ORIGINAL ARTICLE

Evidence of serologic activity in chronic hepatitis B after surface antigen (HBsAg) seroclearance documented by conventional HBsAg assay

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Abstract

Background Possible serologic activity after hepatitis B surface antigen (HBsAg) seroclearance documented by conventional assays in chronic hepatitis B (CHB) has not been thoroughly investigated.

Methods We determined the levels of serum hepatitis B virus (HBV) DNA, hepatitis B core-related antigen (HBcrAg), and linearized HBsAg (CLEIA prototype) in 329 CHB patients (72.0% male) after HBsAg seroclearance was documented by a conventional HBsAg assay.

Results The median interval between presentation and HBsAg seroclearance was 69.4 months. The median age at HBsAg seroclearance was 50 years. Assays for serum HBV DNA, HBcrAg, and linearized HBsAg were performed at a median time interval of 11.2 months after HBsAg loss. Linearized HBsAg and HBcrAg were detectable in 85 (25.8%) and 69 (21%) patients, respectively, and one or both serologic markers were detectable in 133 patients (40.4%). Serum HBV DNA was detectable in only 7 patients (2.1%). There was no correlation between linearized HBsAg and HBcrAg levels ($r = 0.095$,

$p = 0.924$). The incidences of detectable linearized HBsAg and HBcrAg did not differ between patient samples taken at 6–12 and >12 months after HBsAg seroclearance ($p = 0.146$ and 0.079, respectively). Among patients with detectable serologic markers, median levels of linearized HBsAg ($p = 0.581$) and HBcrAg ($p = 0.951$) did not significantly change with time after HBsAg seroclearance.

Conclusion Using novel HBcrAg and linearized HBsAg assays, viral serologic activity after HBsAg seroclearance was demonstrated in more than 40% of CHB patients. These tests have potential applications in diagnosing and prognosticating CHB patients with HBsAg seroclearance.

Keywords HBsAg · Linearized HBsAg · Serology · Seroclearance · HBcrAg

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Introduction

Seroclearance of the hepatitis B surface antigen (HBsAg) is an uncommon event in the natural history of chronic hepatitis B (CHB), with its incidence ranging from 0.1 to 2.26% per year throughout the world [1–3]. Despite being the ultimate treatment endpoint for CHB, HBsAg seroclearance is only seen in 7% of patients after pegylated interferon therapy [4] and 1.4 to 8%, after long-term nucleoside analogue therapy [5, 6]. Even after HBsAg seroclearance, the hepatitis B virus (HBV) is still present at a low replicative level [7, 8], and patients are still at risk to the development of hepatocellular carcinoma (HCC) [9, 10].

An important determinant in the rates of HBsAg seroclearance would be the sensitivity of the HBsAg assay. The standard method used currently in commercial assays is the enzyme-linked immunoassay (ELISA), which has the

advantage of simplicity. Over the past two decades, there has been a gradual improvement in the assay's sensitivity, with the majority of commercial assays achieving a lower limit of detection of 0.05 IU/mL [11, 12]. However, the detection of HBsAg is still not flawless. Conventional HBsAg assays only target one epitope, i.e., the common determinant "a"; excluding other potential epitopes, which might lower the assay's sensitivity [13]. A second factor determining the assay's sensitivity would be its capability in detecting HBsAg mutants [14], in which amino acid substitution within the "a" determinant could give false negative results. The development of the hepatitis B core-related antigen (HBcrAg) assay has resulted in an additional option in the serologic monitoring of CHB [15]. Based on the simultaneous detection of both hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg), the HBcrAg assay has been shown to correlate well with serum HBV DNA, intrahepatic HBV DNA, and covalently closed circular DNA (cccDNA), reflecting actual histologic severity in CHB [16]. It is not affected by HBeAg status or the emergence of HBeAg-negative precore mutations [17].

A recent study employed an innovative and highly sensitive chemiluminescent enzyme immunoassay (CLEIA) for the quantitative detection of HBsAg (prototype) [18]. Using a combination of monoclonal antibodies, targeting both the exposed common determinant "a" of the surface antigen and the epitope embedded inside the lipid bilayer of the viral envelope, this linearized HBsAg assay is able to identify HBsAg mutants that evade detection by current serologic assays [13]. Linearized HBsAg has been shown to demonstrate good correlation with conventional HBsAg assays, and in patients receiving nucleoside analogue therapy, was able to detect HBsAg in the serum after documented HBsAg seroclearance by conventional assays [19]. In addition, linearized HBsAg is 10 times more sensitive than current conventional HBsAg assays, with a lower limit of detection of 0.005 IU/mL. In our current study, we propose studying the serologic activity of CHB patients after documented HBsAg seroclearance by a conventional HBsAg assay using both the HBcrAg and linearized HBsAg assays.

Methods

In the Department of Medicine, the University of Hong Kong, Queen Mary Hospital, Hong Kong, all CHB patients followed up at our clinic underwent tests for serum HBsAg, antibody to HBsAg (anti-HBs), HBeAg, antibody to HBeAg (anti-HBe), liver biochemistry, and alpha-fetoprotein every 6 months. From September 1990 to April 2010, we recruited patients who having had documented HBsAg positivity for at least 6 months were noted to have loss of serum HBsAg by a conventional HBsAg assay (Abbott Laboratories, Chicago, IL, USA) at 2 time points at least 6 months apart, with or without the appearance of serum antibody to HBsAg (anti-HBs). The interval between the last HBsAg-positive result and HBsAg seroclearance for all patients was 6 months. All clinical and biochemical data at initial presentation and during follow-up were recorded. For patients positive for HBeAg at the initial presentation, the date of HBeAg seroconversion was recorded.

The conventional HBsAg assay has a lower limit of detection of 0.05 IU/mL. Anti-HBs, HBeAg, and anti-HBe were measured using commercially available immunoassays (Abbott Laboratories, Chicago, IL, USA). All patients with concomitant chronic hepatitis C and D, evidence of Wilson disease, autoimmune hepatitis, primary biliary cirrhosis, and significant intake of alcohol (20 g/day for women and 30 g/day for men) were excluded. This study was approved by the Institutional Review Board, the University of Hong Kong and West Cluster of Hospital Authority, Hong Kong.

Serum HBV DNA levels, HBcrAg, and linearized HBsAg were measured at a single time point at least 6 months after HBsAg loss was first documented. Serum HBV DNA levels were measured by Cobas Taqman assay (Roche Diagnostics, Branchburg, NJ, USA) with a lower limit of detection of 20 IU/mL. Serum HBcrAg was measured using the CLEIA described previously [15, 16]. Briefly, sodium dodecyl sulfate pre-treated serum was incubated with monoclonal antibodies against denatured HBcAg and HBeAg. After washing and incubation with alkaline phosphatase-labeled secondary antibodies, the relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by comparing with a standard curve generated using known concentrations of recombinant HBeAg-containing peptides. The cut-off value of HBcrAg concentration was 1 kU/mL.

Serum linearized HBsAg was measured using an automated technique based on a CLEIA prototype used in a previous study by Matsubara et al. [13]. Briefly, serum or plasma samples with denatured HBsAg were added to micro-ferrite particles coated with anti-HBs monoclonal capture antibodies recognizing both the outer epitope determinant "a" and an inner (normally embedded) epitope. Following incubation and washing, 200 μL substrate [AMPPD; 3-(2′-spiroadamantan)-4-methoxy-4-(3′-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) solution was added and incubated at 37°C for 5 min. The relative intensity of chemiluminescence was measured, and the HBsAg concentration was calculated by comparison with an international standard curve. The assay range of HBsAg concentration in this reagent was 0.005–150 IU/mL, and retest was acceptable by the 200-fold dilution of sample

Fig. 1 The difference in viral components measured by different serologic assays used
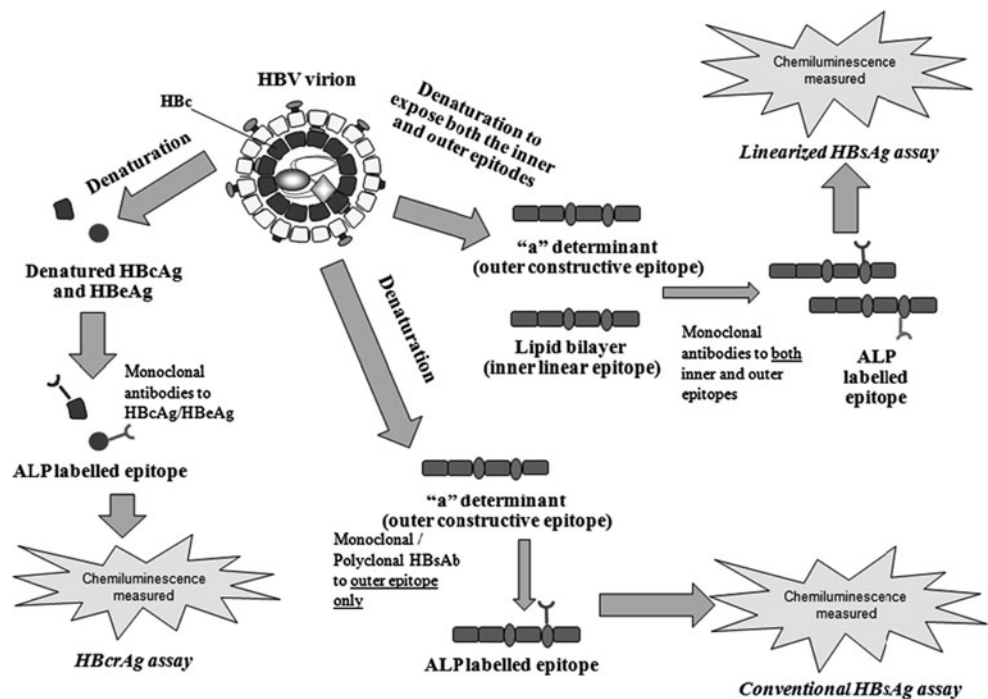
which showed assay range over. In the present study, the cut-off value of HBsAg concentration was set at 0.005 IU/mL. The entire process was automatically conducted in Lumipulse G1200 (FujiRebio Inc., Tokyo, Japan).

The differences in the viral components measured by conventional HBsAg, linearized HBsAg, and HBcrAg assays are depicted in Fig. 1.

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA). The Kruskal–Wallis test was used for continuous variables with a skewed distribution; Chi square test was used for categorical variables. Correlation between serum linearized HBsAg, HBcrAg, and other clinical parameters was tested using Spearman's bivariate correlation. A two-sided p value <0.05 was considered statistically significant.

Results

A total of 388 patients referred to our hospital with prior positive HBsAg result were noted to have HBsAg seroclearance using the conventional HBsAg assay during the recruitment period. By applying the exclusion criteria mentioned above, the following patients were excluded: no positive HBsAg result recorded in our center ($n = 33$), acute HBV infection ($n = 12$), subsequent HBsAg reversion ($n = 11$), documented HBsAg negativity <6 months ($n = 2$), and HCV co-infection ($n = 1$). Three hundred and twenty-nine patients were eventually recruited for this study. Their baseline demographics and liver biochemistry upon presentation are depicted in Table 1. The median

Table 1 Demographics and biochemistry of the studied population at initial presentation

Number of patients	329
Male (%)	237 (72.0)
Age (years)	42.7 (1.9–79.3)
HBeAg-positive (%)	47 (14.3)
Albumin (g/dL)	45 (29–56)
Bilirubin (μmol/L)	11 (3–132)
ALT (U/L)	33 (4–1522)
Cirrhosis (%)	8 (2.3)

Continuous variables expressed in median (range)

ALT alanine aminotransferase, *HBeAg* hepatitis B e antigen

interval between initial presentation, i.e., first documented date of HBsAg positivity at our clinic, and HBsAg seroclearance was 69.4 months (range 6.2–284.2 months).

Forty-seven patients (14.3%) were initially HBeAg-positive, with HBeAg seroconversion noted after a median period of 16.3 months (range 1.2–99.6 months). Concerning treatment of CHB, three patients were treated with conventional interferon-alpha for 52 weeks, with all three patients achieving HBsAg seroclearance at least 6 years after completion of interferon therapy. Fourteen patients (13 on lamivudine and 1 on entecavir) had exposure to nucleoside analogues. The remaining 312 patients were all treatment-naïve.

The median age at HBsAg seroclearance was 50 years (range 4.1–84.7 years), with 48.9% ($n = 161$) patients eventually developing anti-HBs, as assessed at the time of

their last follow-up. The median interval between HBsAg seroclearance and the appearance of anti-HBs was 20.3 months (range 0–165.7 months). Twelve patients achieved HBsAg seroclearance after a median therapy duration of 46 months (range 18–129 months), while two patients achieved HBsAg loss at 21 and 36 months after the termination of nucleoside analogues.

Linearized HBsAg, HBcrAg, and HBV DNA levels were tested at a median interval of 11.2 months (range 6.0–186.9 months) after HBsAg loss was documented by the conventional HBsAg assay. Sixty-three patients (19.1%) had detectable anti-HBs during the testing. The results are depicted in Fig. 2. Eighty-five (25.8%) and sixty-nine (21%) patients had detectable linearized HBsAg (range 0.005–150 IU/mL) and HBcrAg (range 1–934 kU/mL), respectively, with detectability of either one or both viral proteins in 133 patients (40.4%). Twenty-one patients (6.4%) had both detectable linearized HBsAg and HBcrAg. The serum tests for linearized HBsAg and HBcrAg were performed at a median period of 10 (range 6–118) and 11 (range 6–186.9) months after HBsAg seroclearance, respectively. Among patients with prior nucleoside analogue exposure ($n = 2$) or with nucleoside analogue therapy at the time of HBsAg seroclearance ($n = 12$), six and seven patients had detectable linearized HBsAg and HBcrAg, respectively. Only seven patients (2.1%) within the total patient cohort had detectable HBV DNA (range 20–1,594 IU/mL). Among these seven patients, five had detectable linearized HBsAg levels, while none had detectable HBcrAg levels.

Among the 244 patients without detectable linearized HBsAg, 48 (19.7%) had detectable HBcrAg, with the median interval of measurement after HBsAg seroclearance being 12 (range 6–136.3) months. There were also
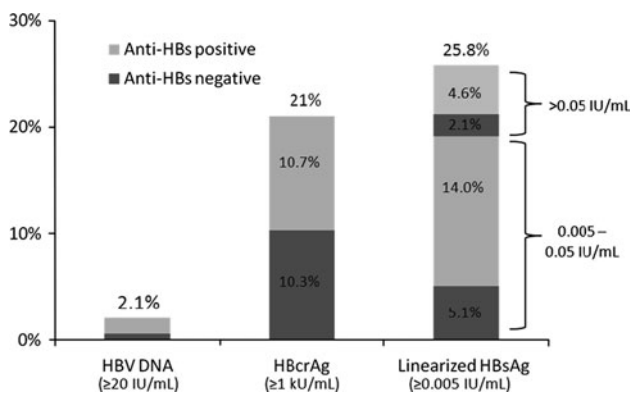

Fig. 2 Percentage of patients with detectable viremia and viral proteins after HBsAg seroclearance documented by a conventional assay ($n = 329$). *HBsAg* hepatitis B surface antigen, *anti-HBs* antibody to the hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Table 2 Percentage of patients with detectable serological markers among patients with and without eventual development of anti-HBs

	Anti-HBs positive patients ($n = 161$)	Anti-HBs negative patients ($n = 168$)	p value
Positive linearized HBsAg	24 (14.9%)	61 (36.3%)	<0.001
Positive HBcrAg	34 (21.1%)	35 (20.8%)	0.949

two patients (0.8%) with detectable serum HBV DNA at 6.5 and 42 months after HBsAg seroclearance.

The association of the eventual appearance of anti-HBs and detectable viral proteins is depicted in Table 2. When compared to anti-HBs positive patients, a significantly higher proportion of anti-HBs negative patients had detectable linearized HBsAg ($p < 0.001$). There was no significant difference in the percentage of patients with detectable HBcrAg between the two groups ($p = 0.949$).

Linearized HBsAg levels showed no correlation with HBcrAg levels ($r = 0.095$, $p = 0.924$). Both linearized HBsAg and HBcrAg levels also showed no correlation with gender ($r = -0.02$ and -0.04, $p = 0.711$ and 0.462, respectively).

The percentage of patients with detectable linearized HBsAg and HBcrAg according to the interval between HBsAg seroclearance and serological testing is shown in Table 3. The incidences of detectable linearized HBsAg and HBcrAg did not significantly differ with time after HBsAg seroclearance at 12, 24, and 36 months ($p > 0.05$). Detectable linearized HBsAg and HBcrAg levels from the time of HBsAg seroclearance are shown in Fig. 3a and b. Among patients with detectable serological markers, the median levels of linearized HBsAg ($p = 0.581$) and HBcrAg ($p = 0.951$) did not change significantly with time after HBsAg seroclearance.

Discussion

Our present study showed that at least 40% of CHB patients with documented HBsAg seroclearance using a conventional HBsAg assay had demonstrable serologic activity indicative of continuous viral transcription.

Older age is an established predisposing factor for HBsAg seroclearance [2, 10]. Despite a low annual incidence rate, the cumulative rate of HBsAg seroclearance after 25 years could reach up to 40% according to a Taiwan study consisting of 1,965 Chinese CHB patients [20]. Therefore, in an endemic region where the prevalence of positive antibody to the hepatitis B core antigen (anti-HBc)

Table 3 Percentage of detectable linearized HBsAg and HBcrAg after HBsAg seroclearance

Time from HBsAg seroclearance (months)	Linearized HBsAg		HBcrAg	
	Detectable percentage (ratio)	p value	Detectable percentage (ratio)	p value
a: 12 months				
≤12	29.1% (52/179)	0.146	24.6% (44/179)	0.079
>12	28.2% (33/150)		20.0% (25/125)	
b: 24 months				
≤24	28.4% (71/250)	0.076	20.0% (50/250)	0.441
>24	17.7% (14/79)		24.1% (19/79)	
c: 36 months				
≤36	25.8% (73/283)	0.901	20.1% (57/283)	0.358
>36	26.1% (12/46)		26.1% (12/46)	

Patients were divided into two groups for analyses based on the time of measurement after HBsAg seroclearance as documented by a conventional HBsAg assay: 12 months (a), 24 months (b), and 36 months (c)

HBsAg hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

is reported to be between 13.5 and 17% [21–23], it may be difficult to differentiate CHB patients with HBsAg seroclearance and those with prior exposure to HBV, especially for persons older than 50 years (the median age of HBsAg loss). Although serum HBV DNA testing is an option for differentiation, the likelihood of a positive HBV DNA result is low as shown in our study (2.1%). Even when the lower limit of detection was improved to 1.1 IU/mL, the chance of HBV DNA positivity was still only 13.4% [9]. The HBcrAg and linearized HBsAg assays would be a useful tool in diagnosing CHB patients with prior HBsAg seroclearance documented by conventional HBsAg assay before the initial clinical presentation, especially since our present study showed the two viral proteins could be detected up to 118 and 187 months after HBsAg seroclearance.

Our present study found that the percentage of detectable serologic markers remained similar in serum samples taken at different time points after HBsAg loss. Moreover, the median levels of both viral proteins among patients with detectable markers after HBsAg seroclearance remained similar over time. Our results suggest that there is persistent low-grade viral transcriptional activity for years after HBsAg seroclearance. In addition, the concept of HBsAg seroclearance might need refinement, given that 25.8% patients with documented HBsAg seroclearance using a conventional assay had a positive linearized HBsAg result. However, it should be noted that these findings are limited by the cross-sectional nature of our study, with the serum of patients taken at different time intervals after HBsAg seroclearance. Longitudinal studies with serial viral protein levels are needed and would be useful to further investigate the relationship of anti-HBs with linearized HBsAg.

The pathophysiology of continued viral protein production after HBsAg seroclearance is not well-defined. The presence of viral escape mutants in the "a" determinant is possible [24]. Although the majority of mutants are detectable by current serologic assays, detection limits of HBsAg mutants vary between different assays [25]. HBsAg production exceeds the required amount for virion assembly of the Dane particle, and can also be secreted as empty subviral particles [26, 27]. These subviral particles have been suggested to be involved in the immune evasion strategy of HBV [28], and could remain in circulation even when the production of virions decreases [29], as in HBsAg seroclearance. In CHB patients achieving HBsAg seroclearance, intrahepatic cccDNA is still detectable at extremely low levels [9]. Serum HBcrAg levels had been previously proven to have good correlation with intrahepatic cccDNA levels [16]. Despite earlier evidence of good correlation between serum HBsAg and intrahepatic cccDNA [30, 31], a recent study did not find such a correlation in HBeAg-negative disease [32], probably since viral integration, a non-essential event in the life cycle of HBV, produces HBsAg in the absence of viral replication [33]. Hence, it is not surprising that studies have shown that there is no significant HBsAg decline in CHB patients treated with nucleoside analogues [34]. Further studies are needed to determine if any correlation exists between linearized HBsAg levels and intrahepatic cccDNA.

Identifying patients with prior HBsAg seroclearance as documented by a conventional assay carries several clinical implications. First, an older age at HBsAg seroclearance is still associated with risk of HCC [9, 10], thus detecting past HBsAg seroclearance would facilitate the enlisting of such patients into HCC surveillance programs. Second, fulminant HBV reactivation is possible in HBsAg-negative but anti-HBc positive patients undergoing immunosuppression or chemotherapy, especially for regimens containing rituximab [35]. Identifying patients with prior HBsAg

Fig. 3 Detectable linearized HBsAg (**a**) and HBcrAg (**b**) levels after HBsAg seroclearance as documented by a conventional HBsAg assay
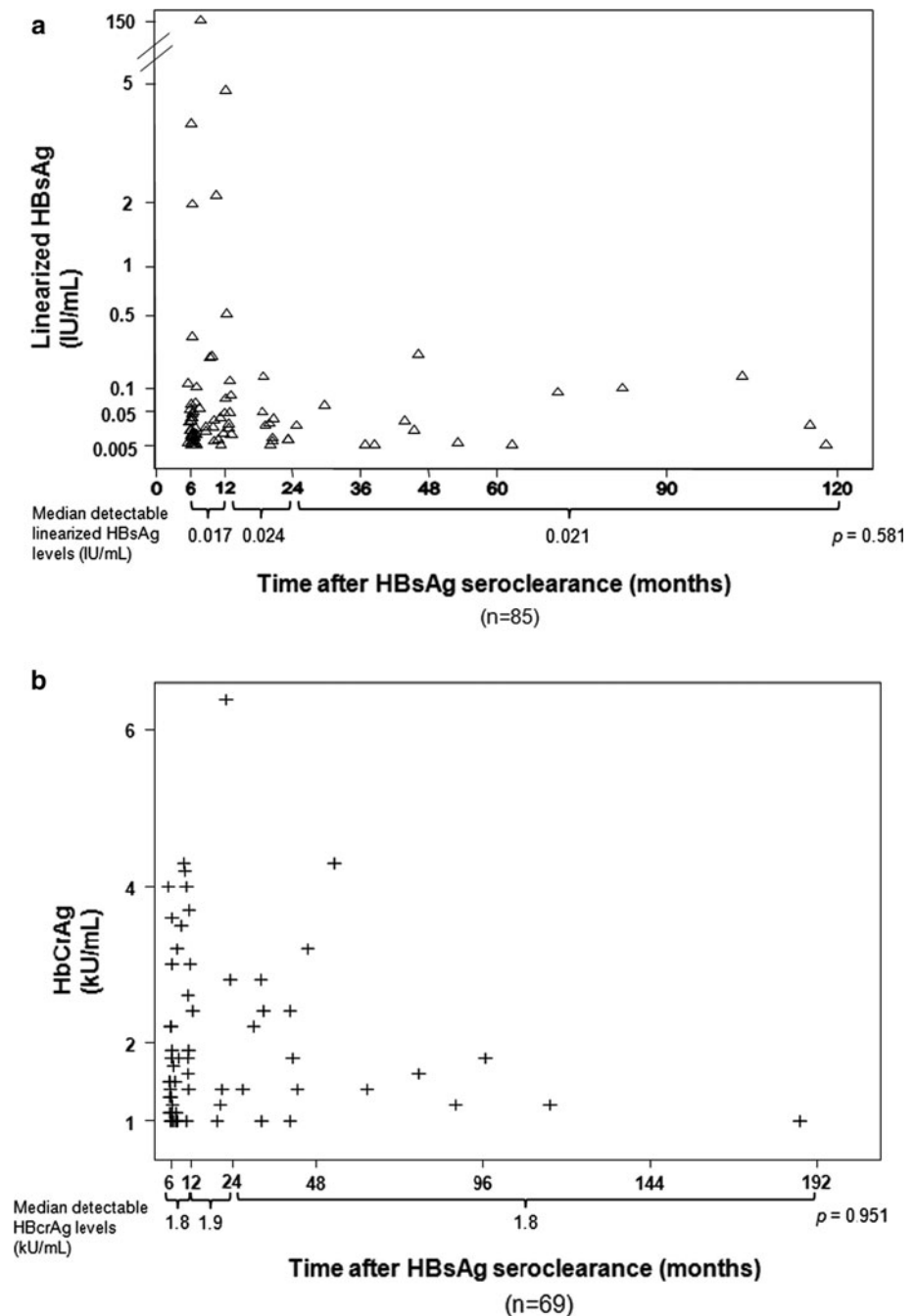
seroclearance would allow either preemptive antiviral therapy or close serologic and virologic monitoring. Third, using both HBcrAg and linearized HBsAg assays, over 40% of CHB patients with prior HBsAg seroclearance could be identified. Detectable HBcrAg and linearized HBsAg in patients considered to have cryptogenic cirrhosis will reveal the actual diagnosis to be occult hepatitis B infection. Future studies could further investigate the relationship of these two novel serologic markers with both serum anti-HBs and anti-HBc.

There are several limitations of this study, the first being its cross-sectional nature as mentioned above. HBV genotypes were not checked in our study. Since genotype-specific changes were found in HBsAg levels during pegylated interferon treatment [36], it would be interesting to determine the effect of genotype on the kinetics of viral protein production after HBsAg seroclearance. Baseline HBV DNA levels were not available in our present study. A previous study in Asian CHB patients found low HBV DNA levels to be predictive of eventual HBsAg seroclearance [2], and therefore, such data should be included in future longitudinal studies concerning these two serologic markers.

In conclusion, the detection of HBcrAg and linearized HBsAg in more than 40% of CHB patients achieving HBsAg seroclearance documented by a conventional HBsAg assay suggests that transcription of viral proteins still exists even when serum HBsAg is undetectable. These two novel assays can assist the diagnosis of CHB patients with prior HBsAg seroclearance. Further longitudinal studies are needed to determine if these two serologic markers have prognostic implications for both treated and untreated CHB patients.

Conflict of interest No conflicts of interest exist for all authors.

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