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Measurement of Cyclin D2 (*CCND2*) Gene Promoter Methylation in Plasma and Peripheral Blood Mononuclear Cells and Alpha-Fetoprotein Levels in Patients with Hepatitis B Virus-Associated Hepatocellular Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Alpha-fetoprotein (AFP) is widely used to screen for hepatocellular carcinoma (HCC). However, the use of this biomarker has been challenged due to its low sensitivity and high rate of false negatives. In this study, we evaluated the diagnostic capability of cyclin D2 (*CCND2*) promoter methylation in patients with HCC related to hepatitis B virus (HBV).

Material/Methods: Using methylation-specific PCR and quantitative real-time PCR, we measured methylation status and mRNA levels of *CCND2* in plasma and peripheral blood mononuclear cells (PBMCs) from 275 subjects: 75 patients with chronic hepatitis B (CHB), 47 with liver cirrhosis (LC), 118 with HCC, and 35 healthy controls (HCs).

Results: The methylation rate of the *CCND2* promoter was significantly higher in HCC patients than in patients without HCC ($P<0.001$). Furthermore, advanced HCC (TNM III/IV) was associated with a significantly higher frequency of *CCND2* methylation and lower *CCND2* mRNA levels than early-stage disease (TNM I/II; $P<0.05$). Combined measurement of *CCND2* methylation and AFP yielded significantly higher sensitivity and area under the curve (AUC) than AFP alone in distinguishing patients with HCC from subjects with LC and CHB ($P<0.001$).

Conclusions: *CCND2* methylation may be useful for predicting HCC progression. In addition, combined measurement of *CCND2* methylation and AFP could serve as a non-invasive diagnostic marker for patients with HBV-related HCC.

MeSH Keywords: **Carcinoma, Hepatocellular • Cyclin D2 • Diagnosis • DNA Methylation**

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Background

Hepatocellular carcinoma (HCC), which constitutes the majority of liver cancers, is the second most common cause of death from cancer around the world [1,2]. In China, HCC is mainly caused by chronic hepatitis B virus (HBV) infection [3]. Although a multitude of diagnostic and therapeutic modalities have been developed for HCC, the long-term prognosis remains poor, and 5 year survival rates are low [4]. A combination of imaging technologies such as ultrasonography (US) and determination of alpha-fetoprotein (AFP) levels is the most widely used method for detecting HCC. However, several studies have shown that low sensitivity and high rates of both false negatives and false positives limit the wide application of serum AFP levels as a marker [5,6]. Farinati et al. reported that only 54% of patients with HCC had abnormal serum AFP levels in a large multicentric survey [7]. AFP has also been questioned for its low specificity, because elevated AFP levels are also found in pregnant women and in patients with active hepatitis and embryonic carcinomas [8]. US-based detection is affected by several factors, including the professional expertise of operators, the physical status of the patient, the presence or absence of cirrhosis, and tumor size [9]. A previous study demonstrated that US alone has a sensitivity of 32% for the diagnosis of early-stage HCC [10]. In addition, US cannot be used to visualize adequately the liver in patients with a nodular liver and does not have the accuracy to distinguish HCC from other lesions [11,12]. Collectively, these parameters determine whether US can effectively detect HCC at the early stage. Potential alternative imaging modalities are unsuitable, as computed tomography (CT) scans and magnetic resonance imaging (MRI) cannot detect small HCC lesions [13]. Hence, effective and non-invasive biomarkers for diagnosis of HCC are urgently required.

DNA methylation plays crucial roles in the progression of several types of human cancer [14,15]. Moreover, changes in DNA methylation patterns are frequently observed in the early stages of disease; for example, Zhang et al. reported that altered DNA methylation could be detected 1–9 years before HCC itself [16]. Several genes have been implicated in the disease stage and clinical outcome of HCC, including *APC* [17], *P15* [18], *IGFBP7* [19], and *GSTP1* [17,20]. Together, these findings suggest that assessment of DNA methylation represents a feasible approach for early diagnosis and prognostic evaluation of HCC.

Cyclin D2 (CCND2) regulates cell-cycle progression [21] and inhibits cell growth. Accordingly, CCND2 levels are elevated in normal human cells under growth arrest. Aberrant expression of CCND2 affects cell-cycle progression, suggesting that CCND2 has an additional function that maintains the non-proliferative state [22–24]. This effect can be relieved by the inhibition of *CCND2* transcription by hypermethylation of the promoter, which frequently occurs in HCC [25]. Based on these observations, we used methylation-specific PCR (MSP) to detect the methylation status of the *CCND2* promoter in both plasma and peripheral blood mononuclear cells (PBMCs). We then assessed the value of *CCND2* promoter methylation as a non-invasive method for diagnosing patients with HBV-associated HCC.

Material and Methods

Patients and controls

A total of 118 patients with HCC, 47 with liver cirrhosis (LC), 75 with chronic hepatitis B (CHB), and 35 healthy controls (HCs) were recruited in the Department of Hepatology, Qilu Hospital of Shandong University, from March 2018 to December 2019. All patients were HBsAg-positive. Figure 1 depicts the selection

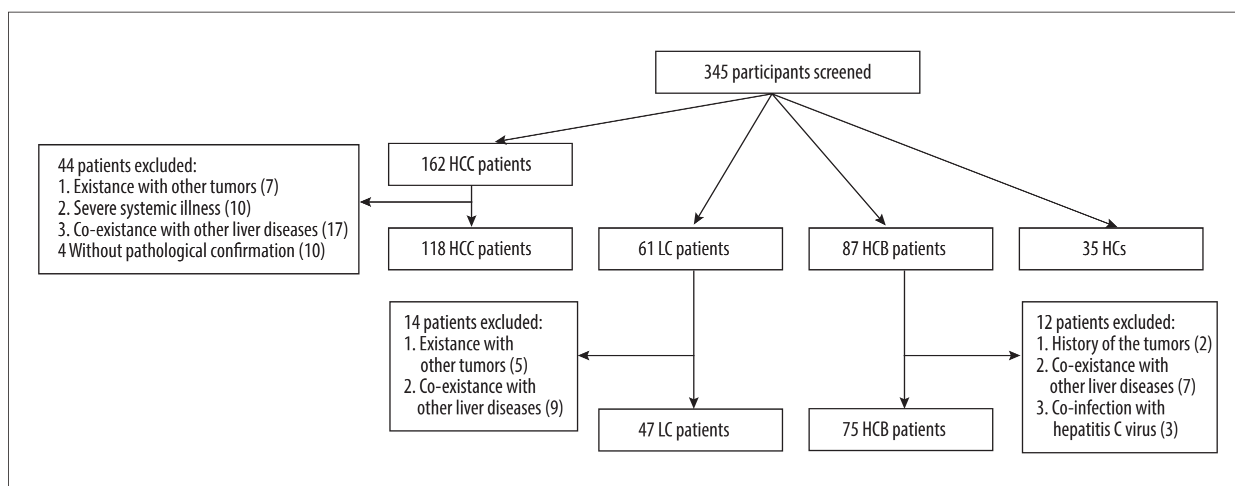

Figure 1. Flowchart describing the participant selection process. HCC – hepatocellular carcinoma; LC – liver cirrhosis; CHB – chronic hepatitis B; HCs – healthy controls.

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process. The Ethics Committee of Qilu Hospital of Shandong University approved the study protocol, and informed consent was obtained from all participants prior to the study.

DNA extraction from plasma and PBMCs

Citrate-anticoagulated peripheral blood (5 mL) was collected from all subjects. DNA was extracted from 400 μL plasma using the QIAamp DNA Blood Mini Kit (Qiagen, Mainz, Germany) and stored at –20°C until use. After centrifugation on a Ficoll-Paque Plus density gradient (GE Healthcare, Uppsala, Sweden), PBMCs were collected from the interface and washed 3 times with phosphate-buffered saline. Genomic DNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted DNA was eluted in 200 μL sterile water and stored at –20°C until use.

Sodium bisulfite modification and MSP

Extracted DNA was modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Treatment with bisulfite converts unmethylated cytosine residues to uracil but does not affect methylated cytosine residues. Modified DNA was dissolved at a final volume of 20 μL and stored at –20°C until use. Bisulfite-modified DNA was amplified using methylated and unmethylated primers specific for the *CCND2* promoter. The primer pairs used for MSP analysis of *CCND2* were described previously [26].
Methylated sequence (276 bp PCR product):
forward, 5'-TACGTGTTAGGGTCGATCG-3';
reverse, 5'-CGAAATATCTACGCTAAACG-3'.
Unmethylated sequence (222 bp PCR product):
forward, 5'-GTTATGTTATGTTTGTTGTATG-3';
reverse, 5'-TAAAATCCACCAACACAATCA-3'.
The MSP reaction mixture had a volume of 25 μL as follows: 10.5 μL nuclease-free water, 12.5 μL PreMix Taq (Zymo Research, Irvine, CA, USA), 0.5 μL of each primer (10 μmol/L), and 1 μL bisulfite-treated DNA. Touchdown PCR conditions were as follows: 95°C for 5 min; ten cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 5 min. Water without DNA was used as a negative control. PCR products (7 μL) were electrophoresed on 2% agarose gels and visualized under UV illumination after staining with Gel Red (Biotium, Fremont, CA, USA).

RNA extraction from PBMCs and quantitative real-time PCR (qRT-PCR)

RNA was extracted from PBMCs by the phenol–chloroform–isopropanol method. Total RNA was resuspended in 20 μL RNase-free water. Subsequently, RNA was converted into cDNA using the PrimeScript RT Reagent Kit (Takara, Shiga, Japan).

Expression of *CCND2* mRNA was detected by qRT-PCR on an Agilent Technologies Stratagene Mx3005P (Stratagene, La Jolla, CA, USA) using SYBR Green PCR Mix (Takara, Shiga, Japan). *ACTB* (encoding β-actin) was used as an endogenous control. The primer pairs used for qRT-PCR analysis of *CCND2* [25] and *ACTB* [19] were described previously. qRT-PCR was performed as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 72°C for 30 s, 65°C for 15 s, and 40°C for 30 s. mRNA levels were calculated using the comparative ($2^{-\Delta\Delta Ct}$) method.

Statistical analysis

All data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U-test was used to compare *CCND2* mRNA levels between the HCC group and other groups, and Spearman's rank correlation was used to assess correlations with clinical features. Differences in *CCND2* methylation frequency between different groups were compared using the Chi-square test. The association between *CCND2* promoter methylation status in HCC patients and clinicopathological parameters was analyzed using the Chi-square test. The diagnostic value of using *CCND2* promoter methylation for distinguishing HCC from LC and CHB was assessed based on the area under the receiver operating characteristic curve (AUC). $P<0.05$ was considered to be statistically significant.

Results

General characteristics

Clinicopathological characteristics of the participants are provided in Table 1.

CCND2 mRNA Levels in PBMCs of HCC

CCND2 mRNA levels were significantly lower in HCC patients than in LC patients ($P<0.001$), CHB patients ($P<0.001$), and HCs ($P<0.001$; Figure 2A). However, there were no significant differences in *CCND2* mRNA levels between LC patients, CHB patients, and HCs ($P>0.05$, respectively).

Moreover, HCC patients with advanced disease (TNM stage III/IV) had significantly lower *CCND2* mRNA levels than patients with early-stage disease (TNM I/II; $P=0.030$; Figure 2B). *CCND2* mRNA levels in the HCC group exhibited no significant relationship with age, HBeAg, gender, smoking status, alcohol use, AFP, tumor number, tumor size, vascular invasion, or CTP staging (Figure 2C–2L).

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Table 1. Characterization of the study participants.

Variable	HCC group (n=118)	LC group (n=47)	CHB group (n=75)	HC group (n=35)
Age (years)	54 (49–62)	52 (42–60)	50 (40–57)	47 (39–55)
Male gender, n (%)	87 (73.73)	35 (74.47)	52 (69.33)	23 (65.71)
HBeAg+, n (%)	43 (36.44)	22 (46.81)	41 (54.67)	NA
ALT (U/L)	37.50 (24.00–69.50)	51.00 (30.00–98.00)	101.00 (57.00–227.00)	NA
AST (U/L)	46.00 (30.75–99.25)	62.00 (36.00–121.00)	61.00 (42.00–121.00)	NA
TBIL (μmol/L)	18.35 (13.93–37.93)	22.50 (15.40–46.40)	20.00 (11.80–49.90)	NA
ALB (g/L)	39.90 (33.98–43.13)	40.80 (34.90–44.60)	41.60 (37.50–44.90)	NA
PT-INR	1.10 (1.02–1.20)	1.12 (1.05–1.21)	1.11 (1.05–1.21)	NA
AFP (ng/mL)	36.41 (4.98–317.78)	12.90 (9.18–115.20)	15.91 (6.21–77.98)	NA
Methylation in PBMCs/plasma, n (%)	55 (46.61)/52 (44.07)	8 (17.02)/5 (10.64)	8 (10.67)/7 (9.33)	4 (11.43)/2 (5.71)

HCC – hepatocellular carcinoma; LC – liver cirrhosis; CHB – chronic hepatitis B; HCs – healthy controls; ALT – alanine aminotransferase; AST – aspartate aminotransferase; TBIL – total bilirubin; ALB – albumin; PT-INR – prothrombin time-international normalized ratio; AFP – alpha-fetoprotein; PBMCs – peripheral blood mononuclear cells; NA – not available.

Methylation frequency of CCND2 and its correlation with gene transcription

The frequency of *CCND2* promoter methylation was significantly higher in HCC patients (55/118, 46.61% in PBMCs; 52/118, 44.07% in plasma) than in LC patients (8/47, 17.02% in PBMCs; 5/47, 10.64% in plasma; $P<0.001$), CHB patients (8/75, 10.67% in PBMCs; 7/75, 9.33% in plasma; $P<0.001$), and HCs (4/35, 11.43% in PBMCs; 2/35, 5.71% in plasma; $P<0.001$; Figure 3A, 3B). However, *CCND2* methylation frequencies did not differ significantly between LC patients, CHB patients, and HCs ($P>0.05$, respectively).

To determine whether altered promoter methylation could affect *CCND2* transcription, we compared *CCND2* mRNA levels in subjects with and without promoter methylation. In the HCC group, the level of *CCND2* mRNA was significantly lower in methylated subjects than in unmethylated subjects ($P=0.041$; Figure 3C). These data support our hypothesis. Figure 3D shows a representative result of agarose gel electrophoresis.

Association between CCND2 promoter methylation and HCC progression

Table 2 shows that the methylation frequency of the *CCND2* promoter in HCC patients was significantly higher in HCC patients with vascular invasion than in those without vascular invasion ($P=0.012$ in PBMCs; $P=0.008$ in plasma). In addition, *CCND2* promoter hypermethylation was more common in HCC patients with advanced disease (TNM III/IV) than in those with early-stage disease (TNM I/II; $P=0.004$ in PBMCs; $P=0.001$ in plasma). The *CCND2* methylation rate increased gradually with TNM stage (Table 3). Together, these results reveal that CCND2 methylation is more frequent in advanced-stage HCC patients. We found no significant correlations between *CCND2* methylation status and other parameters.

Diagnostic utility of CCND2 promoter methylation and AFP level

For discrimination of HCC from LC, *CCND2* promoter methylation had a sensitivity of 46.61% in PBMCs and 44.07% in plasma, and a specificity of 82.98% in PBMCs and 89.36% in plasma. For discrimination of HCC from CHB, *CCND2* promoter methylation had a sensitivity of 46.61% in PBMCs and 44.07% in plasma, and a specificity of 89.33% in PBMCs and 90.67% in plasma (Table 4). Figure 4A and 4B show that the AUC of combined measurement of *CCND2* promoter methylation and AFP level was significantly higher than that of AFP alone (0.698 *vs.* 0.540 in PBMCs, $P<0.001$; 0.694 *vs.* 0.540 in plasma, $P<0.001$) in discriminating HCC from LC. The AUC of combined measurement was also significantly higher than that of AFP alone (0.724 *vs.* 0.571 in PBMCs, $P<0.001$; 0.720 *vs.* 0.571 in plasma, $P<0.001$) in discriminating HCC from CHB (Figure 4C, 4D).

Next, we compared the diagnostic value of combined measurement of *CCND2* methylation and AFP with that of AFP alone for discriminating HCC from LC. As shown in Figure 5, the HCC detection rate in the *CCND2*-methylated group was significantly higher than that in the *CCND2*-unmethylated group,

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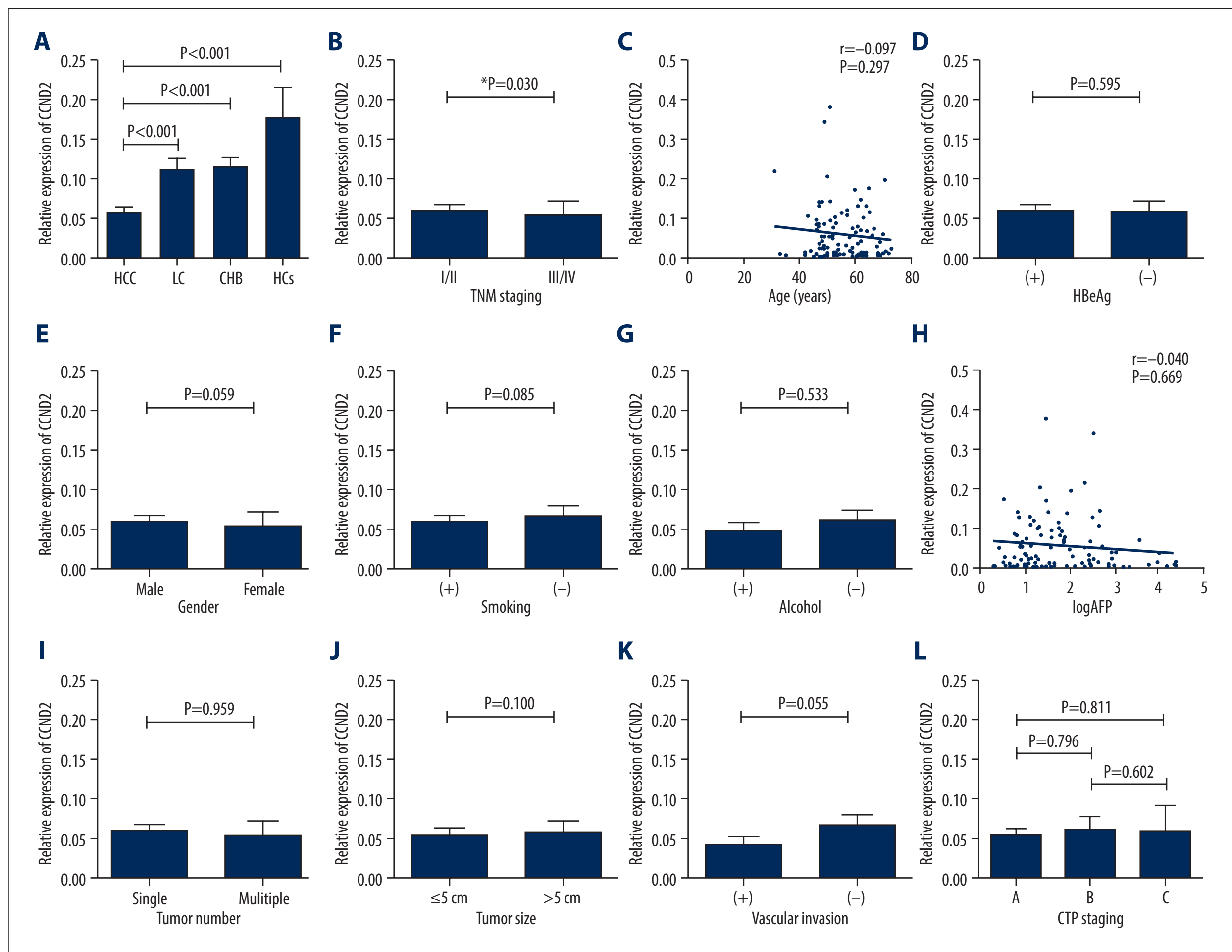

Figure 2. *CCND2* mRNA level in the indicated groups and its correlations with clinical characteristics of HCC. (**A**) *CCND2* mRNA levels differed significantly among the 4 groups (HCC *vs.* LC, $P<0.001$; HCC *vs.* CHB, $P<0.001$; HCC *vs.* HCs, $P<0.001$). (**B**) mRNA levels in HCC patients differed significantly between advanced tumor node metastasis (TNM) stage (III/IV) and early TNM stage (I/II; $P=0.030$). (**C**) mRNA level was not correlated with age in 118 HCC patients ($P=0.297$). (**D–G**) mRNA levels in HCC patients did not significantly differ between HBeAg-positive and HBeAg-negative patients ($P=0.595$), male and female patients ($P=0.059$), smokers and non-smokers ($P=0.085$), or drinkers and non-drinkers ($P=0.533$). (**H**) mRNA levels and serum AFP levels were not significantly correlated in HCC patients ($P=0.669$). (**I–K**) mRNA levels did not significantly differ between HCC patients with a single tumor and those with multiple tumors ($P=0.959$), those with tumors ≤5 cm and those with tumors >5 cm ($P=0.100$), or HCC patients with and without vascular invasion ($P=0.055$). (**L**) mRNA levels did not differ among CTP stages within the HCC group (A *vs.* B, $P=0.796$; A *vs.* C, $P=0.811$; B *vs.* C, $P=0.602$).

regardless of whether the AFP level was ≤20 ng/mL or >20 ng/mL ($\chi^2=14.246$, $P<0.001$, AFP ≤20 ng/mL; $\chi^2=5.499$, $P=0.018$, AFP >20 ng/mL). We defined AFP >20 ng/mL or the presence of *CCND2* methylation as positive. Table 4 revealed that for discrimination of HCC from LC, measurement of *CCND2* methylation plus AFP had a sensitivity of 82.20% (97/118) in PBMCs and 81.36% (96/118) in plasma; a specificity of 57.45% (27/47) in both PBMCs and plasma; a PPV of 82.91% (97/117) in PBMCs and 82.76% (96/116) in plasma; and a negative predictive value (NPV) of 56.25% (27/48) in PBMCs and 55.10% (27/49) in plasma. Notably, the sensitivity of combined measurement was significantly higher than that of AFP alone ($P<0.001$). Similarly, for discriminating HCC from CHB, combined measurement had higher sensitivity (82.20% *vs.* 57.63% in PBMCs, $P<0.001$; 81.36% *vs.* 57.63% in plasma, $P<0.001$) and NPV (69.12% *vs.* 48.98% in PBMCs, $P=0.010$; 68.12% *vs.* 48.98% in plasma, $P=0.014$) than AFP alone (Table 4).

Discussion

CCND2 promoter methylation occurs in multiple types of cancer, including breast, gastric, and prostate cancers [26–28]. In this study, we evaluated the potential utility of *CCND2* promoter methylation in PBMCs and plasma as a non-invasive biomarker for diagnosis of HBV-associated HCC. Our results

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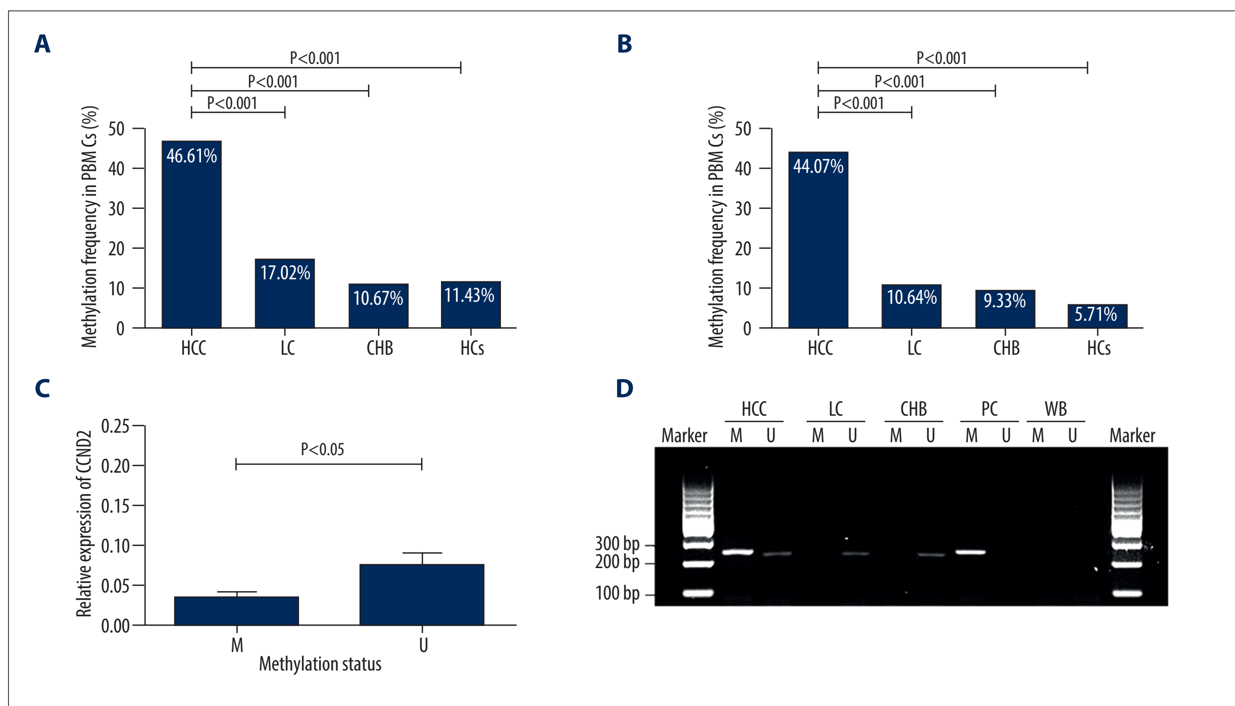

Figure 3. Methylation status of *CCND2* promoter and correlation with expression of *CCND2* mRNA. (**A**) In total, 55/118 (46.61%) hepatocellular carcinoma (HCC) patients, 8/47 (17.02%) liver cirrhosis (LC) patients, 8/75 (10.67%) chronic hepatitis B (CHB) patients, and 4/35 (11.43%) healthy controls (HCs) exhibited aberrant *CCND2* promoter methylation in PBMCs. (**B**) 52/118 (44.07%) HCC patients, 5/47 (10.64%) LC patients, 7/75 (9.33%) CHB patients, and 2/35 (5.71%) HCs exhibited aberrant *CCND2* promoter methylation in plasma. (**C**) *CCND2* mRNA levels differed significantly between the methylation group and non-methylation groups (*P*<0.05). (**D**) Representative measurements of *CCND2* promoter by methylation-specific polymerase chain reaction (MSP). PC – positive control; WB – water blank; M – methylated sequence; U – unmethylated sequence.

revealed that expression of *CCND2* mRNA was significantly lower in patients with HCC than in patients with LC, CHB, and HCs; moreover, *CCND2* methylation frequency was significantly higher in HCC patients than in other groups. In patients with HBV-related HCC, *CCND2* promoter methylation was significantly associated with vascular invasion and negatively correlated with TNM stage. Furthermore, we showed that combined measurement of CCND2 methylation status and serum AFP increased the ability to distinguish HBV-associated HCC from LC and CHB. Therefore, our findings indicate that *CCND2* promoter methylation represents a potentially useful non-invasive biomarker for diagnosis of HBV-related HCC.

CCND2 is involved in cell-cycle regulation, in which its critical function involves the formation of a complex with subunits of CDK6 and CDK4, resulting in phosphorylation of retinoblastoma protein (RB) [21,26]. Overexpression of CCND2 is correlated with progression and poor prognosis of several cancers, indicating that CCND2 should be considered as a proto-oncogene [29,30]. However, silencing of CCND2 expression by promoter methylation is associated with cancer progression, indicating that *CCND2* expression is inhibited by aberrant promoter methylation; thus it would be reasonable to consider CCND2 as a tumor suppressor gene [31,32]. In prostate cancer, elevated methylation of the *CCND2* promoter corresponds with reduced expression of *CCND2* mRNA [33]. When we measured mRNA expression and methylation status of *CCND2* in HCC patients, and analyzed its correlation with other clinicopathological factors, we found that *CCND2* mRNA levels were significantly lower in patients with HCC than in other groups (Figure 2A), whereas the *CCND2* methylation rate was higher in HCC patients (Figure 3A, 3B). Thus, downregulation of CCND2 might be due to promoter methylation, and *CCND2* is more frequently methylated in HCC than in LC, CHB, and HCs; these data are consistent with those of a previous study [25].

More importantly, *CCND2* methylation is closely related to tumor stage in several kinds of human cancer. A recent study of breast cancers found that postmenopausal patients with vascular/lymph invasion exhibit elevated methylation of *CCND2* [34]. Another study showed that increased methylation of the *CCND2* gene is significantly associated with a higher van Nuys grade and is common in early breast cancer development [35]. Consistent with the results of these studies, we observed that advanced-stage (TNM III/IV) HCC patients had a higher frequency of *CCND2* methylation and lower *CCND2* mRNA levels than

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Table 2. Association between *CCND2* methylation status and clinical characteristics of HCC patients.

Parameter	*CCND2* methylation in PBMCs		*P*-value	*CCND2* methylation in plasma		*P*-value
	Methylation, n (%)	No methylation, n (%)		Methylation, n (%)	No methylation, n (%)	
Age (years)			0.802			0.524
≤60	37 (47.44)	41 (52.56)		36 (46.15)	42 (53.85)	
>60	18 (45.00)	22 (55.00)		16 (40.00)	24 (60.00)	
Gender			0.516			0.573
Male	39 (44.83)	48 (55.17)		37 (42.53)	50 (57.47)	
Female	16 (51.61)	15 (48.39)		15 (48.39)	16 (51.61)	
HBeAg			0.713			0.984
Negative	34 (45.33)	41 (54.67)		33 (44.00)	42 (56.00)	
Positive	21 (48.84)	22 (51.16)		19 (44.19)	24 (55.81)	
Smoking			0.703			0.563
No	26 (44.83)	32 (55.17)		24 (41.38)	34 (58.62)	
Yes	29 (48.33)	31 (51.67)		28 (46.67)	32 (53.33)	
Alcohol			0.795			0.717
No	31 (45.59)	37 (54.41)		29 (42.65)	39 (57.35)	
Yes	24 (48.00)	26 (52.00)		23 (46.00)	27 (54.00)	
AFP (ng/mL)			0.080			0.062
≤20	28 (56.00)	22 (44.00)		27 (54.00)	23 (46.00)	
>20	27 (39.71)	41 (60.29)		25 (36.76)	43 (63.24)	
Tumor number			0.851			0.573
Single	41 (47.13)	46 (52.87)		37 (42.53)	50 (57.47)	
Multiple	14 (45.16)	17 (54.84)		15 (48.39)	16 (51.61)	
Vascular invasion			0.012*			0.008*
Negative	24 (36.36)	42 (63.64)		22 (33.33)	44 (66.67)	
Positive	31 (59.62)	21 (40.38)		30 (57.69)	22 (42.31)	
Tumor size			0.992			0.751
≤5 cm	21 (46.67)	24 (53.33)		19 (42.22)	26 (57.78)	
>5 cm	34 (46.58)	39 (53.42)		33 (45.21)	40 (54.79)	
Histological grading			0.566			0.411
Poor	16 (44.44)	20 (55.56)		15 (41.67)	21 (58.33)	
Moderate	27 (44.26)	34 (55.74)		25 (40.98)	36 (59.02)	
Well	12 (57.14)	9 (42.86)		12 (57.14)	9 (42.86)	
TNM staging			0.004*			0.001*
I/II	26 (36.11)	46 (63.89)		23 (31.94)	49 (68.06)	
III/IV	29 (63.04)	17 (36.96)		29 (63.04)	17 (36.96)	

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Table 2 continued. Association between *CCND2* methylation status and clinical characteristics of HCC patients.

Parameter	*CCND2* methylation in PBMCs		*P*-value	*CCND2* methylation in plasma		*P*-value
	Methylation, n (%)	No methylation, n (%)		Methylation, n (%)	No methylation, n (%)	
CTP staging			0.291			0.561
A	29 (41.43)	41 (58.57)		28 (40.00)	42 (60.00)	
B	23 (52.27)	21 (47.73)		22 (50.00)	22 (50.00)	
C	3 (75.00)	1 (25.00)		2 (50.00)	2 (50.00)	

HBeAg – hepatitis B antigen; AFP – alpha-fetoprotein; TNM – tumor node metastasis; CTP – Child-Turcotte-Pugh; * significant difference ($P<0.05$).

Table 3. *CCND2* methylation levels in PBMCs and TNM stage from HCC patients.

Clinicopathological feature	Variable	Number of cases	Methylation	No methylation	Detection rate (%)
TNM stage	I	48	16	32	33.33
	II	24	9	15	37.50
	III	36	23	13	63.89
	IV	10	7	3	70.00
Total		118	55	63	46.61

TNM – tumor node metastasis; PBMCs – peripheral blood mononuclear cells.

Table 4. Diagnostic utility of AFP, *CCND2* promoter methylation, and combined measurement for discrimination of HCC from LC and CHB.

Parameter	*CCND2* methylation		AFP	AFP+*CCND2* methylation		*P*-value	
	PBMCs	Plasma		PBMCs	Plasma	PBMCs	Plasma
HCC *vs.* LC							
Se (%)	55/118 (46.61)	52/118 (44.07)	68/118 (57.63)	97/118 (82.20)	96/118 (81.36)	<0.001*	<0.001*
Sp (%)	39/47 (82.98)	42/47 (89.36)	31/47 (65.96)	27/47 (57.45)	27/47 (57.45)	0.396	0.396
PPV (%)	55/63 (87.3)	52/57 (91.23)	68/84 (80.95)	97/117 (82.91)	96/116 (82.76)	0.722	0.743
NPV (%)	39/102 (38.24)	42/108 (38.89)	31/81 (38.27)	27/48 (56.25)	27/49 (55.1)	0.047	0.061
HCC *vs.* CHB							
Se (%)	55/118 (46.61)	52/118 (44.07)	68/118 (57.63)	97/118 (82.20)	96/118 (81.36)	<0.001*	<0.001*
Sp (%)	67/75 (89.33)	68/75 (90.67)	48/75 (64.00)	47/75 (62.67)	47/75 (62.67)	0.865	0.865
PPV (%)	55/63 (87.30)	52/59 (88.14)	68/95 (71.58)	97/125 (77.60)	96/124 (77.42)	0.307	0.323
NPV (%)	67/130 (51.54)	68/134 (50.74)	48/98 (48.98)	47/68 (69.12)	47/69 (68.12)	0.010*	0.014*

Se – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; * significant difference ($P<0.05$); *P*-value refers to differences between AFP only and a combination of AFP and *CCND2* methylation.

patients with early-stage disease (TNM I/II; $P<0.05$), and that the frequency of *CCND2* methylation increased with TNM stage progression (Table 3). The mechanism linking *CCND2* promoter methylation to HCC progression is unclear. On possibility is that methylation reduces the inhibitory effect of *CCND2* on cell proliferation. *CCND2* has been reported to maintain cells in a non-proliferative state [31,32]. Hypermethylation of the *CCND2* promoter might reduce the expression of *CCND2* and thereby weaken its growth-inhibitory effect. Padar et al. found that prostate cancer patients with a high Gleason score have significantly greater methylation of *CCND2*, those in the higher *CCND2* methylation group had higher mean PSA values, and

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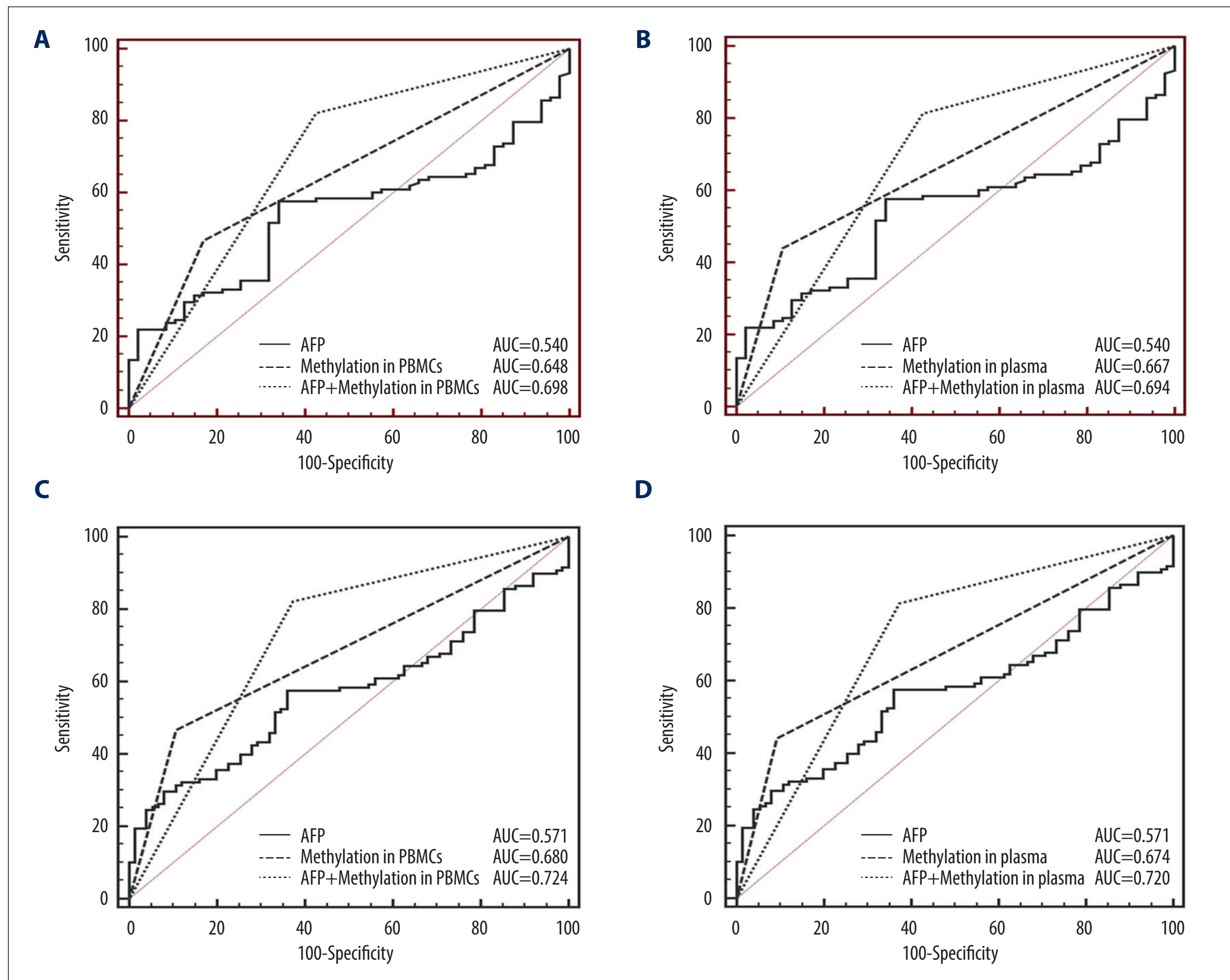

Figure 4. Receiver operating characteristic curves (ROC) of AFP, *CCND2* promoter methylation, and combined measurement for distinguishing HCC from LC and CHB in both PBMCs and plasma. (**A**) The area under the ROC curves (AUC) of combined measurement in PBMCs was significantly higher than that of AFP levels for discriminating HCC from LC patients (AUC 0.698 *vs.* 0.540, *P*<0.001). (**B**) The AUC of combined measurement in plasma was significantly higher than that of AFP levels for discriminating HCC from LC patients (AUC 0.694 *vs.* 0.540, *P*<0.001). (**C**) The AUC of combined measurement in PBMCs was significantly higher than that of AFP levels for discriminating HCC from CHB patients (AUC 0.724 *vs.* 0.571, *P*<0.001). (**D**) The AUC of combined measurement in plasma was significantly higher than that of AFP levels for discriminating HCC from CHB patients (AUC 0.720 *vs.* 0.571, *P*<0.001).

CCND2 methylation is associated with clinicopathological features of poor prognosis [36]. Our results, along with the observations cited above, reveal that elevated *CCND2* methylation rate is correlated with the progression of HCC, suggesting that altered methylation of the *CCND2* promoter might be useful for predicting HCC progression.

Currently, AFP is the biomarker used most widely for HCC screening and diagnosis. However, the sensitivity of serum AFP is only 22–60%, depending on the cut-off point [7,37,38]; consequently, its clinical value is limited. As shown in Figure 5, the rate of HCC detection was significantly higher in the *CCND2*-methylated group than in the unmethylated group, regardless of whether the AFP concentration was greater than or less than 20 ng/mL. This indicates that CCND2 methylation can compensate for deficiencies of AFP and increase the detection rate of HCC. Relative to AFP alone, combined measurement of both markers significantly increased the sensitivity (*P*<0.001) and NPV (*P*<0.05). Although the specificity of combined measurement of *CCND2* promoter methylation and AFP levels is low, it could be used as an initial screening tool for HCC due to its high sensitivity in distinguishing HCC from LC and CHB patients, thereby decreasing the number of missed diagnoses; if screening is positive, follow-up tests (such as ultrasound, CT, or MRI) may be needed to assist diagnosis, or follow-up appointments should be more frequent.

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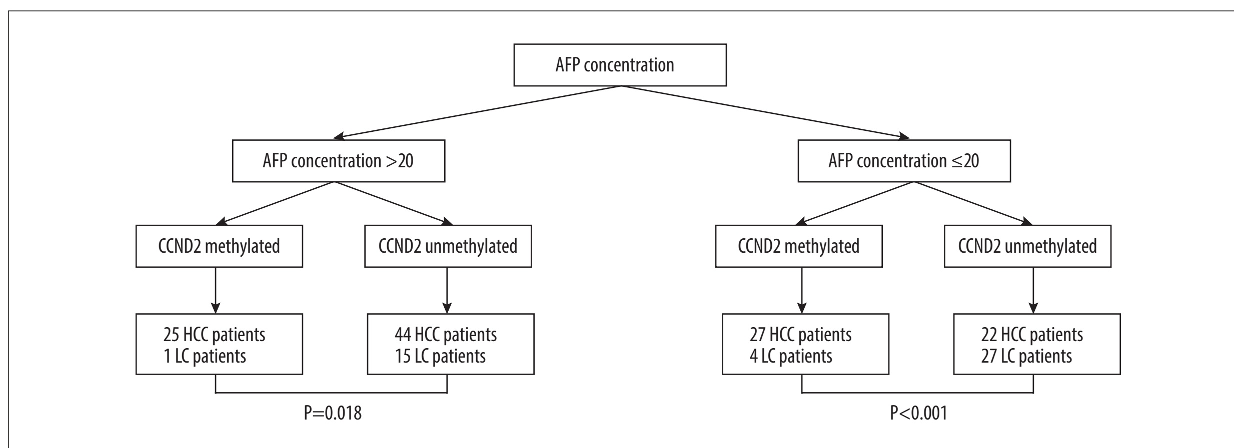

Figure 5. Classification of 118 HCC and 47 LC patients according to AFP concentrations and *CCND2* methylation status in plasma. AFP – alpha-fetoprotein; HCC – hepatocellular carcinoma; LC – liver cirrhosis.

Several studies showed that PBMCs and plasma cell-free DNA are useful for identifying gene mutation and abnormal DNA methylation, and can therefore be used for diagnosis of human cancers [39–41]. In this study, we measured *CCND2* methylation status both in PBMCs and plasma, and obtained consistent results from both types of samples. A previous molecular profiling study [42] revealed that HBV and hepatitis C virus (HCV) cause HCC through different carcinogenetic pathways. Hence, to decrease bias caused by different viruses, we investigated the methylation status of *CCND2* in a cohort comprising only HBV-infected patients.

One weakness of this study is that the MSP method is a qualitative approach for detecting gene methylation. Other methods, such as direct sequencing, may provide more accurate information about methylation. However, MSP can be performed rapidly and easily, enabling most clinical laboratories to carry it out. Although the results of MSP do not provide complete information, the method can still be used to select methylated subjects that may require further examination by sequencing. PIVKA-II, a prothrombin induced by vitamin K deficiency, is increased in malignant hepatocytes [43], suggesting that it may have potential as a biomarker for HCC. A number of studies have revealed that combined determination of PIVKA-II and AFP can improve the diagnostic accuracy of HCC detection compared with either of these biomarkers alone [44,45]. In the present study, we did not assess the diagnostic value of PIVKA-II alone or in combination with *CCND2* methylation in patients with HCC. A future study will be required to address this point.

Conclusions

Methylation of the *CCND2* promoter is common in patients with HCC. Combined measurement of *CCND2* methylation plus serum AFP levels increased the diagnostic value of AFP for discrimination of HCC from LC and CHB, indicating that combined detection of *CCND2* methylation and AFP has potential as a robust and non-invasive biomarker for diagnosis of HCC. Furthermore, *CCND2* promoter methylation was observed more frequently in HCC patients with advanced TNM stage and vascular invasion, suggesting that this marker might be used to predict the progression of HBV-associated HCC.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Medical Ethics Committee of Shandong University Qilu Hospital) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest

None.

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