The Relationship Between Collagen Proportionate Area and Hepatitis B Surface Antigen Levels in E Antigen Positive Hepatitis B Cirrhosis

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ABSTRACT

Background: *Quantitative serum hepatitis B surface antigen (HbsAg) has been widely used as a biomarker for treatment response and prognosis in chronic hepatitis B infection, and has recently been found associated with liver histology in e-antigen positive patients. A histological measurement as a continuous variable—collagen proportionate area (CPA)—is appropriate to assess liver fibrosis degree and substages cirrhosis. We, therefore, aimed to explore the association between serum quantitative HBsAg and CPA in e antigen-positive hepatitis B cirrhosis.*

Methods: *Liver fibrosis staging was evaluated by METAVIR semiquantitative scoring system, only patients with METAVIR fibrosis stage 4 were included. All liver sections were stained with picroSirius red for determination of collagen quantification by digital image analysis.*

Results: *Mean CPA value was 23.46%. The percentage of patients with different classification of CPA (<20%, 20-30%, >30%) were 25.8%, 57.8%, and 16.4%, respectively. A modest correlation was found between CPA and serum HBsAg level (r = –0.306, P =.001). Hepatitis B surface antigen level is independently associated with CPA in multivariable linear regression analyses.*

Conclusion: *Serum HBsAg levels can predict liver fibrosis determined by CPA in HBeAg-positive hepatitis B cirrhosis.*

Keywords: *HBsAg, liver fibrosis, collagen proportionate area, chronic hepatitis B*

INTRODUCTION

About 50 years ago, hepatitis B virus (HBV) infection was identified by detection of serum hepatitis B surface antigen (HBsAg) that was awarded the Nobel prize.[1] To this day, the detection of HBsAg is widely used for HBV infection diagnosis. Although HBsAg quantification has been introduced for more than 30 years, only lately it has been significantly improved and widely used by new commercial automated assays that started the research interest in serum HBsAg quantification as a biomarker for treatment response and prognosis in chronic HBV infection.[2] While quantitative HBsAg has been clinically used for monitoring of peg-interferon treatment and nucleos(t)ide analog treatment,[3] for identifying disease stage[4,5] and for distinguishing patients with HBeAg negative disease from inactive carriers,[6,7] there have been studies that focused on the clinical significance of serum quantitative HBsAg and association with liver histology in patients with hepatitis B e antigen-positive hepatitis B.[8,9]

In a study of chronic hepatitis B (CHB) patients, an association between lower quantitative HBsAg and the presence of significant fibrosis (METAVIR fibrosis stage 2-4) was found in e antigen-positive, treatment-naive patients.[8] In another cohort from Hong Kong, high quantitative HBsAg can predict fibrosis score ≤1 (Ishak scoring system) among HBeAg-positive patients with alanine aminotransferase (ALT) ≤2 ULN.[9] However, the commonly used hepatitis histological scoring systems use similar principles to classify the liver disease stage.[10-12] In the above-mentioned systems, histological stage scores comprise features that mainly depend on architectural changes, with little reference to the direct cause of liver fibrosis—the quantity, quality, and distribution of collagen. Additionally, in advanced liver disease, each of the descriptive morphological stages contains a wide range of quantitative fibrosis values, another limitation of the current morphological category is that no subclassification was contained.[13] Collagen proportionate area (CPA) calculated by digital image analysis (DIA) was

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established to assess the proportion of collagen to tissue, have been confirmed as an appropriate a measurement to assess the fibrosis degree continuously in advanced stage liver disease, substages cirrhosis effectively as well as improves the description of liver disease stage.[14-18]

Currently, although there have studies that focused on the clinical significance of serum quantitative HBsAg on liver histology, it is still unclear about the association between quantitative HBsAg and CPA at any specific time point. In this cross-sectional, retrospective study, we, therefore, aimed to discuss the relationship between quantitative HBsAg and liver fibrosis assessed by the amount of liver collagen by the picroSirius red stain in HBeAg-positive CHB patients with liver cirrhosis and to predict liver fibrosis by using serum HBsAg levels.

MATERIALS AND METHODS

Patients

One hundred twenty-eight treatment-naive hepatitis B e antigen-positive patients with liver biopsy approved cirrhosis between 2003 and 2015 were enrolled in our study. Liver biopsy was performed within 1 month before or after the serum tests in all patients. Inclusion criteria for our study were chronic HBV infection (positive hepatitis B surface antigen (HBsAg) $\geq$ 6 months), both treatment-naive for interferon and nucleos(t)ide analogs, age $\geq$ 18 years. Exclusion criteria were (a) hepatocellular carcinoma or AFP $\geq$ 50 ng/ml; (b) advanced liver cirrhosis (Child-Pugh score > 6); (c) other causes of liver disease; (d) human immunodeficiency virus co-infection; and (e) severe chronic disease or malignant disease. The study was approved by the institution's human research committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Diagnostic Tests

Serum HBsAg levels and HBV DNA levels (expressed as $\log_{10}$ IU/ml) were measured using the Elecsys® HBsAg II quant assay (Roche Diagnostics, Mannheim, Germany) and the Cobas TaqMan® assay (Roche Diagnostics), respectively.

Liver Histology

Liver biopsy specimens were stained with HE trichrome and with Masson's trichrome stain, liver fibrosis staging was evaluated by METAVIR semiquantitative scoring system, only patients with METAVIR stage 4 were included in our study.[10]

All liver histological specimens were restained by picroSirius red for CPA calculation. After the whole digital image segmentation, CPA was calculated by Image-Pro plus 6.0 software. The CPA determination need to eliminate structural collagen that does not represent disease-related collagen such as large portal tracts) and image artifacts; unfilled natural spaces (lymphoid aggregates and vascular cavities) were also not included in the measurement. CPA is expressed as a proportion (%) of collagen area to the tissue area.

Statistical Analysis

The data analysis was performed using the statistical software SPSS (version 19.0; SPSS, Inc., Chicago, IL, USA). Categorical variables are presented as frequencies (%) while continuous variables are presented as the mean ± standard deviation. Hepatitis B surface antigen and HBV-DNA levels were transferred to $\log_{10}$ IU/ml. Chi-square was used for categorical variables. Kruskal-Wallis or Mann-Whitney test was used for a similar comparison of nonparametric data. Spearman correlation was used for the evaluation of the correlation between variables. Multivariable linear regression analysis was applied to determine the independent association between HBsAg level and CPA. Age and gender were adjusted in model 2, further adjusted for PLT, bilirubin, and HBV DNA in Model 3. A *P* value <.05 was considered statistically significant.

Main Points

- *Studies have focused on the clinical significance of serum HBsAg levels and association with liver histology in HBeAg-positive chronic hepatitis B.*
- *Collagen proportionate area is a suitable histological measurement to assess the degree of fibrosis and substage cirrhosis.*
- *Serum HBsAg levels can predict liver fibrosis determined by CPA in e antigen-positive hepatitis B cirrhosis.*

RESULTS

Study Population

One hundred twenty-eight consecutive, treatment-naive patients with liver cirrhosis by METAVIR score were included in our study. There were 36 (28%) females and 92 (72%) males. The clinical and histological characteristics of all the patients are presented in Table 1.

The average mean of CPA in the assessable cohort was 23.46 ± 0.66%. 25.8% patients had CPA< 20%, 57.8%

Table 1. Demographic, Clinical and Histological Features of 128 Patients With Liver Cirrhosis

Factors	
Patients (n, %)	128
Male/female	92/36
Age (years)	30.27 ± 9.36
ALT level (U/L)	229.90 ± 209.20
Platelets ($\times10^{12}$/L)	165.14 ± 47.86
WBCs ($\times10^{9}$/L)	5.66 ± 1.76
Hb (g/L)	139.76 ± 14.56
Total bilirubin (μmol/L)	21.20 ± 17.94
Albumin (g/L)	39.58 ± 4.20
HBV DNA level ($\log_{10}$ IU/mL)	6.63 ± 1.08
HBsAg level ($\log_{10}$ IU/mL)	3.67 ± 0.84
CPA (%)	23.46 ± 6.66

Data are shown as mean ± standard deviation.
ALT, alanine aminotransferase; WBCs, white blood cells; Hb, hemoglobin; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; CPA, collagen proportionate area.

patients had CPA at 20-30%, 16.4% had CPA >30%, respectively.

Characteristics of Patients With Different CPA Stratification

Patients were divided into three groups according to the cut-off values of CPA (<20%, 20-30%, > 30%, respectively), and their characteristics were compared. Patients with a higher CPA group (>30%) had significantly higher levels of bilirubin and lower levels of HBsAg, however, among the three groups the mean values of age, platelet count and HBV DNA showed no significant differences (Table 2).

Correlation Between CPA, HBsAg, and HBV DNA

There was a modest correlation observed between CPA and serum HBsAg levels (r = –0.306, P = .001), serum HBsAg levels and HBV DNA levels (r = 0.322, P < .001); but poor correlation between CPA and quantitative HBV DNA (r = –0.031, P = .728) (Figure 1).

Multivariable Regression Analyses

To evaluate whether the level of HBsAg is independently associated with CPA, multivariable linear regression analyses were conducted to adjust for important clinical factors (Table 3). The association between HBsAg level and CPA remained significant in model 1 (unadjusted) and model 2 (adjusted with gender and age) (all standardized β = –0.022; P = .002) and persisted with slight attenuation in β value even after adjustment with PLT, Bilirubin, and HBV DNA level (standardizedβ= –0.022 to –0.021; P = .004).

DISCUSSION

Liver stage and fibrosis have always been confused in histological assessment, liver disease staging is necessary for

Table 2. Characteristics of Patients With Different CPA Stratification

Factors	<20% (n = 33)	CPA 20-30% (n = 74)	>30% (n = 21)	*P*
Age	30.67 ± 10.62	30.07 ± 9.68	30.33 ± 5.69	.803
Gender (%)				.467
Female	36.4%	25.7%	23.8%	
Male	63.6%	74.3%	76.2%	
Hb (g/L)	138.34 ± 12.35	140.92 ± 15.58	138.00 ± 14.36	.432
WBC ($\times10^{9}$/L)	5.78 ± 1.40	5.67 ± 1.88	5.39 ± 1.80	.474
PLT ($\times10^{12}$/L)	168.33 ± 40.30	166.18 ± 50.58	156.76 ± 50.23	.547
ALT (U/L)	216.91 ± 222.43	269.88 ± 258.75	165.86 ± 178.09	.091
Bilirubin (μmol/L)	16.06 ± 11.06	22.92 ± 18.58	23.21 ± 23.11	.025
Albumin (g/L)	40.25 ± 4.27	39.42 ± 4.01	39.05 ± 4.91	.386
Creatinine (mmol/L)	76.47 ± 17.84	73.36 ± 15.63	73.45 ± 15.42	.853
PT (s)	13.11 ± 1.32	13.45 ± 2.13	13.56 ± 1.26	.430
HBV-DNA ($\log_{10}$ IU/ml)	6.55 ± 1.28	6.69 ± 1.09	6.35 ± 1.06	.462
HBsAg ($\log_{10}$ IU/mL)	3.95 ± 0.93	3.70 ± 0.73	3.19 ± 0.92	.001

CPA, collagen proportionate area; Hb, hemoglobin; WBC, white blood cells; ALT, alanine aminotransferase; PT, prothrombin time; HBsAg, hepatitis B surface antigen.

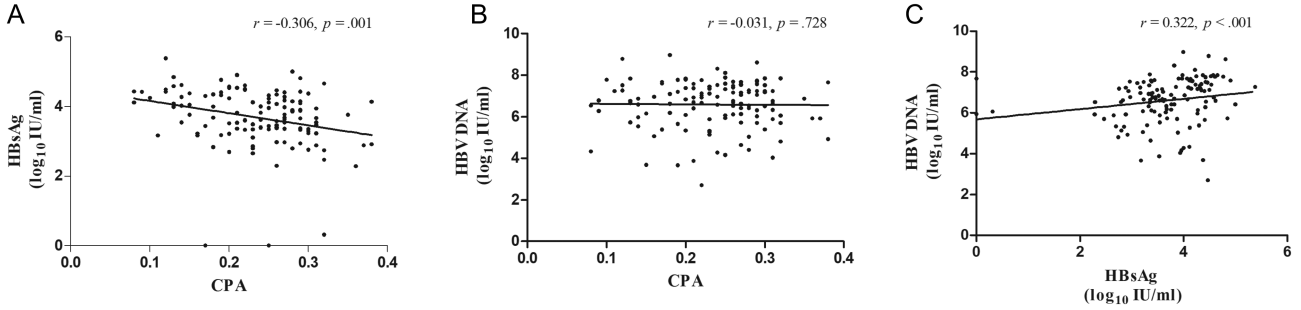

Figure 1. A modest correlation was observed between collagen proportionate area and serum HBsAg (a), serum HBsAg and HBV DNA (b); but the poor correlation between collagen proportionate area and serum HBV DNA (c). CPA, collagen proportionate area; HBsAg, hepatitis B surface antigen.

Table 3. Linear Regression of HBsAg (log_{10} IU/mL) on CPA in the 128 Patients with Advanced Cirrhosis

	B	95% CI	*P*
HBsAg			
Model 1 = unadjusted	−0.022	−0.035 to −0.008	.002
Model 2 = adjusted with age, gender	−0.022	−0.035 to −0.008	.002
Model 3 = Model 2+PLT, bilirubin and HBV DNA	−0.021	−0.035 to −0.007	.004

HBsAg, hepatitis B surface antigen; CPA, collagen proportionate area; PLT, platelets.

routine histopathological assessment while liver fibrosis measurement is another process.[13] The "number" of the stage score is neither continuous variable nor quantitatively related so that cannot be used as a measurement. For example, METAVIR stage 1 does not equivalent to half as much fibrosis as stage 2. However, we always confuse the two items that led to data misinterpretation in the literature. Thus, a histological technique that calculates fibrosis quantitatively in advanced liver disease is of great interest in liver pathology. DIA measurement assisted by computer fulfills the requirement that directly and quantitatively measures liver fibrosis using histological specimens stained by picroSirius red which identifies the tissue collagen primarily.[19] DIA measures both the area of the tissue and the stained collagen, CPA is calculated as a proportion of collagen to tissue, which represents liver fibrosis degree more accurately than the histological liver stage. In addition, the "liver cirrhosis" stage category has poor diagnostic power, in patients with Ishak stages 5 and 6, CPA had a good correlation with hepatic vein pressure gradient and had a higher sensitivity for distinguishing "early" from "late" liver cirrhosis. Thus, CPA assesses the fibrosis degree continuously and subclassifies cirrhosis, as a description improvement of the liver disease stage. This is the first study that evaluated the relationship between quantitative assessment of fibrosis on liver biopsies by measurement of the CPA and qHBsAg in treatment-naive HBeAg-positive patients with liver cirrhosis.

In this cohort, there was an inverse correlation between CPA and serum HBsAg, and a modest correlation was observed between serum HBsAg and HBV DNA, but a poor correlation between CPA and serum HBV DNA. Previously, a stronger correlation between HBV DNA and HBsAg has been described in HBeAg-positive patients.[4,5,20] HBsAg circulates in serum in a wide array of particulate forms: competent virions and non-infectious sub-viral particles (SVPs) such as spherical or long filamentous forms.[21] In the serum of chronically HBV infected patients, the SVPs were found in great excess over competent virions. Until now, HBsAg has been identified from two sources: integrated DNA and covalently closed circular DNA (cccDNA). Covalently closed circular DNA can provide both SVPs and whole virions that are needed for HBV replication, but integrated sequences can generate only SVPs.[3] Currently, the three HBsAg particles (whole virions, spheres, and filaments) cannot be distinguished by commercially available quantitative HBsAg assays. A different relationship between serum HBV DNA, HBsAg, and CPA may represent differential regulation of molecular source and expression of HBV DNA and HBsAg.

We found HBsAg level is associated with CPA independently in patients with liver cirrhosis. The mechanisms why increasingly fibrosis is associated with lower serum HBsAg in our cohort is unclear. First, the pre-S/S mutations region might impair HBV replication, impact virion secretion, or change the binding ability of HBsAg to antibodies, resulting in a decreased detectable levels of serum HBsAg.[22] While under selective pressure during immune clearance appears higher frequency of pre-S/S mutants,[23] patients with advanced-stage liver disease had pre-S/S regions mutation more frequently.[24] Both of these contribute to lower detectable HBsAg levels in patients with advanced fibrosis. Secondly, the host immune system may be activated and increasingly targets HBsAg production in disease progress, or HBsAg might be partially masked in immune complexes, with the underlying fibrosis development continued. In addition, CPA increases reflect hepatic parenchyma cells' volume decreases, which may diminish the host's ability to produce HBsAg. Moreover, HBsAg particles retention within hepatocyte but not secretion can also cause qHBsAg decline with increasing severity of fibrosis.

To our knowledge, our study firstly demonstrated an association between quantitative HBsAg and liver fibrosis severity determined by CPA in CHB patients with liver cirrhosis. Nonetheless, there have been a few limitations in our study. In previous studies, elder patients always have more severe liver fibrosis.[25-27] However, there is no significant correlation between age and CPA in patients with cirrhosis at multivariate analysis in our cohort. Our patients were relatively young, the median age was 29 years, when patients have clinical indications for liver biopsy to diagnose significant liver fibrosis or cirrhosis, those with significant signs of liver cirrhosis were already excluded at baseline. Thus one limitation of our study is a selection bias with early cirrhosis. Secondly, HBV genotype was not analyzed although the predominant HBV genotypes in Asia are genotypes B and C. The role of different genotypes in qHBsAg is not fully described,[28] future studies involving patients with various genotypes are needed to further validate the finding. Moreover, the pre-S/S region mutations may impair the HBsAg generation type and detectable values of HBsAg, the pre-S/S region mutations were not evaluated in our study. Of note, high HBsAg level has also been demonstrated to be positively associated with hepatocellular carcinoma occurrence and long-term liver cirrhosis,[25,29-31] indicating that high serum HBsAg levels were not always advantageous in the process of chronic hepatitis B. Thus, further large, longitudinal trials enrolling patients in different phases of CHB with different HBV genotypes are required to explore the potential mechanism of HBsAg in HBV infection.

CONCLUSION

In conclusion, in our cohort with antigen-positive patients, serum HBsAg levels can predict liver fibrosis determined by CPA in liver cirrhosis.

Ethics Committee Approval: This study was approved by the first affiliated hospital of Zhengzhou University Human Research Ethics Committee.

Informed Consent: Participants provided informed consent prior to participating in the study.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept – S.Q., W.C.; Design – S.Q., W.C.; Materials: Y.R.; Data Collection and/or Processing –Y.R.; Writing Manuscript – S.Q., W.C.; Critical Review – S.Q., W.C., Y.J., Z.L.

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