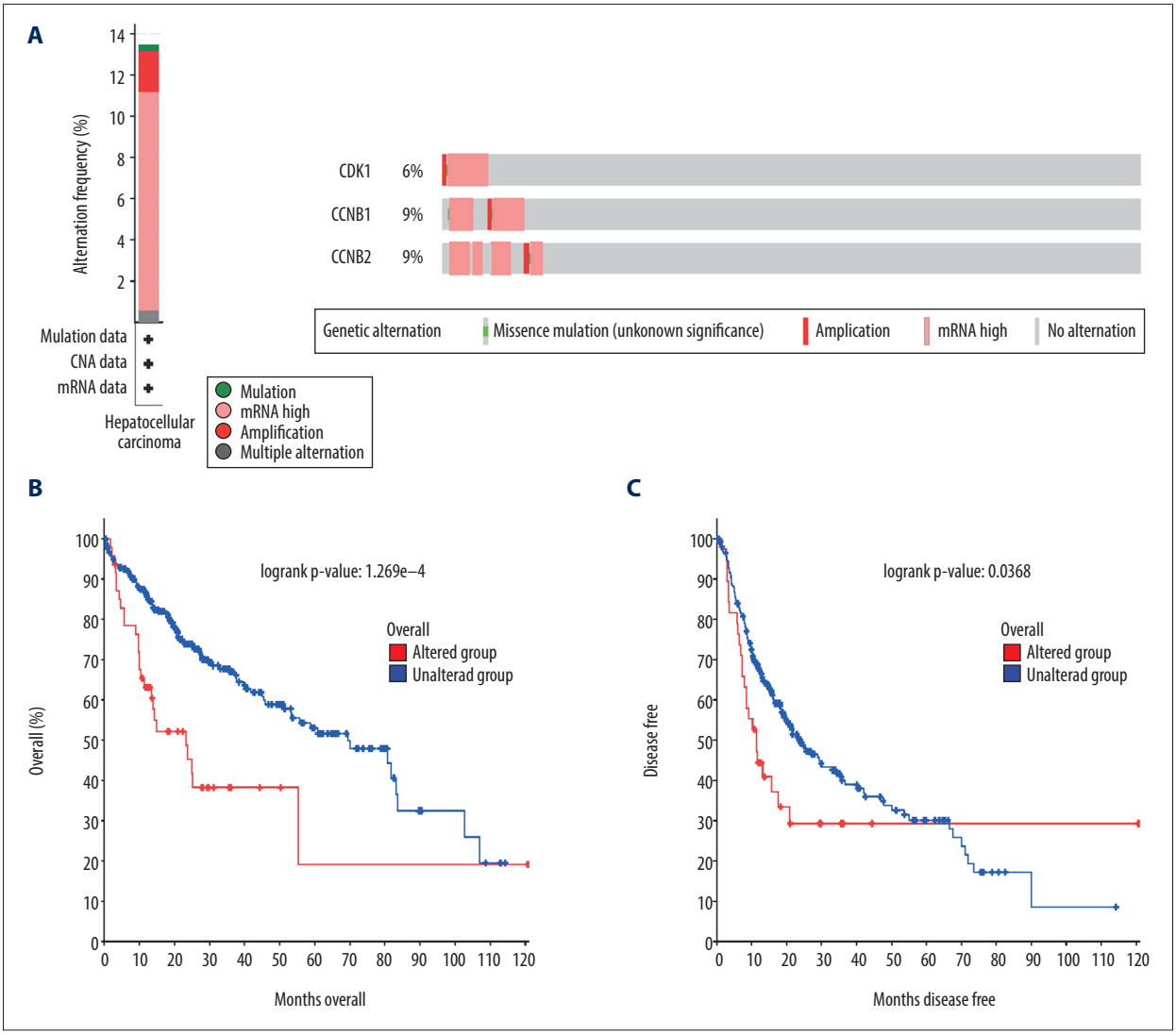

Figure 6. (**A**) *CDK1*, *CCNB1*, and *CCNB2* mutation rates were 6%, 9%, and 9%, respectively. (**B, C**) Genetic alterations in *CDK1*, *CCNB1*, and *CCNB2* were associated with shorter overall survival (OS) and disease-free survival (DFS) of HCC patients.

Interestingly, the correlations between markers for DCs and the expression levels of *CDK1*, *CCNB1*, and *CCNB2* were significantly positively associated. The results further confirmed that higher expression of these genes increased infiltration by DCs. In addition, positive correlations between the expression levels of these genes and the gene markers for Tregs, Th1, and Th2 were also observed in the results. However, no significant correlation was detected in the gene markers for NK cells. Furthermore, positive correlations between the expression levels of *CDK1*, *CCNB1*, and *CCNB2* and the expression of gene markers in exhausted T cells, such as PD-1, PDL-1, CTLA4, TIM-3, LAG3, and GZMB, were observed in HCC. Therefore, these results further demonstrate that higher expression levels of *CDK1*, *CCNB1*, and *CCNB2* were correlated with various infiltrating immune cells, which suggests an important role in immune escape.

Discussion

Orderly G2/M transition is controlled by the CDK1/CCNB complex, which plays a critical role in governing the cell cycle of mammalian cells [8]. Recent studies reported that the mRNA expression levels of *CDK1*, *CCNB1*, and *CCNB2* were significantly higher in several types of cancer and were associated with poor prognosis [9]. Moreover, the relationships between mRNA expression of these genes and tumor cell malignancy were confirmed in HCC [10–12]. However, the specific molecular alterations of these genes and their biological functions in HCC were not completely understood. Therefore, we further explored the roles of these genes in HCC, especially in terms of the immune environment.

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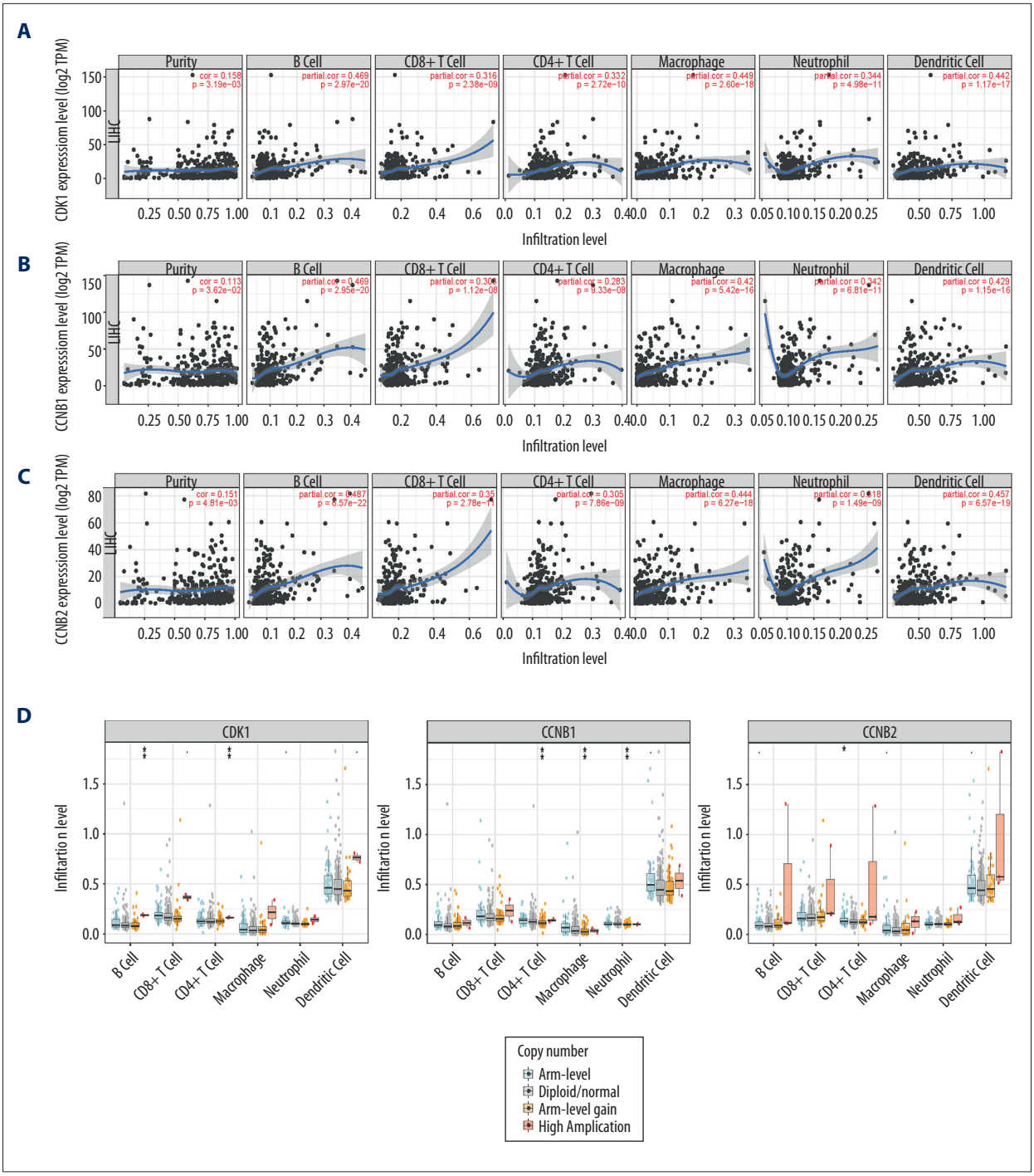

Figure 7. (**A–C**) *CDK1*, *CCNB1*, and *CCNB2* expression levels were significantly related to tumor purity and significant positive correlations existed with immune infiltration cells including CD4⁺ T cells, CD8⁺ T cells, B cells, neutrophils, macrophages, and DCs in HCC. (**D**) CNA of *CDK1* had significant correlations with immune infiltration cells including CD4⁺ T cells and B cells. CNA of *CCNB1* had significant correlations with CD4⁺ T cells, neutrophils, and macrophages. CNA of *CCNB2* had a significant correlation with CD4⁺ T cell. CNA, copy number alteration.

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Table 2. Correlation analysis between *CDK1*, *CCNB1*, *CCNB2*, and related immune markers.

Description	Gene marker	CKD1		CCNB1		CCNB2	
		Cor	P	Cor	P	Cor	P
TAM	*CCL2*	0.143	**	0.107	*	0.166	**
	CD68	0.324	***	0.34	***	0.3	***
	IL10	0.329	***	0.339	***	0.344	***
Monocyte	*CD86*	0.438	***	0.438	***	0.461	***
	CD115	0.27	***	0.274	***	0.288	***
M1 Macrophage	*NOS2*	–0.015	0.776	–0.019	0.728	–0.008	0.880
	ROS	0.084	0.119	0.079	0.144	0.1	0.063
M2 Macrophage	*CD163*	0.17	**	0.159	**	0.156	**
	VSIG4	0.191	***	0.181	***	0.177	***
	CSF1R	0.27	***	0.274	**	0.288	**
DCs	*CD11C*	0.465	***	0.442	***	0.434	***
	CD1C	0.219	***	0.188	***	0.229	***
	NRP1	0.266	***	0.208	***	0.217	***
Neutrophils	*CCR7*	0.226	***	0.143	**	0.205	***
	ITGAM	0.359	***	0.383	***	0.361	***
	CD59	0.085	0.114	0.069	0.2	0.066	0.221
Th1	*STAT4*	0.326	***	0.271	***	0.31	***
	STAT1	0.42	***	0.413	***	0.41	***
	TBX21	0.184	***	0.121	*	0.185	***
	CD4	0.308	***	0.267	***	0.325	***
	IFNG	0.35	***	0.334	***	0.151	**
Th2	*GATA3*	0.336	***	0.281	***	0.351	***
	STAT6	0.106	*	0.055	0.306	0.041	0.443
	CXCR4	0.453	***	0.387	***	0.433	***
	CCR4	0.295	***	0.245	***	0.256	***
Treg	*FOXP3*	0.239	***	0.212	***	0.24	***
	CCR8	0.484	***	0.444	***	0.452	***
	STAT5B	0.236	***	0.179	***	0.189	***
	TGFB1	0.379	***	0.378	***	0.394	***
NKs	*KIR3DL1*	0.021	0.704	–0.018	0.735	0.021	0.700
	KIR2DL1	–0.052	0.332	–0.05	0.355	–0.047	0.389
	KIR2DS4	0.066	0.218	0.03	0.580	0.041	0.449
B cell	*CD19*	0.349	***	0.289	***	0.348	***
	CD79A	0.27	***	0.216	**	0.29	***
	KRT20	0.178	***	0.219	***	0.204	***

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Table 2 continued. Correlation analysis between *CDK1*, *CCNB1*, *CCNB2*, and related immune markers.

Description	Gene marker	CKD1		CCNB1		CCNB2	
		Cor	P	Cor	P	Cor	P
T-cell exhaustion	*PD1 (PDCD1)*	0.433	***	0.398	***	0.457	***
	CTLA4	0.475	***	0.427	***	0.455	***
	TIM-3	0.457	***	0.454	***	0.464	***
	LAG3	0.339	***	0.332	***	0.401	***
	GZMB	0.165	**	0.135	*	0.186	***
PDL1	*CD274*	0.284	***	0.257	***	0.245	***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Our study further proved overexpression of *CDK1*, *CCNB1*, and *CCNB2* in diverse types of cancer. We also found that HCC patients with higher levels of mRNA expression of these genes had a significantly poorer prognosis, which was confirmed by HCC cohorts from TCGA and ICGC. Furthermore, the mRNA expression levels of these genes were significantly associated with different pathological grades and individual cancer stages in HCC. Genes that were co-expressed with *CDK1*, *CCNB1*, and *CCNB2* in HCC patients were extracted for GO and KEGG enrichment analysis. As expected, the co-expressed genes were found to be primarily related to cell cycle functions and pathways. Lower levels of methylation of these genes in HCC might cause them to be overexpressed. Moreover, genomic alterations of these genes were found to be significantly correlated with poorer clinical outcomes of patients with HCC. Thus, the results strongly indicate that *CDK1*, *CCNB1*, and *CCNB2* are potential therapeutic targets in HCC.

Interestingly, another important finding from our study was that the mRNA expression levels of *CDK1*, *CCNB1*, and *CCNB2* were correlated with immune infiltration levels and various immune markers. Significantly positive relationships were found between expression levels of these genes and infiltration levels of $CD4^+$ T cells, $CD8^+$ T cells, B cells, neutrophils, DCs, and macrophages, particularly B cells, macrophages, and DCs. In addition, the correlations between the expression levels of these genes and the immune markers suggested that these genes function in regulating tumor immunity in HCC. First, immune markers of M2 macrophages, such as CD163, VSIG4, and CSF1R, demonstrated significant correlations with expression of these genes, but immune markers of M1 macrophages, such as NOS2 and ROS, did not show significant correlations. The results indicated that *CDK1*, *CCNB1*, and *CCNB2* also possessed a potential mechanism to polarize TAMs in HCC. In tumor tissues, M1 macrophages had antitumor effects, while M2 macrophages had immunosuppressive effects that could promote tumor progression, including HCC [25,26]. Furthermore, this study also revealed that these genes could potentially activate Tregs and cause T-cell exhaustion through PD-1, PDL-1, TIM-3, and CTLA4. The increasing number of Tregs and dysfunctional T cells would suppress immune system response against cancer cells, which would in turn promote tumor recurrence, metastasis, and treatment resistance in neoplasms [27–29]. In addition, significant correlations were also observed between expression of these genes and diverse markers of Th1, Th2, and DCs in HCC. A previous study had shown that a dysfunctional immune defense is partly related to intrahepatic DCs [30]. In general, the results indicate that *CDK1*, *CCNB1*, and *CCNB2* might participate in the recruitment and regulation of infiltration cells in the immune microenvironment, which would lead to immune escape in HCC.

Clinical trials of immune checkpoint inhibitors in HCC, such as tremelimumab (anti-CTLA4), nivolumab (anti-PD1), and durvalumab (anti-PDL1), have shown positive responses [31]. Our results indicate a poorer prognosis with higher expression levels of *CDK1*, *CCNB1*, and *CCNB2* and a potential mechanism for inducing immune escape in HCC. We suggest that these genes can be potentially used to evaluate the infiltration levels of immune cells in HCC and predict the response to immunotherapy. CDK inhibitors such as Dinaciclib and Riviciclib are in clinical trials for treating several types of cancer [32–36]. Moreover, the combination of a CDK4/6 inhibitor such as Palbociclib with immune checkpoint inhibitors (PD-1 and CTLA4) and PI3Ka has been confirmed to induce complete and durable regressions (>1 year) of triple-negative breast cancer *in vivo* [37]. Two phase II clinical trials (NCT02778685 and NCT03147287) that combine Palbociclib with endocrine therapy and immunotherapy are currently underway [38]. We further propose that combining inhibitors of CDK1/CCNB with immunotherapy might improve the survival time of HCC patients. However, our study lacked experiments needed to verify the conclusions, and further experiments by testing clinical samples are needed.

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Conclusions

Our study provides multi-pronged evidence of the impacts of *CDK1*, *CCNB1*, and *CCNB2* in the survival of HCC and the potential of these genes as therapeutic markers. The results confirm that upregulation of these genes is correlated with poor prognosis and is affected by methylation and genetic alterations. In addition, our results indicate a potential immune regulatory role of these genes in tumor immunity. Therefore, *CDK1*, *CCNB1*, and *CCNB2* may potentially be able to predict the response to immunotherapy, and combining immunotherapy with inhibitors of these genes may improve the curative effect.

Conflicts of interest

None.

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