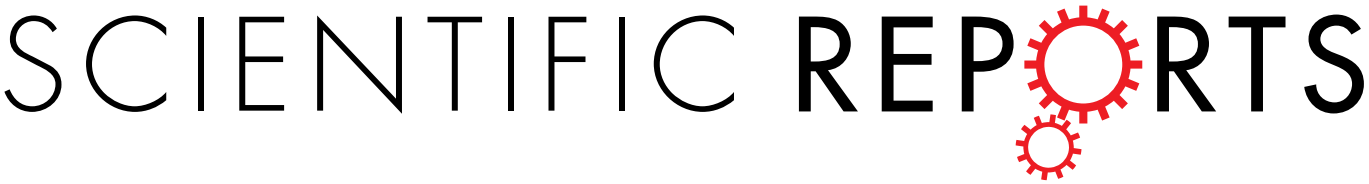

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Serum hepatitis B core antibody as a biomarker of hepatic inflammation in chronic hepatitis B patients with normal alanine aminotransferase

Received: 25 November 2016
Accepted: 25 April 2017
Published online: 05 June 2017

Jiyuan Zhou[1], Liuwei Song[2], Hong Zhao[1], Linlin Yan[1], Anlin Ma[3], Shibin Xie[4], Xuqing Zhang[5], Dazhi Zhang[6], Qing Xie[7], Guo Zhang[8], Jia Shang[9], Jun Cheng[10], Weifeng Zhao[11], Zhiqiang Zou[12], Mingxiang Zhang[13], Ningshao Xia[2] & Guiqiang Wang[1,14]

Our previous studies unexpectedly indicated that the level of serum hepatitis B core antibody (anti-HBc) was positively correlated with the serum alanine aminotransferase (ALT) level. The aim of this study was to determine whether anti-HBc could serve as a potential biomarker for the detection of liver inflammation in chronic hepatitis B (CHB) patients, especially in patients with normal ALT levels. Serum anti-HBc levels were quantified in 655 treatment-naïve CHB patients, including 45 patients who underwent two liver biopsies (baseline phase and the 78th weeks of antiviral-treatment). Serum anti-HBc levels increased significantly along with the increasing histology activity index (HAI) score. After antiviral-treatment, patients with HAI score reduction had significant decline in serum anti-HBc level. Multivariate analysis showed that anti-HBc was independently associated with moderate-to-severe hepatic inflammation in patients with normal ALT level. Furthermore, serum anti-HBc showed a high diagnostic accuracy for predicting moderate-to-severe inflammation in both hepatitis B e antigen (HBeAg)-positive and HBeAg-negative CHB patients with normal ALT levels (area under the curve, AUC = 0.87 and 0.75; respectively). Thus, anti-HBc may be a strong indicator for assessing the hepatic inflammatory degree and used for antiviral treatment decisions in CHB patients with normal ALT levels.

Hepatitis B virus (HBV) infection is the most common chronic viral infection, and is the major cause of hepatocellular carcinoma (HCC), one of the most frequent cancers in Asian-Pacific region especially in China[1,2].

HBV is not cytopathogenic and is indirectly involved in the occurrence of hepatocyte damage and necroinflammation by immune-mediated responses[3]. In the progression of chronic hepatitis B (CHB), the persistent

[1]Department of Infectious Disease, Center for Liver Disease, Peking University First Hospital, Beijing, China. [2]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health, Xiamen University, Xiamen, China. [3]Department of Infectious Disease, China-Japan Friendship Hospital, Beijing, China. [4]Department of Infectious Disease, The Third Affiliated Hospital Sun Yat-Sen University, Guangzhou, Guangdong, China. [5]Department of Infectious Diseases, South West Hospital affiliated to Third Military Medical University, Chongqing, China. [6]Department of Infectious Diseases, Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. [7]Department of Infectious Diseases, Rui Jin Hospital Shanghai Jiao Tong University School of Medicine, Shanghai, China. [8]Department of Infectious Diseases, The People's Hospital of Guang Xi Zhuang Autonomous Region, Nanning, Guangxi, China. [9]Department of Infectious Diseases, The People's Hospital of He Nan Province, Zhengzhou, Henan, China. [10]Department of Infectious Diseases, Di Tan Hospital affiliated to Capital Medical University, Beijing, China. [11]Department of Infectious Diseases, Xinxiang Medical University Third Hospital, Xinxiang, Henan, China. [12]Department of Infectious Diseases, Yan tai City Hospital for Infectious Disease, Yan tai, Shandong, China. [13]Department of Infectious Diseases, Shenyang Sixth People's Hospital, Shenyang, Liaoning, China. [14]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou, Zhejiang, China. Correspondence and requests for materials should be addressed to N.X. (email: nsxia@xmu.edu.cn) or G.W. (email: john131212@126.com)

Parameters	HBeAg (+) Patients (n = 404)			HBeAg (−) Patients (251)		
	Normal ALT (n = 98)	ALT > ULN (n = 306)	*P* value	Normal ALT (n = 95)	ALT > ULN (n = 156)	*P* value
Sex distribution, males	75.5% (74/98)	80.8% (248/306)	0.25	63.2% (60/95)	84% (131/156)	<0.001
age, years	37.56 ± 10.07	35.90 ± 10.4	0.14	42.26 ± 10.61	41.25 ± 8.98	0.37
BMI (kg/m^2)	22.7 ± 2.54	24.31 ± 16.69	0.12	23.87 ± 3.04	23.49 ± 2.71	0.23
HBsAg (log_{10} IU/ml)	3.86 ± 0.95	3.82 ± 0.76	0.42	3.17 ± 0.65	3.15 ± 0.77	0.99
HBV DNA (log_{10} IU/ml)	6.78 ± 1.69	6.97 ± 1.65	0.35	4.43 ± 1.61	5.09 ± 1.61	0.002
Anti-HBc (log_{10} IU/ml)	3.94 ± 1.09	4.41 ± 0.82	<0.001	4.30 ± 0.65	4.58 ± 0.55	<0.001
ALT (U/L)	27.35 ± 8.36	131.4 ± 151.4	<0.001	27.53 ± 8.59	112.8 ± 122.8	<0.001
AST (U/L)	28 ± 13.76	77.6 ± 86.5	<0.001	27.50 ± 10.92	74.32 ± 83.46	<0.001
ALP (U/L)	69.34 ± 22.33	84.68 ± 28.18	<0.001	76.79 ± 23.95	86.34 ± 30.71	0.03
GGT (U/L)	30.42 ± 31.18	62.27 ± 63.20	<0.001	33.15 ± 33.92	64.76 ± 62.30	<0.001
Tbil	14.42 ± 7.01	17.36 ± 17.34	0.15	19.48 ± 41.59	17.87 ± 11.82	0.07
Albumin (g/L)	44.04 ± 4.39	43.64 ± 5.36	0.28	44.89 ± 5.39	44.92 ± 6.44	0.41
PT s	12.76 ± 1.65	12.81 ± 1.45	0.47	12.33 ± 1.41	12.68 ± 1.52	0.08
PTA %	98.66 ± 19.58	96.60 ± 16.64	0.58	98.97 ± 14.89	95.30 ± 17.96	0.2
INR	1.04 ± 0.15	1.04 ± 0.12	0.42	1 ± 0.09	1.04 ± 1.11	0.03
Platelet counts ($x10^9$/L)	186.5 ± 57.61	176.3 ± 55.37	0.17	167.7 ± 52.46	153.6 ± 56.45	0.04
Histology (n, %)						
HAI Score 0–4	64.3% (63/98)	30.1% (92/306)	<0.001	63.2% (60/95)	32.7% (51/156)	<0.001
≥5	35.7% (35/98)	69.9% (214/306)		36.8% (35/95)	67.3% (105/156)	
Fibrosis Score 0–2	66.3% (65/98)	62.4% (191/306)	0.55	68.4% (65/95)	53.8% (84/156)	0.02
≥3	33.7% (33/98)	37.6% (115/306)		31.6% (30/95)	46.2% (72/156)	

Table 1. Clinical characteristics of patients with chronic hepatitis B virus infection. MBI, body mass index; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBc, hepatitis B core antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; Tbil, total bilirubin; PT, prothrombin time; PTA, prothrombin time activity; INR, international normalized ratio; HAI, histology activity index.

inflammation burden of the liver is not only the main risk factor for the development of liver cirrhosis and hepatocellular carcinoma (HCC), but it also leads to ineffective HBV clearance[3–5]. Clinically, serum alanine aminotransferase (ALT) has been widely used for evaluating the severity of hepatic inflammation in liver disease. However, numerous studies have reported that some CHB patients with normal ALT levels have severe liver damage and require antivirus treatment according to current guidelines[6,7]. Therefore, the accurate assessment and monitoring of the severity of liver inflammation plays an important role not only in the control of disease progression, but in the therapy decision for patients with normal ALT levels.

Recently, quantitative antibodies to hepatitis B core antigen (anti-HBc) levels have been reported to predict the treatment response for CHB patients receiving antiviral therapies[8–10]. Patients with high baseline anti-HBc levels had a significantly higher response than patients with low baseline anti-HBc level. Furthermore, our previous studies also suggested that the anti-HBc level was positively correlated with ALT and that the proposed serum anti-HBc level could be a potential biomarker for hepatic inflammatory activity in CHB patients[9]. However, no direct evidence has reported.

Therefore, we aimed to determine whether serum anti-HBc could serve as a potential biomarker for the detection of the severity of liver inflammation, and used for antiviral treatment decisions CHB patients with normal ALT levels.

Results

CHB patients' characteristics. 655 CHB patients were enrolled in the cohort study, which included 404 hepatitis B e antigen (HBeAg) -positive (HBeAg [+]) and 251 HBeAg-negative (HBeAg [+]) CHB patients. In 98 HBeAg (+) patients with normal ALT levels, 35 (35.7%) patients had at least moderate inflammation, and 33 (33.7%) had significant fibrosis. In 95 HBeAg (−) patients with normal ALT levels, 35 (36.8%) patients had at least moderate inflammation, and 30 (31.6%) had significant fibrosis. The CHB patient's characteristics at the time of liver biopsy are summarized in Table 1.

Significantly increased levels of serum anti-HBc correlate with histological inflammation in CHB patients. In the HBeAg (+) CHB patients, serum anti-HBc levels increased significantly along with the increasing histology activity index (HAI) score (mean ± SD; HAI 0–4: 3.80 ± 1.10 vs. HAI 5–6: 4.45 ± 0.60 vs. HAI 7–9: 4.71 ± 0.55 vs. HAI 10–18: 5.02 ± 0.30, $P < 0.001$ by ANOVA) (Fig. 1a). In the HBeAg (−) CHB patients, serum anti-HBc levels also differed significantly between HAI score (HAI 0–4: 4.21 ± 0.53 vs. HAI 5–6: 4.54 ± 0.60, $P < 0.001$; HAI 5–6 vs. HAI 7–9: 4.74 ± 0.56, $P < 0.05$; HAI 7–9 vs. HAI 10–18: 5.06 ± 0.37, $P < 0.05$) (Fig. 1b). Spearman's correlation analysis indicated that serum anti-HBc levels were positively correlated with the HAI score in both HBeAg (+) and HBeAg (−) CHB patients (r = 0.549 and 0.494, respectively, $P < 0.001$).

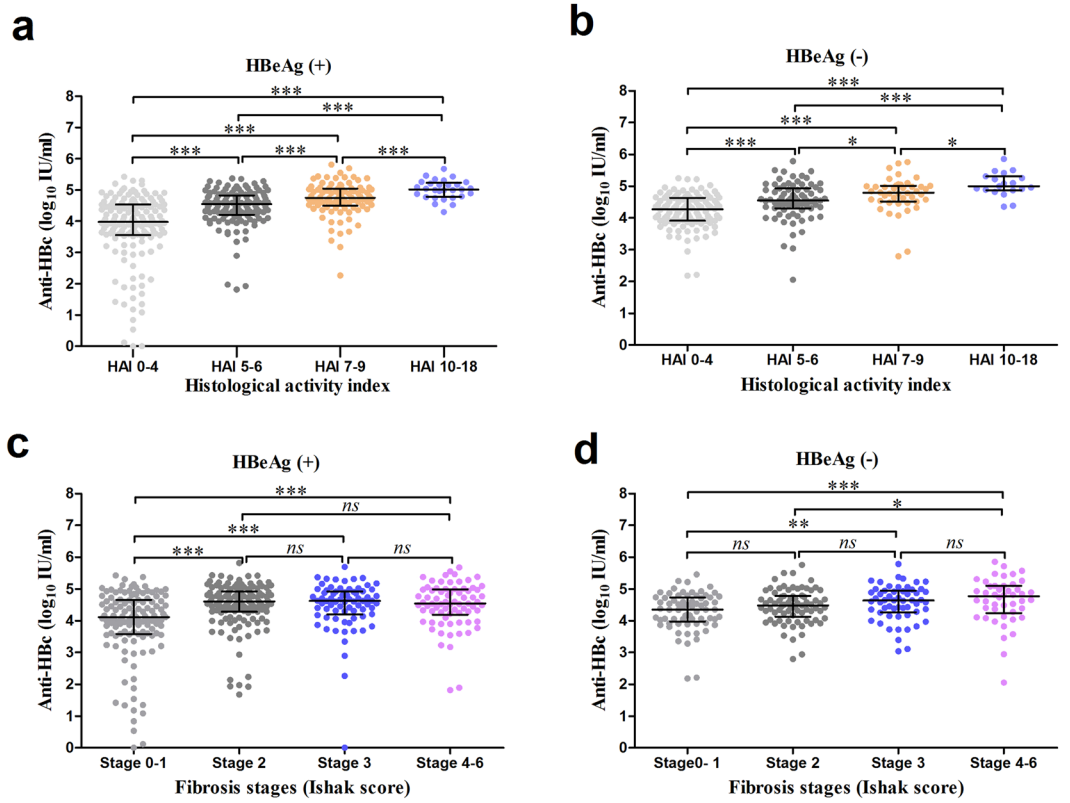


Figure 1. Serum anti-HBc level increased significantly along with increasing histology activity index score. Serum anti-HBc level in different stages of histology activity index score in total HBeAg (+) (**a**) and HBeAg (−) (**b**) CHB patients. Serum anti-HBc collected from patients with different stages of CHB-related liver inflammation was quantified for the levels of anti-HBc using ELISA. Graph showing correlation between serum anti-HBc level and stages of liver fibrosis HBeAg (+) (**c**) and HBeAg (−) (**d**) CHB patients. Interquartile ranges with medians presented. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and *ns*, no significance.

Serum anti-HBc were significantly correlated with liver fibrosis stage in both HBeAg (+) and HBeAg (−) patients (r = 0.233 and 0.240, respectively, $P < 0.001$), whereas there was no significant difference in the anti-HBc level between fibrosis stage score more than 2 (Fig. 1c,d).

The level of serum anti-HBc decreased along with alleviated histological inflammation in CHB patients receiving antivirus treatment. To evaluate the correlation between anti-HBc and the severity of liver inflammation in a longitudinal cohort, of the aforementioned 655 patients, 45 CHB patients (29 HBeAg [+] and 16 HBeAg [−]) undergoing second liver biopsies after 78 weeks of antiviral-treatment were further analyzed. The levels of serum anti-HBc decreased in accordance with the alleviated histological inflammation, and the levels were significantly different between the baseline and 78th week time point ($P < 0.001$, Fig. 2a–d). However, in this study, the fibrosis score remained stable after 78 weeks antiviral therapy (Supplementary Figure S1).

As shown in Fig. 2e–f, there was a significant correlation of between the anti-HBc level and HAI score both in the first liver biopsy (baseline) and the second liver biopsy (78th week) (r = 0.430 $P = 0.022$ and r = 0.461 $P = 0.001$; respectively), suggested that serum anti-HBc levels were positively correlated with severity of hepatic inflammation in the longitudinal cohort study.

Serum anti-HBc was better than ALT in correlation with histological inflammation in CHB patients. Clinically, ALT as a noninvasive marker has been widely used for detecting the severity of hepatic inflammation in liver disease. To compare anti-HBc to ALT, the correlation between ALT and HAI score was further analyzed. In both HBeAg (+) and HBeAg (−) patients, serum ALT levels were significantly correlated with HAI score in patients with ALT >2 × ULN (r = 0.207 $P = 0.01$ and 0.311 $P < 0.01$; respectively), whereas that was no significance in patients with normal ALT levels (r = 0.126 $P = 0.21$ and r = 0.156 $P = 0.13$; respectively) and patients with 1 × ULN < ALT ≤ 2 × ULN (r = 0.122 $P = 0.12$ and r = 0.080 $P = 0.46$; respectively). Nevertheless, in both HBeAg (+) and HBeAg (−) CHB patients, the correlation between serum anti-HBc and HAI score in patients with normal ALT levels (r = 0.617 and 0.378, respectively; all $P < 0.001$) and patient with ULN < ALT ≤ 2 × ULN (r = 0.427 and 0.428, respectively; all $P < 0.001$) were much better than that in patients with ALT >2 × ULN (r = 0.263 $P < 0.01$ and 0.232 $P = 0.06$; respectively) (Table 2). Therefore, serum anti-HBc could be better than ALT in correlation with HAI score in patients with ALT ≤2 × ULN.

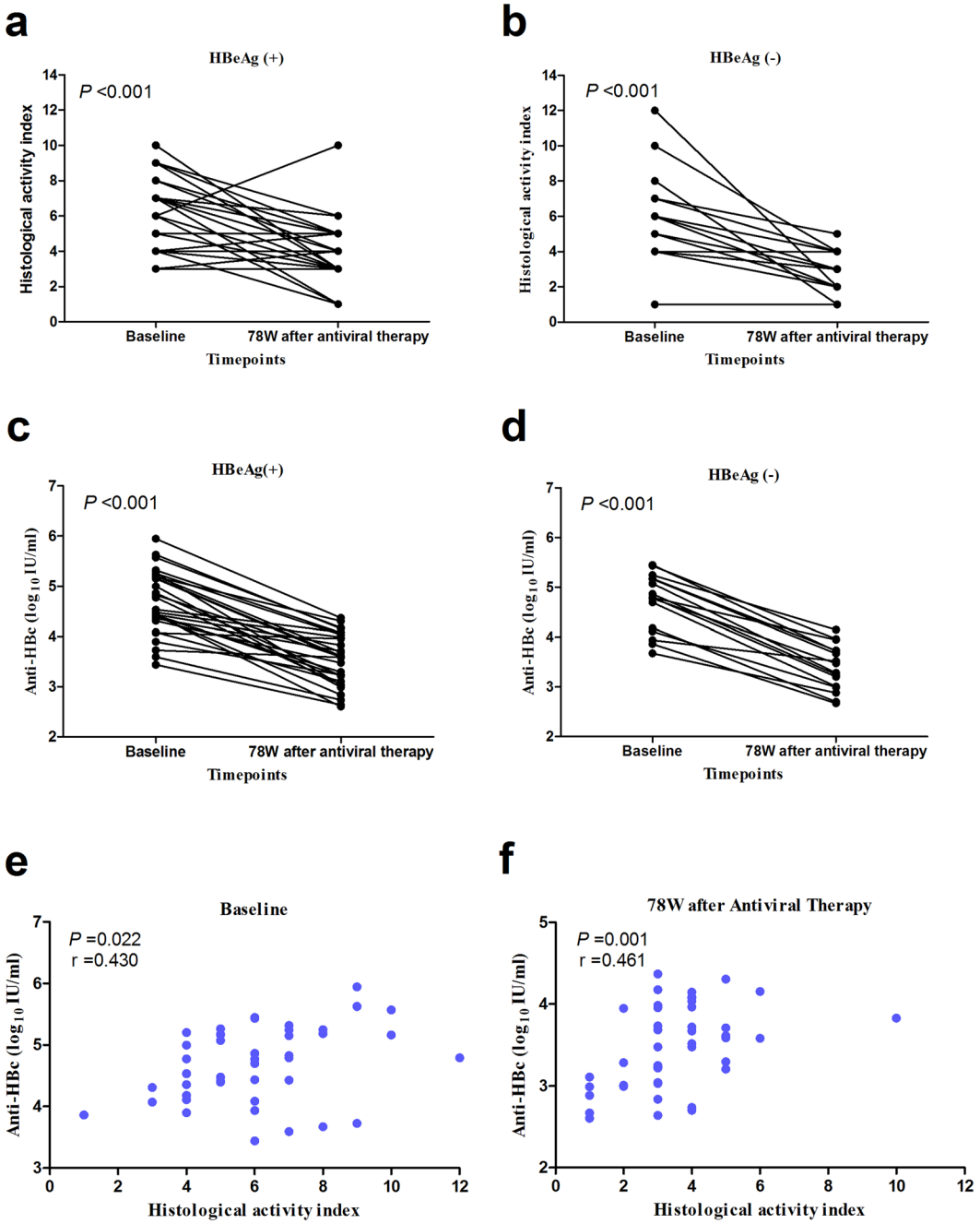


Figure 2. The level of serum anti-HBc decreased along with alleviated histological inflammation in CHB patients receiving antivirus treatment. Dynamic changes of histological activity index score in HBeAg (+) (**a**) and HBeAg (−) (**b**) CHB patients receiving antiviral treatment after a second liver biopsy. Dynamic changes of serum anti-HBc levels in HBeAg (+) (**c**) and HBeAg (−) (**d**) CHB patients receiving antiviral treatment after a second liver biopsy. The correlation between serum anti-HBc levels and HAI score in the baseline (**e**) and 78th week time point (**f**).

Serum anti-HBc can significantly differentiate between mild or no inflammation and moderate-to-severe inflammation in patients with normal ALT levels. Patients with moderate-to-severe inflammation had significantly higher levels of HBsAg, HBV DNA, anti-HBc, ALT, AST, ALP, GGT and Albumin than the patients with mild or no inflammation. Multivariate analysis indicated that anti-HBc was independently associated with moderate-to-severe inflammation in both HBeAg (+) and HBeAg (−) CHB patients (Supplementary Tables S2 and 3). However, in this study, anti-HBc was not independently associated with significant fibrosis in CHB patients by univariate and multivariate analysis (Supplementary Tables S4 and 5).

Furthermore, we further divided the recruited patients into the following four groups based on the Asian-Pacific consensus statement on the management of chronic hepatitis B: Low normal group, ALT $\leq 0.5 \times$ ULN; High normal group, ALT 0.5–1 $\times$ ULN; Minimally raised group, ALT 1–2 $\times$ ULN and raised groups,

	Correction	ALT stratum (*Spearman's r; P* value)		
		ALT < 1 × ULN	1 × ULN < ALT < 2 × ULN	ALT > 2 × ULN
HBeAg + (404)	ALT vs. anti-HBc	0.044; 0.66	0.138; 0.08	0.250; <0.01
	ALT vs. HAI	0.126; 0.21	0.122; 0.12	0.207; 0.01
	anti-HBc vs. HAI	0.617; <0.001	0.427; <0.001	0.263; <0.01
HBeAg− (251)	ALT vs. anti-HBc	0.191; 0.06	0.195; 0.06	0.219; 0.03
	ALT vs. HAI	0.156; 0.13	0.080; 0.46	0.311; <0.01
	anti-HBc vs. HAI	0.378; <0.001	0.428; <0.001	0.232; 0.06

Table 2. Correlation between histology activity index score and anti-HBc levels, ALT levels according to the ALT stratum. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen ULN, upper limit of normal; HAI, histology activity index.

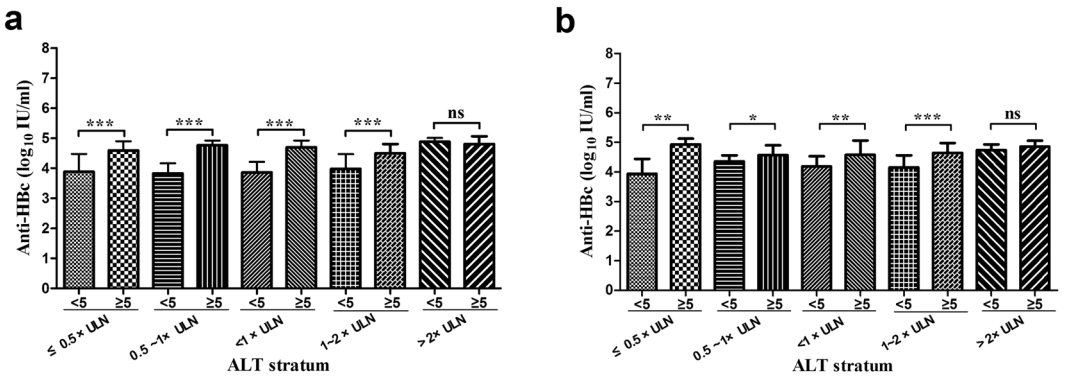


Figure 3. Correlation between Anti-HBc levels and histological scores of activity in HBeAg (+) (**a**) and HBeAg (−) (**b**), according to the ALT stratum. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and *ns*, no significance. Interquartile ranges with medians presented. ALT, alanine aminotransferase; ULN, upper limit of normal; HAI, histology activity index.

ALT >2 × ULN[4]. As shown in Fig. 3, apart from raised groups, patients with moderate-to-severe inflammation had significantly higher levels of serum anti-HBc than the patients with mild or no inflammation in all groups. Altogether, these results indicate that serum anti-HBc as a potential biomarker could be used for differentiating between mild or no inflammation and moderate-to-severe inflammation in patients with ALT ≤2 × ULN, especially in patients with normal ALT levels.

Serum anti-HBc was an independent predictors of moderate-to-severe inflammation in patents with normal ALT levels. To investigate whether anti-HBc is independently associated with moderate-to-severe inflammation in patents with normal ALT levels, the univariate and multivariate analysis of clinical parameters, anti-HBc with liver inflammation was further analyzed. In HBeAg (+) CHB patients, the serum levels of HBsAg, HBV DNA, anti-HBc, AST, GGT, TBil and platelet counts were significantly correlated with moderate-to-severe inflammation based on the univariate analysis, whereas the multivariate logistic regression analysis indicated that the anti-HBc ($P = 0.009$, odd ratio = 4.78), HBV DNA ($P = 0.04$, odd ratio = 0.64) and TBil ($P = 0.03$, odd ratio = 1.02) could be used to independently predict moderate-to-severe inflammation (Table 3).

In HBeAg (−) CHB patients, the univariate analysis identified anti-HBc, AST and GGT were significantly associated with moderate-to-severe inflammation. The multivariate analysis indicated that only anti-HBc ($P < 0.001$, odd ratio = 5.40) was an independent predictors of moderate-to-severe inflammation (Table 4).

Serum anti-HBc holds a better predictive value for differentiating between mild or no and moderate-to-severe in patients with normal ALT levels. ROC curves were created to evaluate the diagnostic efficiency of serum anti-HBc for differentiating between mild or no inflammation and moderate-to-severe inflammation in CHB patients with normal ALT levels. As shown in Fig. 4, the diagnostic value of anti-HBc was assessed. In HBeAg (+) CHB patients, the AUC of anti-HBc for the prediction of moderate-to-severe inflammation was 0.87. Maximizing the sum of sensitivity and specificity, the optimal cut-offs of anti-HBc was 4.47 $\log_{10}$ IU/mL. According to obtaining a specificity of at least 95%, the cut-offs of anti-HBc was 4.67 $\log_{10}$ IU/mL. In HBeAg (−) CHB patients, the AUC of anti-HBc for the prediction of moderate-to-severe inflammation was 0.75. The optimal cut-offs of anti-HBc was 4.47 $\log_{10}$ IU/mL with a maximizing the sum of sensitivity and specificity and 5.00 $\log_{10}$ IU/mL with a specificity of 95%.

It is worth noting that the AUCs of AST and GGT were significantly less effective than anti-HBc for differentiating mild or no inflammation from moderate-to-severe inflammation. Furthermore, the combination index was constructed to explore whether considering HBV DNA and TBil to anti-HBc levels in addition to anti-HBc

Parameters	Univariate analysis		*P* Value (Univariate)	Multivariate analysis	*P* Value (Multivariate)
	HAI < 5 (n = 63)	HAI ≥ 5 (n = 35)		Odds Ratio (95% CI)	
Sex distribution, males	74.6% (47/63)	77.14% (27/35)	1		
age, years	37.11 ± 10.38	38.37 ± 9.58	0.39		
BMI (kg/m^2)	22.86 ± 2.64	22.40 ± 2.34	0.47		
HBsAg (log_{10} IU/ml)	4.05 ± 1.05	3.51 ± 0.61	<0.001	1.27 (0.59–2.76)	0.54
HBV DNA (log_{10} IU/ml)	7.16 ± 1.60	6.12 ± 1.66	0.001	0.64 (0.42–0.98)	0.04
Anti-HBc (log_{10} IU/ml)	3.89 ± 0.89	4.44 ± 0.71	<0.001	4.78 (1.48–15.43)	0.009
ALT (U/L)	26.92 ± 7.98	28.13 ± 9.07	0.34		
AST (U/L)	24.10 ± 6.25	35.23 ± 19.86	<0.001	1.08 (1.00–1.17)	0.33
ALP (U/L)	67.59 ± 20.17	72.67 ± 26.00	0.65		
GGT (U/L)	23.77 ± 16.37	42.64 ± 45.63	0.003	1.02 (0.99–1.06)	0.56
Tbil	12.52 ± 4.57	17.93 ± 9.17	<0.001	1.02 (0.99–1.07)	0.03
Albumin	44.34 ± 4.02	43.47 ± 5.01	0.36		
PT s	12.74 ± 0.96	12.82 ± 2.48	0.49		
PTA %	100.2 ± 19.76	95.88 ± 19.23	0.67		
INR	1.007 ± 0.089	1.06 ± 0.20	0.47		
Platelet counts (x10^9/L)	199.3 ± 54.26	163.3 ± 56.93	0.003	1.00 (0.99–1.01)	0.78

Table 3. Univariate and multivariate analysis of clinical parameters, cytokines with histological activity index in HBeAg (+) patients with normal ALT levels (n = 98). MBI, body mass index; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBc, hepatitis B core antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; Tbil, total bilirubin; PT, prothrombin time; PTA, prothrombin time activity; INR, international normalized ratio.

would lead to a better diagnosis of HBeAg (+) CHB patients with moderate-to-severe inflammation using binary logistic regression. The best formula for predicting moderate-to-severe inflammation was (2.081 × log_{10}Anti-HBc IU/ml) − (0.345 × log_{10}HBV DNA IU/ml) + (0.134 × TBil IU/ml) −9.008. However, the addition these variables to anti-HBc did not improve its diagnostic efficiency.

Discussion

To our knowledge, this is the first study to evaluate the serum anti-HBc as a strong indicator for assessing the hepatic inflammatory degree in CHB patients with normal ALT levels. The results of these analyses are supported by the large, multicentral cohorts and strict pathological assessments. The results indicated that the levels of anti-HBc increased significantly along with the increasing HAI score in patients with normal ALT levels. In addition, compared to other predictors, the diagnostic performance of serum anti-HBc for the differentiating between mild or no inflammation and moderate-to-severe inflammation was excellent in both HBeAg (+) and HBeAg (−) CHB patients with normal ALT levels (AUC = 0.87 and 0.75; respectively).

Active significant hepatic inflammation is the main risk factor for developing cirrhosis and HCC in CHB patients[2,4]. The latest guidelines suggested that early control of developing liver inflammation should be the priority for the detection of liver fibrosis[11]. Thus, accurate evaluation of the initial stage of liver inflammation and progression over time represents a high priority and growing medical need. To date, percutaneous liver biopsy followed by histological analysis remains the gold standard for evaluating the severity of liver injury. Because it is a costly and invasive procedures with inherent risks, the clinical use of this diagnostic method has been restricted[12,13]. Over the past decades, several non-invasive methods have been developed or are being validated, especially the diagnosis of liver fibrosis[14,15]. However, there have been few novel markers for assessing the severity of liver inflammation. In clinical practice, serum ALT is an easily accessible surrogate parameter and is commonly used for monitoring the state of liver inflammation[16]. The present study indicated that approximately 36.6% (70/193) of CHB patients with normal ALT levels have moderate-to-severe hepatic inflammation (Table 1), which is higher than the previous reports[4,6]. For these patients, recent guidelines recommend that CHB patients with moderate-to-severe hepatic inflammation (HAI ≥5) should be considered for antiviral therapy immediately[4,11]. Thus, use of ALT could be weakness in assessing liver injury and may underestimates the proportion of patients who urgently need antiviral therapy in patients with normal ALT levels. Recent studies have noted the possibility of lowering ULN to 30 IU/L for men and 19 IU/L for women[17,18]. However, the majority of countries in Asia including China, still use an ALT of 40 IU/ml as ULN[4,19]. The results of our study may provide support for decreasing the ULN of ALT in Chinese patients, and reducing the portion of patients with moderate-to-severe hepatic inflammation.

Anti-HBc is one of the most universal serological markers for HBV infection and can generally persist throughout a patient's life, regardless of acute, chronic or past HBV infections[20,21]. In this study, serum anti-HBc levels were not only correlated positively with the severity of liver inflammation in baseline of total patients, but decreased along with alleviated histological inflammation in CHB patients receiving antivirus treatment. Furtherly, we analyzed the correlation between HAI score and serum anti-HBc, ALT in CHB patients, and the results indicated that serum anti-HBc was better than ALT in correlation with severity of liver inflammation in

Parameters	Univariate analysis		*P* Value (Univariate)	Multivariate analysis	*P* Value (Multivariate)
	HAI < 5 (n = 60)	HAI ≥ 5 (n = 35)		Odds Ratio (95% CI)	
Sex distribution, males	65% (39/60)	60% (21/35)	0.66		
age, years	39.68 ± 11.54	37.89 ± 9.23	0.64		
BMI (kg/m^2)	22.95 ± 2.62	22.43 ± 2.54	0.24		
HBsAg ($\log_{10}$ IU/ml)	3.66 ± 0.92	3.43 ± 0.57	0.33		
HBV DNA ($\log_{10}$ IU/ml)	4.34 ± 1.33	4.57 ± 2.02	0.58		
Anti-HBc ($\log_{10}$ IU/ml)	3.81 ± 1.02	4.79 ± 0.64	<0.001	5.40 (1.91–15.29)	<0.001
ALT (U/L)	26.78 ± 8.22	28.8 ± 9.17	0.2		
AST (U/L)	25.65 ± 10.35	30.67 ± 11.29	0.004	1.01 (0.99–1.09)	0.13
ALP (U/L)	75.07 ± 26.11	79.85 ± 24.29	0.35		
GGT (U/L)	26.74 ± 27.54	44.61 ± 41.07	<0.001	1.01 (0.99–1.03)	0.45
Tbil	15.16 ± 7.38	26.88 ± 67.82	0.84		
Albumin	45.21 ± 4.54	44.34 ± 6.66	0.21		
PT s	12.36 ± 1.41	12.29 ± 1.45	0.62		
PTA %	99.59 ± 15.91	97.94 ± 13.19	0.93		
INR	1.00 ± 0.09	1.02 ± 0.10	0.63		
Platelet counts (x10^9/L)	172.2 ± 48.31	160.0 ± 58.75	0.07		

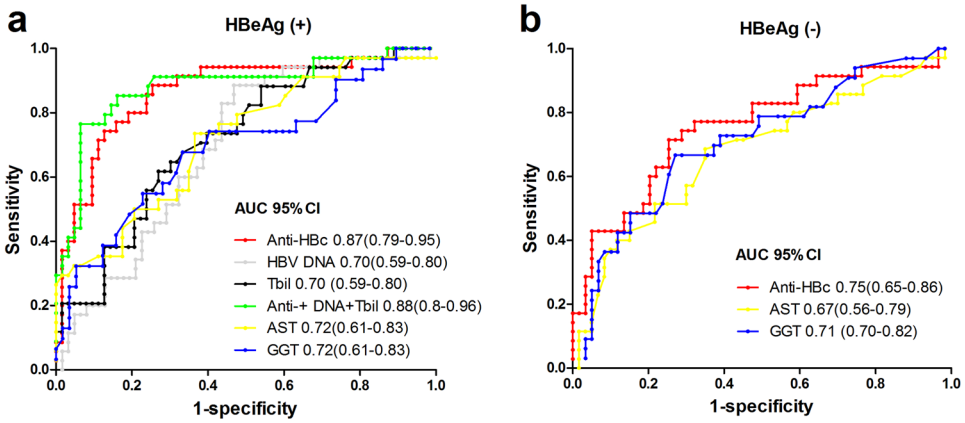
Table 4. Univariate and multivariate analysis of clinical parameters, cytokines with histological activity index in HBeAg (−) patients with normal ALT levels (n = 95). MBI, body mass index; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBc, hepatitis B core antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; Tbil, total bilirubin; PT, prothrombin time; PTA, prothrombin time activity; INR, international normalized ratio.

patients with ALT ≤2 × ULN, especially in patients with normal ALT levels (Table 2). In addition, anti-HBc were significantly different between moderate-to-severe inflammation and no mild inflammation in different groups from patients with normal ALT level. Thus, based on the aforementioned results, we hypothesized that the level of serum anti-HBc could be a potential biomarker for accurate assessment of the severity of liver inflammation in patients with normal ALT levels.

In the present study, we are first to report that increasing levels of serum anti-HBc were independently associated with moderate-to-severe hepatic inflammation in both HBeAg (+) and HBeAg (−) CHB patients with normal ALT levels (odd ratio = 4.78 $P = 0.009$ and odd ratio = 5.40 $P < 0.001$; respectively) based on the multivariate analysis (Tables 3 and 4). The possible mechanism underlying the roles of anti-HBc in hepatic inflammation throughout the progression of chronic HBV infection is still unknown. Chronic HBV infection involves different cells of the innate and adaptive immune responses[1]. During a cellular immune response, the T lymphocytes subgroups such as $CD4^+$ and $CD8^+$ cells respond to this virus and can directly cause the persistent inflammation of chronic hepatitis B[1]. However, the role of B-lymphocytes in the severity of chronic hepatitis B is unclear[22]. A recent study reported that high prevalence of activated B cells plays a crucial role in the progression of CHB infection by B lymphocytes-mediated immune response to HBV[23]. Hepatitis B core protein (HBcAg) is the most immunogenic HBV antigen, and its antibody, anti-HBc secreted via the activated antibody-secreting B cells directly play an important role in the severity of chronic hepatitis B by hepatocytotoxic response[22]. Furthermore, Li *et al*. found that circulating $CXCR5^+CD4^+$ T cells can help naïve B cells to produce anti-HBc via IL-21 in patients with chronic HBV infection[24]. The percentage of circulating $CXCR5^+CD4^+$ T cells was positively correlated with serum levels of ALT and AST, suggesting that the frequency and phenotype of CXCR5+CD4+ T cells is associated with HBV-related liver damage[25]. Therefore, we hypothesized that the anti-HBc could play an important role in liver inflammation of CHB patients through the hepatocytotoxic effects of anti-HBc-secreting B-lymphocytes. Anti-HBc may be a strong indicator for liver damage during a certain cellular immune response in CHB patients, and further studies on the mechanism of anti-HBc involvement hepatocellular injury will be worthwhile.

In HBeAg (+) CHB patient with normal ALT levels, 81.63% of the patients with moderate-to-severe inflammation were correctly identified using anti-HBc specific low cutoff value of 4.47 $\log_{10}$ IU/mL (which was established by maximizing the sum of sensitivity and specificity). And using anti-HBc specific high cut-off of 4.67 $\log_{10}$ IU/mL (which established by obtaining a specificity of at least 95%), 95.24% of the patients with mild or no inflammation (PPV 85.71%) were correctly excluded. In HBeAg (+) patient with normal ALT levels, using anti-HBc specific low cut-off value of 4.47 $\log_{10}$ IU/mL, 67.47% of the patients with moderate-to-severe inflammation were correctly distinguished. 95% of the patients with mild or no inflammation (PPV 75%) were correctly excluded using the cut-off of 5.00 $\log_{10}$ IU/mL. Therefore, to evaluate liver inflammation caused by HBV infection, the measurement of serum anti-HBc levels may provide accurate evidence to determine the antiviral choice in patients with normal ALT levels.

In conclusion, we are the first to demonstrate that serum anti-HBc is significantly corrected with hepatic inflammation in CHB patients with normal ALT. Serum anti-HBc is a promising noninvasive clinical biomarker that exhibits a high diagnostic accuracy for moderate-to-severe hepatic inflammation in patients with normal ALT levels.



	Anti-HBc Cutoff Value (log_{10}IU/ml)	Liver inflammation HAI < 5	Liver inflammation HAI ⩾ 5	Se (%)	Spe (%)	PPV (%)	NPV (%)	Correctly classified (%)
HBeAg (+)								
	> 4.47	10	27	77.14	84.13	72.97	86.89	81.63
	⩽4.47	53	8					
	> 4.67	3	18	51.43	95.24	85.71	77.92	79.59
	⩽4.67	60	17					
HBeAg (-)								
	> 4.47	16	22	62.86	73.33	57.89	77.19	69.47
	⩽4.47	44	13					
	> 5.00	3	9	25.71	95	75	68.67	69.74
	⩽5.00	57	26					

Figure 4. Serum anti-HBc holds a better diagnostic value for differentiating between mild or no (HAI < 5) and moderate-to-severe (HAI ≥ 5) in HBeAg (+) (**a**) and HBeAg (−) (**b**) patents with normal ALT. AUROC, area under receiver operating characteristics curve; GGT, gamma glutamyl transpeptidase; AST, aspartate aminotransferase; Se, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value.

Patients and Methods

Patients. A total of 655 consecutive treatment naïve patients with chronic HBV infection who underwent liver biopsy in 24 teaching hospitals located in mainland of China were enrolled into this study between October 2013 and February 2016. Patients recruited in the cohort study met the following criteria: (1) age 18–75 years; (2) HBsAg seropositive status beyond 6 months; (3) treatment naïve; (4) negative serum levels of anti-HAV IgM, anti-HCV, anti-HEV IgM/IgG, anti-EBV IgM, and anti-CMV IgM; and (5) off potential transaminase-lowering agents such as bicyclol for at least 2 weeks prior to blood sampling biochemistries. Exclusion criteria for this study included overlapping etiologies for hepatitis including HCV, human immunodeficiency virus (HIV), alcohol abuse, autoimmune, genetic, drug-induced and nonalcoholic fatty liver disease. Patients with decompensated cirrhosis or hepatocellular carcinoma were also excluded.

Additionally, of the aforementioned 655 patients, 45 CHB patients who underwent second liver biopsies after 78 weeks of antiviral-treatment were also enrolled.

All patents provided written informed consent for the scientific use of their clinical data and sample. The study was approved by the local ethics committee of Peking University First Hospital, and all methods were performed in accordance with the relevant guidelines and regulations. The complete protocol for the clinical trial has been registered at clinicaltrials.gov (NCT01962155) and chictr.org (ChiCTR-DDT-13003724).

Histologic staging. Ultrasonographic-guided liver biopsies with a length ≥20 mm were routinely performed on all patients according to a standardized protocol after receiving the patient's written informed consent. Pathological assessments were conducted in the Department of Pathology at the You-An Hospital affiliated with Capital Medical University. Each section was blindly and independently assessed by 2 pathologists. When discrepancies occurred, the samples were reviewed by experienced pathologists who were also responsible for reassessment in a randomly selected 10% of samples[26]. Disease activity grade was staged using the modified histology activity index (HAI), and hepatic fibrosis was assessed using the Ishak fibrosis score. For analysis, HAI ≥ 5 was considered moderate-to-severe inflammation, and F ≥ 3 was considered significant fibrosis[27,28].

Laboratory measurements. At the time of liver biopsy, biochemical tests, blood cell and coagulation tests were performed using routine automated analyzers. Serum levels of hepatitis B surface antigen (HBsAg) and hepatitis B e (HBeAb) were quantified using commercially available enzyme immunoassays (Roche Diagnostics, Penzberg, Germany). Serum HBV DNA levels (range 2.0×10^1–1.7×10^8 IU/ml) were measured via a COBAS AmpliPrep/COBAS TaqMan method as previously described[29].

Anti-HBc measurement. Sandwich enzyme-linked immunosorbent assays for serum anti-HBc level were performed as described previously[30].

Statistical analysis. All statistical analyses were performed with SPSS ver.16.0 (Chicago, IL, USA). Quantitative variables were expressed as the mean ± standard deviation (SD). The chi-square test was used to analyze relationships between categorical variables, and Student's *t* test was used to analyze single specific differences of biological interest. Spearman's rank tests were used to analyze associations between variables and stages of hepatic pathology. A logistic regression was performed to analyze whether anti-HBc was an independent risk factor for significant inflammation in CHB patients.

The diagnostic abilities of anti-HBc and combination between anti-HBc and different variables were evaluated based on the estimated ROC curves and by calculations of the sensitivity, specificity, positive predictive values (PPV) and negative predictive value (NPV) for cut-off values. Statistical significance was defined as $P < 0.05$ (two-tailed).

References

1. Liaw, Y. F. & Chu, C. M. Hepatitis B virus infection. *Lancet* **373**, 582–592, doi:10.1016/s0140-6736(09)60207-5 (2009).
2. Trepo, C., Chan, H. L. & Lok, A. Hepatitis B virus infection. *Lancet* **384**, 2053–2063, doi:10.1016/s0140-6736(14)60220-8 (2014).
3. Bertoletti, A., Maini, M. & Williams, R. Role of hepatitis B virus specific cytotoxic T cells in liver damage and viral control. *Antiviral Res* **60**, 61–66 (2003).
4. Sarin, S. K. *et al.* Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatology international* **10**, 1–98, doi:10.1007/s12072-015-9675-4 (2016).
5. Berasain, C. *et al.* Inflammation and liver cancer: new molecular links. *Annals of the New York Academy of Sciences* **1155**, 206–221, doi:10.1111/j.1749-6632.2009.03704.x (2009).
6. Chao, D. T., Lim, J. K., Ayoub, W. S., Nguyen, L. H. & Nguyen, M. H. Systematic review with meta-analysis: the proportion of chronic hepatitis B patients with normal alanine transaminase </=40 IU/L and significant hepatic fibrosis. *Alimentary pharmacology & therapeutics* **39**, 349–358, doi:10.1111/apt.12590 (2014).
7. Alam, S. *et al.* Evaluation of normal or minimally elevated alanine transaminase, age and DNA level in predicting liver histological changes in chronic hepatitis B. *Liver international: official journal of the International Association for the Study of the Liver* **31**, 824–830, doi:10.1111/j.1478-3231.2011.02491.x (2011).
8. Yuan, Q. *et al.* Quantitative hepatitis B core antibody level may help predict treatment response in chronic hepatitis B patients. *Gut* **62**, 182–184, doi:10.1136/gutjnl-2012-302656 (2013).
9. Hou, F. Q. *et al.* Quantitative Hepatitis B Core Antibody Level Is a New Predictor for Treatment Response In HBeAg-positive Chronic Hepatitis B Patients Receiving Peginterferon. *Theranostics* **5**, 218–226, doi:10.7150/thno.10636 (2015).
10. Fan, R. *et al.* Baseline quantitative hepatitis B core antibody titre alone strongly predicts HBeAg seroconversion across chronic hepatitis B patients treated with peginterferon or nucleos(t)ide analogues. *Gut*, doi:10.1136/gutjnl-2014-308546 (2015).
11. Terrault, N. A. *et al.* AASLD guidelines for treatment of chronic hepatitis B. *Hepatology (Baltimore, Md.)* **63**, 261–283, doi:10.1002/hep.28156 (2016).
12. Regev, A. *et al.* Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *The American journal of gastroenterology* **97**, 2614–2618, doi:10.1111/j.1572-0241.2002.06038.x (2002).
13. Cadranel, J. F., Rufat, P. & Degos, F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology (Baltimore, Md.)* **32**, 477–481, doi:10.1053/jhep.2000.16602 (2000).
14. Afdhal, N. H. & Nunes, D. Evaluation of liver fibrosis: a concise review. *The American journal of gastroenterology* **99**, 1160–1174, doi:10.1111/j.1572-0241.2004.30110.x (2004).
15. Martinez, S. M., Crespo, G., Navasa, M. & Forns, X. Noninvasive assessment of liver fibrosis. *Hepatology (Baltimore, Md.)* **53**, 325–335, doi:10.1002/hep.24013 (2011).
16. Kim, W. R., Flamm, S. L., Di Bisceglie, A. M. & Bodenheimer, H. C. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology (Baltimore, Md.)* **47**, 1363–1370, doi:10.1002/hep.22109 (2008).
17. Prati, D. *et al.* Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Annals of internal medicine* **137**, 1–10 (2002).
18. Yapali, S., Talaat, N. & Lok, A. S. Management of hepatitis B: our practice and how it relates to the guidelines. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association* **12**, 16–26, doi:10.1016/j.cgh.2013.04.036 (2014).
19. Liao, B. *et al.* Significant fibrosis is not rare in Chinese chronic hepatitis B patients with persistent normal ALT. *PloS one* **8**, e78672, doi:10.1371/journal.pone.0078672 (2013).
20. Hoofnagle, J. H., Seeff, L. B., Bales, Z. B. & Zimmerman, H. J. Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. *The New England journal of medicine* **298**, 1379–1383, doi:10.1056/nejm197806222982502 (1978).
21. Ollier, L., Laffont, C., Kechkekian, A., Doglio, A. & Giordanengo, V. Detection of antibodies to hepatitis B core antigen using the Abbott ARCHITECT anti-HBc assay: analysis of borderline reactive sera. *J Virol Methods* **154**, 206–209, doi:10.1016/j.jviromet.2008.09.006 (2008).
22. Zgair, A. K., Ghafil, J. A. & Al-Sayidi, R. H. Direct role of antibody-secreting B cells in the severity of chronic hepatitis B. *Journal of medical virology*. doi:10.1002/jmv.24067 (2014).
23. Oliviero, B. *et al.* Enhanced B-cell differentiation and reduced proliferative capacity in chronic hepatitis C and chronic hepatitis B virus infections. *J Hepatol* **55**, 53–60, doi:10.1016/j.jhep.2010.10.016 (2011).
24. Li, Y. *et al.* Circulating chemokine (C-X-C Motif) receptor 5(+) CD4(+) T cells benefit hepatitis B e antigen seroconversion through IL-21 in patients with chronic hepatitis B virus infection. *Hepatology (Baltimore, Md.)* **58**, 1277–1286, doi:10.1002/hep.26489 (2013).
25. Hu, T. T. *et al.* Expansion of circulating TFH cells and their associated molecules: involvement in the immune landscape in patients with chronic HBV infection. *Virol J* **11**, 54, doi:10.1186/1743-422x-11-54 (2014).
26. Deng, Y. Q. *et al.* Selected Cytokines Serve as Potential Biomarkers for Predicting Liver Inflammation and Fibrosis in Chronic Hepatitis B Patients With Normal to Mildly Elevated Aminotransferases. *Medicine* **94**, e2003, doi:10.1097/md.0000000000002003 (2015).
27. Wai, C. T. *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology (Baltimore, Md.)* **38**, 518–526, doi:10.1053/jhep.2003.50346 (2003).
28. Hui, A. Y. *et al.* Identification of chronic hepatitis B patients without significant liver fibrosis by a simple noninvasive predictive model. *The American journal of gastroenterology* **100**, 616–623, doi:10.1111/j.1572-0241.2005.41289.x (2005).
29. Jia, W. *et al.* Antibody to hepatitis B core antigen levels in the natural history of chronic hepatitis B: a prospective observational study. *Medicine* **93**, e322, doi:10.1097/md.0000000000000322 (2014).
30. Li, A. *et al.* Novel double-antigen sandwich immunoassay for human hepatitis B core antibody. *Clinical and vaccine immunology: CVI* **17**, 464–469, doi:10.1128/cvi.00457-09 (2010).

Acknowledgements

This study was supported by China Mega-Project for Infectious Diseases (grant numbers 2013ZX10002005, 2012ZX10002006, 2013ZX10002004 and 2012ZX10005005), the Project of Beijing Science and Technology Committee (grant number D121100003912002), the Complications of severe hepatitis, HRS attenuated by improving HRS through IP3R-IP3 (grant number F15-139-9-08), study on the optimization scheme of integrated traditional Chinese and Western Medicine for severe hepatitis, and the National Key Clinical Program of the Ministry of Health.

Author Contributions

Jiyuan Zhou designed and did the ELISA experiments, analyzed data, and contributed to writing and revising the manuscript; Liuwei Song did the ELISA experiments and contributed to discussion; Hong Zhao analyzed data and contributed to discussion; Linlin Yan, Anlin Ma, Shibin Xie, Xuqing Zhang, Dazhi Zhang, Qing Xie, Guo Zhang, Jia Shang, Jun Cheng, Weifeng Zhao, Zhiqiang Zou and Mingxiang Zhang recruited patients; Ningshao Xia and Gui-Qiang Wang designed experiments, analyzed data and provided overall direction. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-03102-3

Competing Interests: The authors declare that they have no competing interests.

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