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Original Article

MeCP2 level is associated with hepatocellular carcinoma development in chronic hepatitis B patients under antiviral therapy

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Abstract: Aim: Hepatocellular carcinoma (HCC) is a common and aggressive malignant tumor with especially high prevalence in Asia. This present study aimed to investigate the association of MeCP2 with HCC development in patients with undetectable HBV DNA by antiviral therapy. Methods: We retrospectively reviewed the 258 patients that were recruited into the present study. The control patients were matched with the HCC patients by age, gender, hepatitis e antigen (HBeAg) status, and duration of NA therapy in a 1:1:1 ratio. Area under ROC curve (AUC) was also used to compare diagnostic significance of MeCP2 using the Hanley and McNeil method. Results: For the entire cohort of 258 patients, MeCP2 was overexpressed in HCC tissues, which was significantly higher than that in cirrhosis and non-cirrhosis tissues (P<0.001). MeCP2 significantly increased in HCC cell lines compared with the control group of THLE-2 including SMMC-7721 (P<0.001), Huh-7 (P<0.001), and Hep3B (P<0.001). Overexpression of MeCP2 was closely related to liver cirrhosis (P=0.001) and TNM stage (P=0.017). The AUROC for the entire cohort, cirrhotic patients and non-cirrhotic patients, was 0.741 (95% CI: 0.629-0.804), 0.682 (95% CI: 0.526-0.782), and 0.776 (95% CI: 0.646-0.903), respectively. The predictive accuracies of MeCP2 in different groups of patients were further compared. For the whole cohort, this test had a high specificity in identifying patients without HCC development (85%). Among patients without cirrhosis, this test had a high sensitivity in identifying patients with future HCC development (83%). Conclusions: We found that MeCP2 was expressed significantly higher in HCC tissues compared with cirrhosis and non-cirrhosis tissues. MeCP2 could be a novel risk marker to predict HCC development in CHB patients with profound viral suppression under NA therapy. MeCP2 measurement may serve as a useful strategy for risk stratification in terms of follow up interval and HCC surveillance.

Keywords: MeCP2, CHB, HCC

Introduction

Hepatocellular carcinoma (HCC) is a common and aggressive malignant tumor with an especially high prevalence in Asia and developing countries and a relatively low prevalence in Europe and North America [1, 2]. Although routine screening of individuals at high risk for developing HCC may extend the life of some patients and the prognosis of patients with HCC has improved recently [3, 4], the survival outcomes of patients with HCC following surgical resection may vary, as several factors are associated with the prognosis of HCC.

Hepatitis B virus (HBV) infection is one of the most common chronic infections and is the main cause of cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) globally [5, 6] with more than 1 million deaths from HBV-related diseases annually [7]. Among various adverse outcomes of CHB, developing HCC is the most common and most serious. Cirrhosis and high serum HBV DNA levels (≥2,000 IU/mL) are major risk factors [8, 9]. The risk of HCC is significantly increased in the case of cirrhosis which is due to CHB and the activation of oncogenes, overexpression of growth factors, and inactivation of tumor suppressor genes [10]. The progression to liver cirrhosis in chronic HBV infection is mediated by active virus replication. Annual incidence of cirrhosis in the overall population with CHB is 2%-7%, depending on viral replication status [11]. Therefore, it is of para-

mount importance to assess severity of liver cirrhosis which helps in prognostication and in turn affects management like antiviral treatment initiation, endoscopic screening for varices, and screening for HCC. Currently available non-invasive methods including direct and indirect serum markers, transient elastography, and magnetic resonance (MR) elastography are associated with certain disadvantages [12]. Novel factors are needed to evaluate liver cirrhosis and the possibilities of developing HCC.

DNA hypermethylation plays an important role in silencing the tumor suppressor genes, being one of the most consistent hallmarks of human cancers. The phenomenon is of comparable significance to classic genetic mutations [13]. Especially in recent years, DNA methylation has emerged as an attractive target for cancer therapeutics. DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs) [14]. The previous report has proven that inhibition of DNA methyltransferase activity can strongly inhibit the mutation of tumors. The repressive effects of DNA methylation are mediated in large part by the methyl-CpG binding proteins (MBDs) and are also associated with histone modifications. MBDs such as methyl-CpG-binding protein 2 (MeCP2), methyl-CpG-binding domain 1 (MBD1), and MBD2 could specifically bind to CpG-methylated DNA and are associated with histone deacetylase (HDAC) [15].

MeCP2 is a basic chromosomal protein that binds to symmetrical methylated 5'-CpG dinucleotide sequences [16, 17]. MeCP2 is essential in human brain development and has been linked to several cancer types and neurodevelopmental disorders [18-20]. However, the role of MeCP2 in osteosarcoma disease has not been fully explored. Thus, our present study aimed to investigate the association of MeCP2 with HCC development in patients with undetectable HBV DNA by antiviral therapy.

Materials and methods

Patients and tissue samples

Patients were recruited from the Jingmen First People's Hospital from January 2012 to November 2016. We recruited all CHB patients who developed HCC despite achieving profound viral suppression (i.e. undetectable serum HBV DNA levels by the Cobas Tagman assay) under nucleos(t)ide analogue (NA) therapy for at least 1 year before diagnosis of HCC. Other inclusion criteria included age ≥18 years, no significant alcohol consumption (>30 gram and >20 gram per day for men and women, respectively), no coexisting liver diseases like HCV infection, primary biliary cholangitis (PBC), autoimmune hepatitis (AIH) and Wilson's disease, as well as no previous history of HCC. After excluding cases that did not have available serum samples for measurement of MeCP2 level, 86 patients with HCC were recruited into the present study. Control subjects were NA-treated cirrhotic patients and CHB patients without HCC development. These control patients were matched with HCC patients by age, gender, hepatitis e antigen (HBeAg) status, and duration of NA therapy in a 1:1:1 ratio. The study protocol was approved by the Institutional Review Board, Jingmen First People's Hospital.

Cell lines and culture conditions

Human HCC cell lines (SMMC-7721, Huh-7 and Hep3B cells) were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC-7721, Huh-7, and Hep3B cell lines were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. THLE-2 cells were cultured in BEGM (Bronchial Epithelial Medium, Invitrogen, Carlsbad, CA, USA), supplemented with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I, and 0.01 mg/ml bovine serum albumin dissolved in BEBM medium.

siRNA transfection

HCC cell lines were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. MeCP2-specic siRNAs (si-MeCP2: Sense GAGGGAUGA GGGUGAAGAA and antisense UUCUUCACCCUCAUCCCUC and negative control siRNA (si-NC) were purchased from GenePharma, Shanghai, China.

MTT assay

HCC cells proliferation was also measured by using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were

grown in a 96-well plate for 24 hours, transfected with si-MeCP2 or negative control si-NC, and incubated in normal medium. Cells were seeded in 0.1 mg/mL MTT for 4 hours and lysed in dimethyl sulfoxide (DMSO) at room temperature for 10 minutes. The absorbance in each well was detected by a microplate reader (Bio-Rad, Hercules, CA, USA) at 0, 12, 24, 36, 48, 60 and 72 hours after transfection.

Total RNA isolation

Total RNA was isolated from 300 µl of serum using the mirVana PARIS Kit (Ambion, Austin, TX, 97 USA) according to the manufacturer's instructions. Briefly, for each sample, total RNA was extracted from 300 µl of serum with 2× denaturing solution, acid-phenol: chloroform, and 100% ethanol. After several washings and centrifugation, the RNA was eluted into 60 µl of preheated (95°C) elution solution. RNA quantity and purity were determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA). RNA purity was considered satisfactory with A260/A280 of 1.9-2.1. The RNA samples were stored at -80°C until reverse transcription.

Real-time quantitative PCR

We typically extracted 2 µg to 9 µg of total RNA and OD260/280 ratios typically ranged from 1.8 to 2.0, indicating high RNA purity. 10 ng of total RNA was used for each miRNA quantification. miRNA detection was performed and ran on the Eppendorf Mastercycler EP Gradient S (Eppendorf, Germany) using commercial assays (TaqMan microRNA assays; Applied Biosystems, Foster City, CA, USA) for miRNAs. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$, where Ct is cycle threshold. Normalization was performed with universal small nuclear RNA U6 (RNU6B). Each sample was examined in triplicate and the mean values were calculated. mRNA levels in tumor samples/non-tumorous samples of 0.5-fold was defined as underexpression of the gene whereas a ratio of 2.0-fold was defined as overexpression.

Immunohistochemistry and evaluation of immunostaining

Immunohistochemical staining was performed with the Dako Envision Plus System (Dako, Carpinteria, CA), according to the manufacturer's instructions. The primary antibodies were anti-MeCP2 (Cell Signaling Technology Inc., Beverly, MA, 1:50). Tissues were evaluated as positive for MeCP2 staining when there were more than 10% of tumor cells demonstrating cytoplasmic and/or nucleus immunoreaction deposits. The sections were scored with a four-tier scale: 0 = negative (0-10%), 1 = weak signal (10-20%), 2 = intermediate signal (20-50%), and 3 = strong signal (>50%). 0 and 1 were defined as low while 2 and 3 were defined as high. All sections were scored independently by two observers who did not have any prior knowledge of the clinic-pathologic data. The concordance between scores from different sections of the same tumor was greater than 90%. All discrepancies in scoring were reviewed and a consensus was reached.

Western blotting analysis

Fresh surgical specimens were snap frozen in liquid nitrogen and stored in a deep freezer. Normal tissues and the tumor were lysed in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing proteinase inhibitors (CalBiochem, San Diego, CA). The extracts were collected and centrifuged at 12,000×g for 5 minutes. Protein concentrations were determined using BCA Protein Assay (Pierce), according to the manufacturer's instructions. The following antibodies were used: anti-MeCP2 (Cell Signaling Technology Inc., Beverly, MA). We also used β-actin as a loading control.

Follow up

Postoperative serum AFP and abdominal ultrasound were carried out in all patients, monthly. Patients received abdominal contrast-enhanced CT scan or MRI once every 3 months in the first two years after surgery and once every 6 months thereafter. Further investigations were carried out when clinically indicated or when tumor recurrence was suspected.

Statistical methods

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test. Categorical variables were compared using χ^2 or Fisher's analysis. The predictive performance of plasma and exosomal miRNAs were

Table 1. Demographics of all patients

Variable	HBV group	Cirrhosis group	HCC group
Case, n	86	86	86
Age	61.2 ± 4.1	61.0 ± 3.6	61.3 ± 5.1
Sex			
Female	34	26	33
Male	52	60	53
HBeAg			
Positive	54	67	64
Negative	32	19	22
Liver cirrhosis			
Yes	0	86	63
No	86	0	23
TBL (μmol/l)	12.5 ± 8.3	15.1 ± 7.3	16.1 ± 8.2
ALB (g/dl)	39.4 ± 6.6	38.9 ± 6.5	37.9 ± 4.6
ALT (U/L)	25.7 ± 14.1	50.4 ± 30.2	79.4 ± 66.5
AFP at diagnosis (ng/ml)			
≤400	86	68	61
>400	0	18	25
Tumor size (cm)			
>5 cm	-	-	28
≤5 cm	-	-	58
TNM staging			
I	-	-	12
II	-	-	20
III-IV	-	-	54

BL: total bilirubin; ALB: albumin; ALT: alanine aminotransferase; PT: prothrombin time; PLT: blood platelet; AFP: alpha-fetoprotein.

measured using the area under ROC curve (AUC). AUCs were also used to compare diagnostic significance of MeCP2 using the Hanley and McNeil method [21]. MiRNAs panel was further analyzed by logistic regression model for the differentiation between HCC and HBV groups. Statistical analyses were conducted with SPSS for Windows version 18.0 release (SPSS, Inc., Chicago, IL) and ROC curve analysis was computed using MedCalcV.11.0.3.0 (MedCalc software, Mariakerke, Belgium). A value of $P<0.05$ was considered significant in all analyses.

Results

Patient characteristics

The demographics of the study population (86 HCC patients, 86 patients with cirrhosis, and 86 HBV patients) are illustrated in **Table 1**. There were no significant differences among the three groups in terms of age, gender, HBeAg status, serum HBV DNA level, and duration of therapy. Of all 258 patients recruited into this study, the baseline characteristics of patients divided by MeCP2 levels are summarized in **Table 2**. Overexpression of MeCP2 was closely related to liver cirrhosis ($P=0.001$) and TNM stage ($P=0.017$) (**Table 2**). There was no significant relation between overexpression of MeCP2 with gender, age, HBsAg, HBeAg, and tumor size, etc. Different NA therapies in HCC and control groups were shown in **Table 3**.

MeCP2 was overexpressed in HCC tissues and cell lines

In the mRNA level, MeCP2 was overexpressed in HCC tissues, which was significantly higher than that in cirrhosis and non-cirrhosis tissues ($P<0.001$) (**Figure 1A** and **1B**). MeCP2 significantly increased in HCC cell lines compared with the control group of THLE-2 including SMMC-7721 ($P<0.001$), Huh-7 ($P<0.001$), and Hep3B ($P<0.001$) (**Figure 1C-E**). Meanwhile, we detected the protein expression of MeCP2 by Western blotting. We found increased expression level of MeCP2 in tumor tissues compared with cirrhosis and non-cirrhosis tissues ($P<0.001$) (**Figure 2A**) and protein expression of MeCP2 was high in the three HCC cell lines (**Figure 2B**). We performed immunostaining in the 30 HCC samples and found that 24 (80%) patients identified as MeCP2 overexpression (**Figure 2C**).

Silencing MeCP2 arrested cell proliferation

MTT assay showed that si-MeCP2 remarkably inhibited cell proliferation after transfection of si-MeCP2 and si-NC in SMMC-7721, Huh-7, and Hep3B cell lines ($P<0.001$) (**Figure 3A-C**).

The role of MeCP2 levels in predicting HCC development

The performance of MeCP2 to predict HCC development in CHB patients who achieved undetectable serum HBV DNA while on NA ther-

Table 2. Correlation between MeCP2 expression and clinicopathologic features

Variables		MeCP2 expression		
		Low (n=112)	High (n=144)	*P*
Sex	Female	33	48	0.509
	Male	79	96	
Age	Median	61	60	0.613
	Range	20-70	32-71	
HBeAg	Positive	81	104	0.986
	Negative	31	40	
Liver cirrhosis	Yes	41	108	0.001
	No	71	36	
TBL (umol/l)	Median	15.2	16	0.603
	Range	4.2-56.7	4.6-64.4	
Alb (g/dl)	Median	38.4	40	0.092
	Range	24.5-51.4	23.6-53.8	
ALT (U/L)	Median	54.9	55.5	0.887
	Range	10,6-235.7	8.3-240.3	
Diameter (cm)	≤5	18	36	0.695
	>5	12	20	
AFP level (μg/L)	≤400	14	26	0.983
	>400	16	30	
TNM stage:	I	9	24	0.017
	II	14	10	
	III	7	22	

Abbreviations: AFP, alpha-fetoprotein; HBeAg, Hepatitis E antigen; TBIL total bilirubin; ALB, albumin; ALT, alanine.

Table 3. Different NA therapies in HCC and control groups

Variable	HBV group	Cirrhosis group	HCC group
Entecavir	75 (87.2%)	80 (93.0%)	77 (89.5%)
Telbivudine	5 (5.8%)	2 (2.3%)	3 (3.5%)
Lamivudine	3 (3.5%)	1 (1.2%)	2 (2.3%)
Adefovir	2 (2.3%)	2 (2.3%)	3 (3.5%)
Tenofovir	1 (1.2%)	1 (1.2%)	1 (1.2%)

apy was measured in terms of area under receiver operating curve (AUROC). The AUROC for the whole cohort, cirrhotic patients and non-cirrhotic patients, was 0.741 (95% CI: 0.629-0.804) (**Figure 4A**), 0.682 (95% CI: 0.526-0.782) (**Figure 4B**), and 0.776 (95% CI: 0.646-0.903) (**Figure 4C**), respectively. **Table 4** shows the predictive accuracies of MeCP2 in different groups of patients. For the whole cohort, this test had a high specificity in identifying patients without HCC development (85%). Among patients without cirrhosis, this test had a high sensitivity in identifying patients with future HCC development (83%).

Discussion

Hepatocellular carcinoma is a major health problem worldwide with high morbidity and mortality. In the past decade, HCC incidence has been increasing in Western Europe and Northern America [22, 23]. Outcomes for patients with HCC have improved markedly over the last 30 years due to the presence of various therapeutic modalities and advances in surgical treatment [24]. However, prediction of HCC development is still vital for monitoring tumor recurrence and choosing subsequently adjuvant therapy.

DNA methylation has been shown to be a key contributor to epigenetic regulation of gene expression. Since links between gene silencing and DNA methylation have been demonstrated, the cellular mechanism of methylated DNA recognition by MBPs has emerged as an important research focus in gene regulation. MeCP2 was the first MBP discovered to selectively recognize and bind methylated DNA sequences [25, 26]. An additional role for MeCP2 during carcinogenesis has recently been described. Hyper-methylation of tumor suppressor gene promoters is a well-characterized event in carcinogenesis [27, 28]. The initial evidence of MeCP2 involvement with cancer arose when it was found that methylation of the breast cancer 1 gene (BRCA1) promoter in the presence of MeCP2 resulted in repression. Hypermethylation of additional tumor suppressor genes in cancer has also been shown to be associated with MeCP2 and other MBPs [29, 30]. Further characterization of MeCP2's role in carcinogenesis is needed to elucidate its individual function in tumor suppression.

In our present study, we found that there was a significant difference of MeCP2 level in HCC tissues compared with that in cirrhosis and non-cirrhosis tissues ($P<0.001$). Moreover, expression of MeCP2 was significant higher in cirrhosis than non-cirrhosis tissues ($P<0.001$) which was

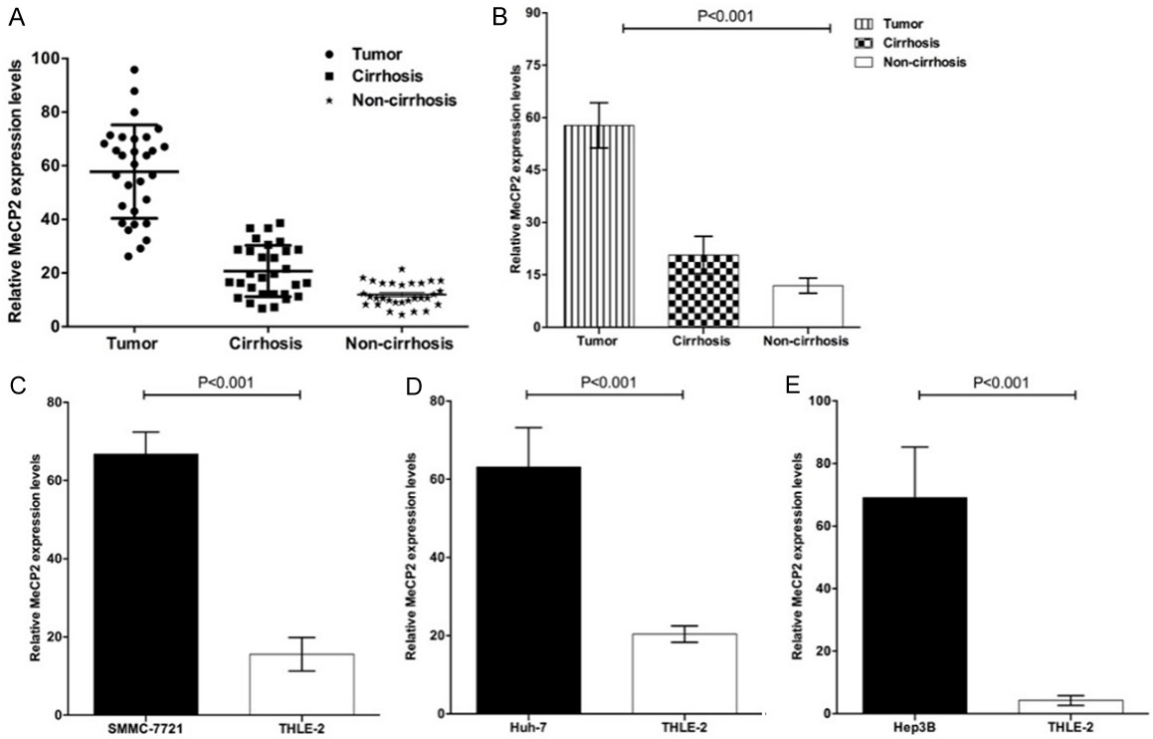

Figure 1. MeCP2 is upregulated in HCC tissues and cell lines. Relative MeCP2 concentration was detected using real-time qPCR. MeCP2 expression levels were higher in HCC tissues than those in cirrhosis and non-cirrhosis tissues. (A) and (B) (P<0.001), SMMC-7721, Huh-7, and Hep3B cell lines than the normal epatic cell line (THLE-2) (C-E) (P<0.001).

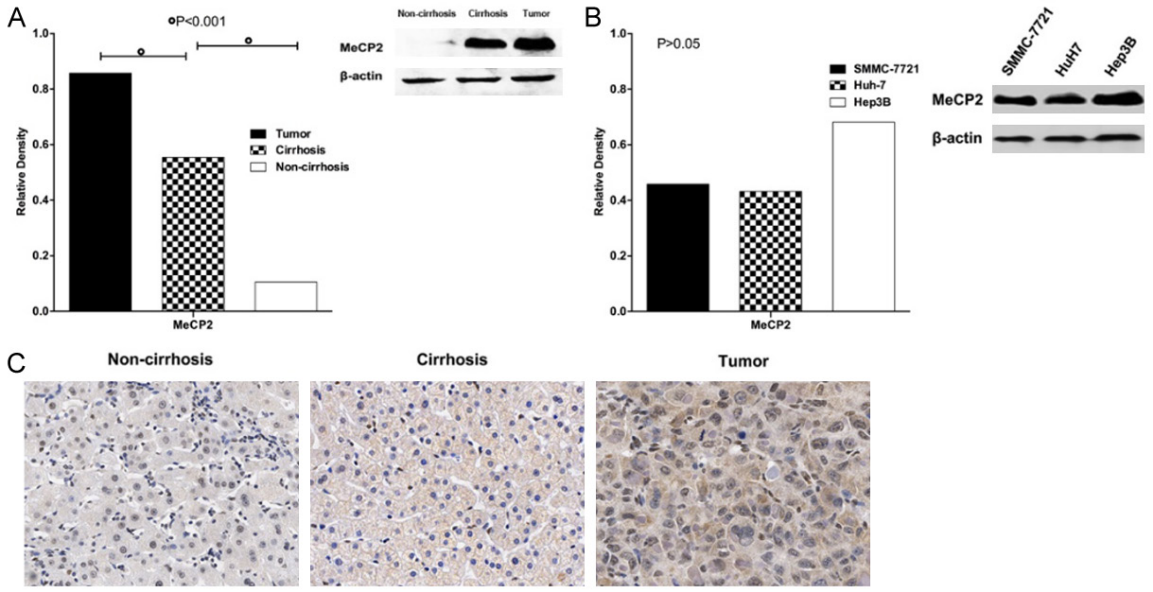

Figure 2. Expression changes of MeCP2 after transfection and silencing MeCP2 inhibits cell proliferation by MTT assay. After transfection of si-MeCP2 or negative control si-NC, OD values were measured. ANOVA was used for the comparison of curves of cell proliferation. Cell proliferation inhibition was observed in HCC cell lines SMMC-7721 (A), Huh-7 (B), and Hep3B (C) cells (P<0.01).

consistent with the results that MeCP2 was closely related to liver cirrhosis (P=0.001). We further detected the performance of MeCP2 in predicting HCC development in CHB patients

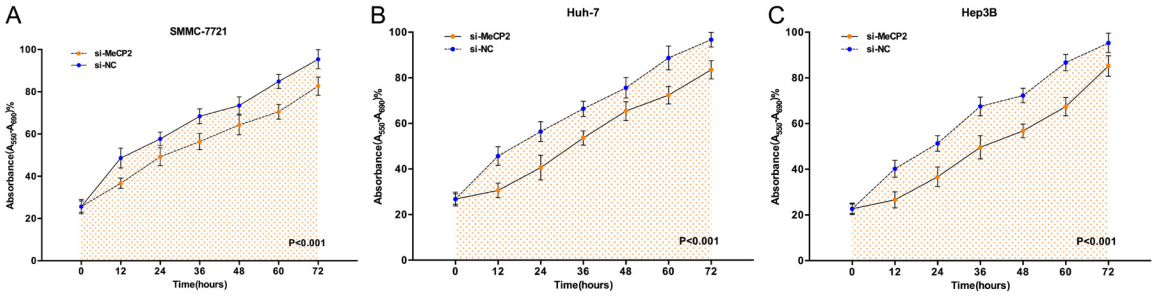

Figure 3. MeCP2 is upregulated in HCC tissues and cell lines. A. Comparison of MeCP2 expression in HCC tissues with those in cirrhosis and non-cirrhosis tissues by Western blotting; B. MeCP2 expression in SMMC-7721, Huh-7, and Hep3B cell lines by Western Blotting; C. MeCP2 expression in HCC tissues with those in cirrhosis and non-cirrhosis tissues by immunohistochemical staining.

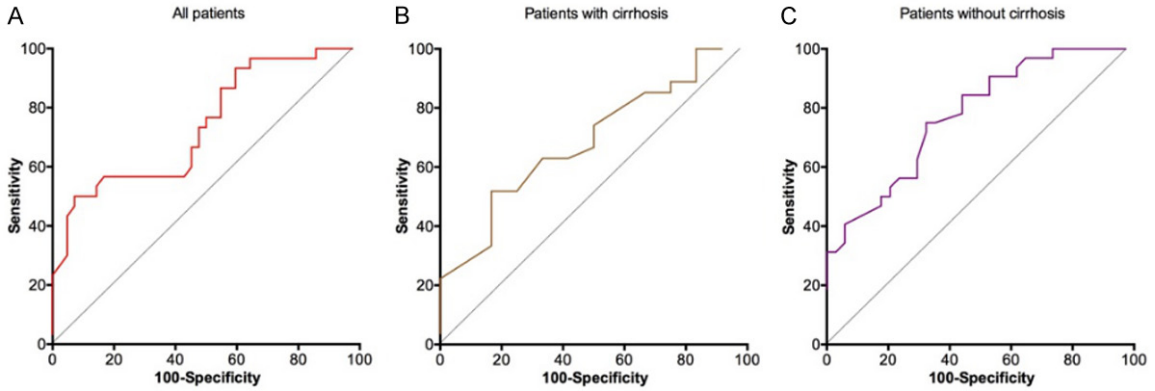

Figure 4. AUROC of pre-treatment MeCP2 for HCC prediction in CHB patients. A. All patients; B. CHB patients with cirrhosis; C. CHB patients without cirrhosis; Abbreviations: AUROC, area under receiver operating curve; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B.

Table 4. Predictive accuracies of MeCP2 for HCC in different groups of patients

Variable	All patients	Cirrhosis positive patients	Cirrhosis negative patients
AUROC	0.741	0.688	0.776
Sensitivity	53%	57%	83%
Specificity	85%	78%	62%
Positive-predictive value	78%	70%	63%
Negative-predictive value	64%	62%	76%

who achieved undetectable serum HBV DNA on NA therapy. The AUROC for the whole cohort, cirrhotic patients and non-cirrhotic patients, was 0.741, 0.682, and 0.776, respectively. For the whole cohort, this test had a high specificity in identifying patients without HCC development (85%). Among patients without cirrhosis, this test had a high sensitivity in identifying patients with future HCC development (83%).

The findings of the present study have significant clinical implications as MeCP2 may help to further stratify the risk of HCC development in patients with cirrhosis as well as those without cirrhosis. This in turn will help to streamline the management plan in terms of follow up interval and HCC surveillance.

The proportion of CHB patients receiving NA therapy has been increasing. For instance, in a nationwide cohort study of Taiwan, it was found that >40% of CHB patients were NA-experienced [31]. With potent nucleos(t)ide analogues (entecavir and tenofovir), the majority of CHB patients are able to achieve profound viral suppression with undetectable serum HBV DNA level. However, NA therapy can reduce but can-

not eliminate the risk of HCC [32]. As of now, there have not been any satisfactory tests to predict HCC in patients under treatment [33], highlighting the importance of exploring alternative predictive factors. Therefore, further follow up studies to improve the predictive power of MeCP2 by adding in other already identified risk factors for the development of HCC such as age and gender with other viral markers like HBsAg and hepatitis B core-related antigen (HBcrAg) are highly recommended [34, 35].

However, there are limitations to this study: (1) the sample size was too small and a further larger sample study is needed to confirm the present experimental results; (2) whether overexpression of MeCP2 has the optimal specificity and sensitivity for HCC diagnosis and prognosis also needs future confirmation.

In conclusion, we found MeCP2 to be expressed significantly higher in HCC tissues compared with cirrhosis and non-cirrhosis tissues. MeCP2 could be a novel risk marker in predicting HCC development in CHB patients with profound viral suppression under NA therapy. MeCP2 measurement may serve as a useful strategy for risk stratification in terms of follow up interval and HCC surveillance.

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Disclosure of conflict of interest

None.

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