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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

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

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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

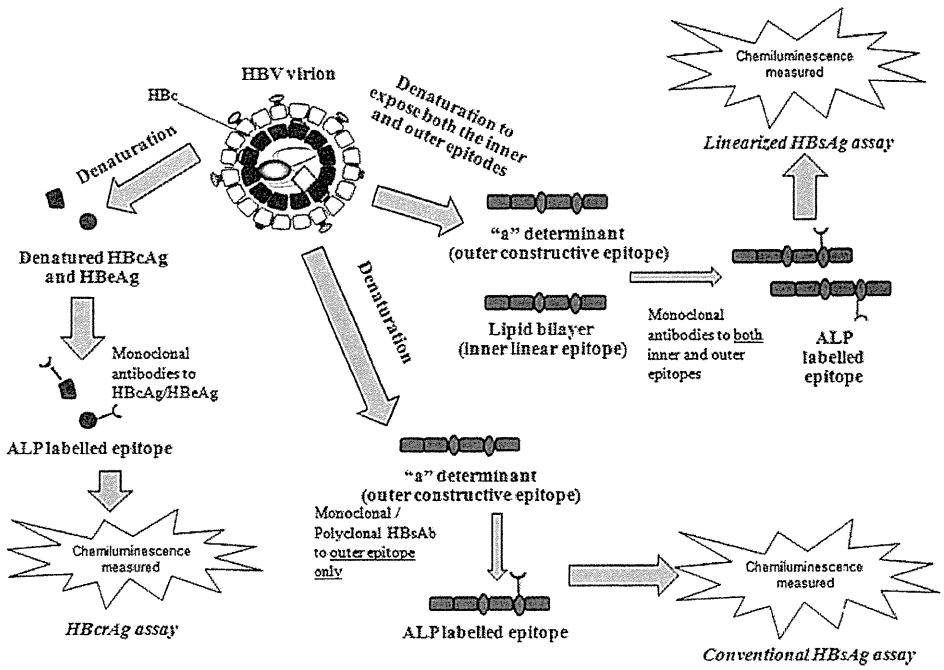

Fig. 1 The difference in viral components measured by different serologic assays used

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 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).

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

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

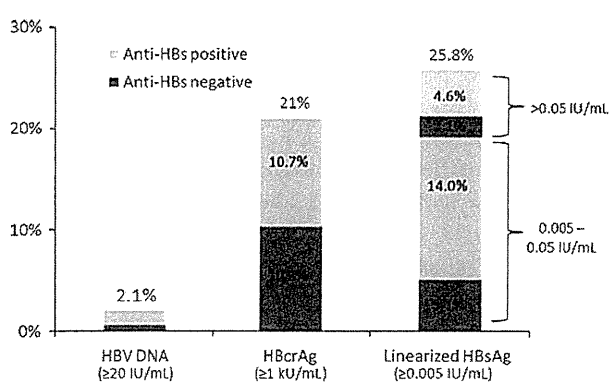

Fig. 2 Percentage of patients with detectable viremia and viral proteins after HBsAg seroclearance documented by a conventional assay ($n = 329$). *HBsAg* hepatitis B surface antigen, *anti-HBs* antibody to the hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

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

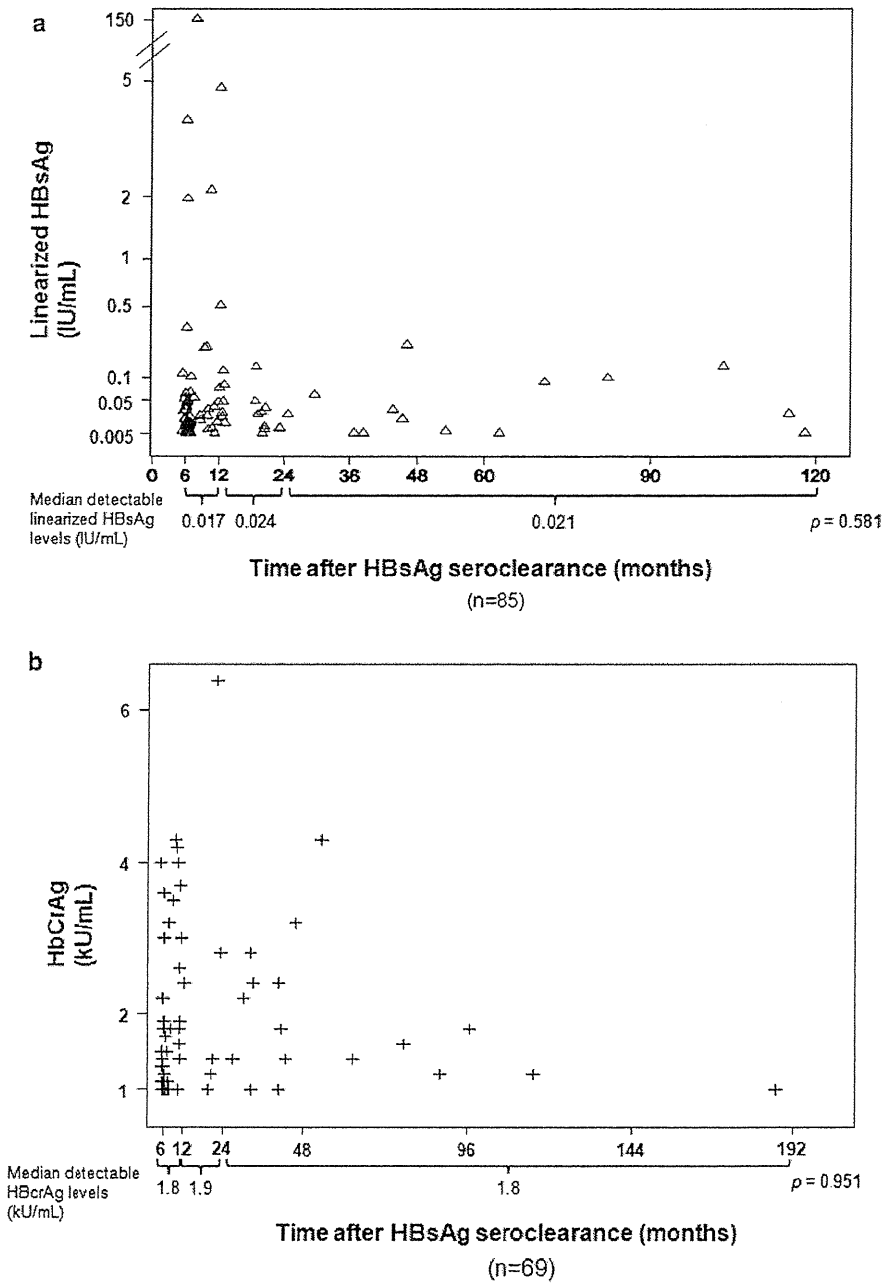

Fig. 3 Detectable linearized HBsAg (**a**) and HBcrAg (**b**) levels after HBsAg seroclearance as documented by a conventional HBsAg assay

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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III. B 型肝炎

B型肝炎の病態

HBV遺伝子型と臨床像

Clinical implication of hepatitis B virus genotype

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はじめに

B型肝炎患者は無症候性キャリアも含めて現在，全世界で3億5千万人と推定され，世界中で年間約100万人がB型肝炎ウイルス(HBV)感染に関連して死亡している．我が国においてはHBV感染症例は150万人前後存在するとされている．1986年から母児感染予防事業によりB型肝炎ワクチン，抗B型肝炎ヒト免疫グロブリンの投与が行われ，ほぼ完全に垂直感染を抑えることに成功した．また，核酸増幅検査導入により輸血後B型肝炎は極めてまれになった．しかし近年性行為による水平感染が増加しており，その中で従来self-limitingな疾患であると考えられていた急性肝炎患者の慢性化が報告されるようになり，問題となっている．更に，B型肝炎患者の中には様々な治療を行ってもなかなか奏効せず，病状が悪化，進行する例も少なくない．このように臨床の場において同じHBV感染者であるにもかかわらず，その経過においては大きな差があることがしばしば経験されている．その原因の一つとしてHBV遺伝子型(genotype)が注目されている．

そこで本稿では，HBV genotypeによる臨床像の違いについて述べる．

1. HBV genotype

一般的にDNAウイルスはRNAウイルスに比べて遺伝子変異が少ないとされているが，HBVは逆転写過程をもつために高率に変異を起こすことが知られており，DNAウイルスの中では変異しやすいウイルスと考えられている．

HBVは近年の分子系統解析の進歩に伴い，現在までにgenotype A型からJ型までの10種に分類されている．これらのHBV genotype分布には地域特異性が存在し，genotype A，Dはヨーロッパ諸国および地中海沿岸に広く分布する一方，genotype B，Cは東アジアを中心に広く分布している．またgenotype Eは主に西アフリカに分布し，genotype F，Hは中南米に分布している．genotype Gは今のところフランス，ドイツ，USなどの一部の地域においてのみ報告されている．

日本全国13施設の共同研究により解析可能であった720例のHBV genotypeの分布状況は，genotype Cが約85％，genotype Bが約12％，genotype Aが約2％であり，我が国における持続感染者はgenotype Cとgenotype Bがほとんどである．日本国内においてもこの2つのgenotype間には地域特異性があり，ほとんどの地域ではHBV/Cが大多数を占めるのに対し，

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沖縄と東北地方ではHBV/Bの割合が非常に高い[1]．近年，我が国においてB型急性肝炎が増加傾向であり，なかでもgenotype Aの増加が目立っている．成人のHBV初感染による急性肝炎は，我が国ではself-limitingな疾患であり慢性化はまれであると考えられていたが，欧米では急性肝炎の約10％が慢性化しており，これがHBV genotypeの違いによることがわかってきている[2]．欧米にみられていたgenotype Aの初感染からの慢性化が我が国においても報告されるようになり，現在genotype Aによる慢性肝炎の増加が報告されている[3]．

一方でgenotype間の病態の違いに関してはそのgenotypeの地域分布に偏りがあるため，すべてのgenotypeを横断的に検討することは困難である．

またgenotypeと病態に関しては，genotypeに依存した頻度の多い変異が存在している．病態の一部には変異が関与していると思われるため，それを踏まえて記述する．

2. Genotype Bおよびgenotype C

前述のとおり，我が国におけるHBVは主にgenotype BとCである．我が国の持続感染のほとんどは母児感染による垂直感染がほとんどであり，思春期頃よりALTの上昇をきたす．そして，HBe抗原陽性からHBe抗体陽性へとセロコンバージョンを起こし，ウイルスの排除には至らないものの，持続感染のまま病態は安定化する．しかし一方で，セロコンバージョンを起こさず肝炎が持続し，肝硬変，肝癌，あるいは肝不全へと病状が進行してしまう例や，セロコンバージョンを起こしたにもかかわらず肝炎が持続する例もみられる．HBV genotype別に検討してみると，genotype Bの患者は比較的若年からセロコンバージョンを起こし，無症候性キャリアとなりやすいが，genotype Cの患者はHBe抗原陽性のまま病態が進行してしまう例が多いことがわかる[4]．

我が国においてはgenotype Bの予後は良好であるが，一方台湾では若年肝癌がHBV/Bで多く[5]，同じgenotypeであるにもかかわらず病態に大きな相違がみられた．そこで我が国を含むアジアから得られた検体を用いてgenotype Bの全塩基配列を決定し比較してみると，アジア諸国に分布するsubgenotype Ba(Asia型)と我が国固有のsubgenotype Bj(Japan型)の2つの亜型が存在することが確認できた．面白いことに，subgenotype Baはプレコアからコア領域の部分がgenotype Cとウイルス遺伝子組換えを起こしていた[6]．

これらsubgenotype Ba，subgenotype Bj，genotype C(C2/Ce(極東アジア型))について，年齢，性別，病態をマッチさせたケースコントロールスタディを行ったところHBe抗原陽性率はHBV/C(C2/Ce)で最も高く，HBV/Bjが最も低かった．更に，HBe抗原産生に関連しているウイルス遺伝子変異の検討を行った結果，basal core promoter(BCP)の2重変異(A1762T/G1764A)はHBV/Cで高率に認められ，HBV/Bjは最も低率であった．逆にプレコア変異(G1896A)はgenotype Bjで高率であり，genotype Cは低率であった．subgenotype Baはsubgenotype Bjとgenotype Cのちょうど中間的な性質を示していた[7](**表1**)．subgenotype Baはgenotype Cとのウイルス遺伝子組換えによりgenotype Bとgenotype Cの両方の性質を獲得したのかもしれない．

我が国における劇症肝炎と急性肝炎のケースコントロールスタディでは，劇症肝炎に関与する因子にgenotype Bjであることや，genotype Bjに頻度が高いG1896A変異が報告されている[8]．またgenotype Baに関してもA1762T/G1764A，G1896A変異が劇症肝炎に関係する可能性[9]がある．なお，genotype Cとのウイルス遺伝子組換えを起こしていないgenotype BはBj以外に北極圏にみられるsubgenotype B6がある．その臨床的特徴はsubgenotype Bjとよく似ていた[10]．

また慢性肝炎の急性増悪に関しては，subgenotype BaにおいてはA1762T/G1764Aが，genotype C2/CeにおいてはT1753V，A1762T/G1764A，G1896A，G1899Aが有意差をもって慢性肝炎に対して頻度が高かった[11]．培養細胞

表1 HBV genotype BとCの特徴

	HBV genotype		
	Bj	Ba	C
HBe抗原陽性率	18%	35%	50%
コアプロモーター変異(nt.1762/1764)	15%	33%	63%
プレコア変異(nt.1896)	50%	18%	13%
ATG initiator(nt.1809-1812)	CCAC	CCAC	CCAC
encapsidation signal(nt.1858)	T	T	T

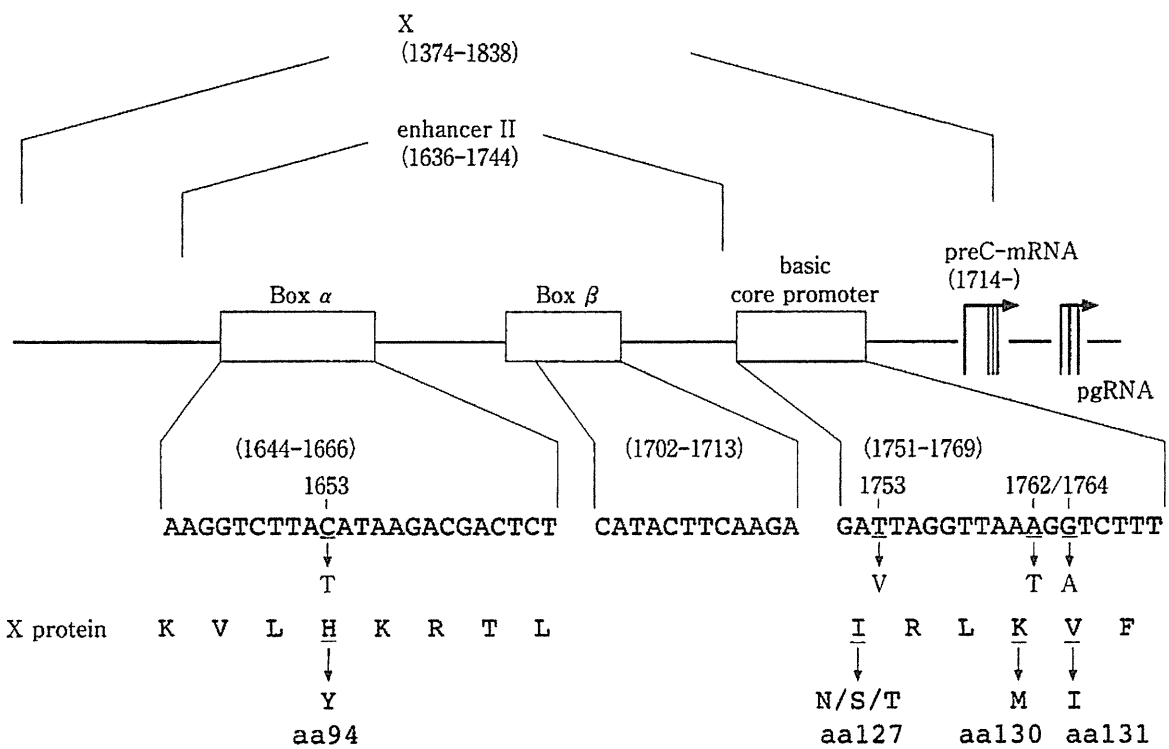

図1 HBV subgenotype C1/Cs(東南アジア型)とC2/Ce(極東アジア型)における enhancer II/コアプロモーター，プレコア領域の遺伝子変異

株とヒト肝細胞置換uPA/SCIDマウスを用いたHBV genotype A，B，Cの感染実験において，genotype Cの感染マウスに肝線維化の進展がみられたように，genotype CはBに比べて肝硬変，肝癌への進展がみられることが多い[12]．実際に台湾のコホートでもgenotype Cはgenotype Bの2倍の発癌リスクが示された[13]．我が国のHBV関連肝癌のgenotype分布をみると，多くがgenotype Cであり，subgenotype Bjは高齢者肝癌の一部に散見されるのみであった[14]．

genotype BとCを主とした肝発癌に関係するHBVの遺伝子変異はpre-S欠失，C1653T，T1753V，A1762T/G1764A変異がメタアナリシスで報告されている．

これらの変異の頻度をgenotype BとCとで比較してみると，Cにおいて頻度が高くなっている．subgenotype C2の遺伝子変異と肝癌との関係については様々な報告があるが，代表的なものとしてはBCP変異(でありHBXタンパクのアミノ酸変異でもある(図1))のC1653T，A1762T/G1764A変異がある[15-17]．また，subgenotype C2を主とした集団において経時的に

表2 HBV genotype AとCの特徴

	HBV genotype		
	Aa	Ae	D
HBe抗原陽性率	31%	49%	37%
コアプロモーター変異(nt.1762/1764)	50%	44%	25%
プレコア変異(nt.1896)	0%	0%	48%
ATG initiator(nt.1809-1812)	TCCT	CCAC	CCAC
encapsidation signal(nt.1858)	C	C	T

検討すると，A1762T/G1764A変異は必須であるがT1653やV1753が加わることで発癌にかかわるとする報告もある[18]．

HBVのpre-S欠失に関しては，HBVのpre S-S大タンパク質を過剰発現するトランスジェニックマウスで肝障害を起こし，肝細胞癌が多発することが報告されている[19]．pre-S欠失はgenotype BよりgenotypeCに多くみられ，しかも病態進展(肝硬変，肝細胞癌)に有意に関与していることが報告されている[20]．すなわち，pre-S欠失はgenotypeにより差がみられ，病態の進行に伴い頻度が増加しており，genotypeによる病態の違いの原因の一つである可能性が考えられた．

3. genotype Aおよびgenotype D

我が国において今後HBV/Aの水平感染による慢性肝炎の増加が危惧されることは前に述べた．我が国に主に存在していたHBV genotypeはBjとCであり，これらの水平感染の場合では慢性化をきたすことは少ない．Yotsuyanagiらの報告によると，HBV/AによるB型急性肝炎の肝障害の程度はHBV/Cと比較して軽いが，HBs抗原の消失までの期間も長く，HBV/Aの慢性化率はHBV/Cと比較して高くなる傾向にあることを報告した[21]．またSuzukiらは，genotype Aの急性肝炎後の慢性化は31例中7例(23%)と高頻度に認めたと報告している[22]．我が国における多施設共同研究においても同様に遺伝子型Aの高い慢性化率を報告している[2,22,23]．このように，我が国におけるgenotype Aの急性肝炎の増加と他の遺伝子型と比較して慢性化率が高いことから，今後はgenotype Aのキャリアの増加が予想される[3]．

HBV/Aは欧州，米国，アフリカ，インド，フィリピンなど広く分布しており，世界で最も感染者が多いといわれている．一方，HBV/Dも地中海を主として世界に広く分布している．genotype Aは更にアフリカや東南アジアに分布するsubgenotype Aa(Asia/Africa型)と，欧米に広く存在するsubgenotype Ae(Europe型)の2つの亜型に分類される[24]．このsubgenotype Aaとsubgenotype Aeの間にもsubgenotype Ba，subgenotype Bjと同様に臨床的な違いが認められる．subgenotype Aa感染者は比較的若年でセロコンバージョンする一方で[25]，アフラトキシンの修飾の可能性もあるが肝癌の若年発症が高率に認められる[26]．subgenotype Aeは，成人の初感染による急性肝炎後の慢性化が約10%に認められるが，肝癌の発生は少ないとされる．

subgenotype Aaとsubgenotype Aeのウイルス学的特徴を明らかにするために，欧米に多いgenotype Dを加えたcase studyを行い，背景および臨床データを比較検討した．その結果，subgenotype Aaはsubgenotype AeやHBV/Dに比べHBe抗原陽性率が有意に低く，特に30歳未満でその傾向が顕著に認められた[27](表2)．subgenotype Aaではプレコア/コア領域の直前にあり，プレコア/コアタンパクの翻訳を制御しているATGイニシエーター(Kozak配列)に特異的な変異がみられ，そのためHBe抗原前駆体タンパクの翻訳効率が非常に低下したと考えられる[27,28]．

また，genotype Aはgenotype Dに比べ，プレ

コア領域変異を起こしにくいといわれているが，この理由としてεループと呼ばれる部位の2次構造にみられる特徴のためと考えられる．この立体構造はDNA合成のイニシエーションとなる部位で，途中で折り返すと多くの塩基が互いに相補的となり，ペアリングを形成して安定な形態をとる．この部分に変異が起こると立体構造が不安定化しDNAの複製効率が非常に低下することがわかっている．このループの中のプレコア領域1,896番の塩基は1,858番と対になっており，subgenotype Aa，Aeでは1,858番がCのため1,896番はGの方が安定でありAには変化しにくくなっている[28,29]．したがってsubgenotype Aa，Aeではプレコア領域変異を起こしにくいことがわかる．

またgenotype Aだからといって肝癌にならないわけではなく，我が国のgenotype Aの慢性肝炎においての検討ではHBe抗原のセロコンバージョン率が低く，肝硬変や肝癌の発症率もgenotype Bよりも高い可能性がある[3]．genotype AとDの比較では，慢性肝炎においてHBe抗原のセロコンバージョン後の生化学的な消退やHBV DNAの陰性化，HBs抗原の陰性化はgenotype Aで頻度が高い[30]．

genotype Dは比較的若年でプレコア変異によりHBe抗原のセロコンバージョンを起こす．遺伝子変異と病態に関しては，G1764T/C1766GはBCP変異の中で頻度が高く(29.2％)，HBe抗原陰性例の39.3％にみられた．A1752CやT1753V変異は肝癌患者に有意差をもって多く認められ，更にgenotype DにおいてA1762T/G1764A変異はもともと頻度が低いが，血中HBVウイルス量が高値な肝癌患者にはみられた[31]．また，genotype Dに関してはsubgenotype D1-D5において変異のパターンが異なるとの報告もあり[32]，今後の検討が待たれる．

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原　　著

HBs 抗原定量値の臨床的有用性の検討

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Evaluation of the Clinical Utility of Quantitative Measure of Hepatitis B Surface Antigen

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There are 1.5 million hepatitis B virus (HBV)-infected patients in Japan. Anti-viral therapy is important for chronic hepatitis B patients to prevent hepatocellular carcinoma. Recently, HBs antigen (HBsAg) quantification has been reported to be useful for not only HBV screening but also for monitoring of anti-viral treatment. In this paper, we evaluated the clinical utility of quantitative assay of HBsAg by HISCL HBsAg kit. Although there can be a significant difference in age, HBeAg positive/negative and viral genotype, there is not in the disease stage. Moreover, the weak correlation was confirmed between HBsAg and HBV-DNA levels with or without anti-virus treatment. In the clinical practice, as HBV-DNA becomes undetectable immediately by anti-viral therapy such as entecavir, it may be difficult to evaluate the efficacy. The monitoring of the HBsAg concentration in addition to HBV DNA would be useful for the evaluation. Hence, the clinical role of HBsAg concentration could spread widely in Japan.

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B 型肝炎ウイルス (HBV) 感染者は全世界で 4 億 2 千万人，日本においては 150 万人程度存在するといわれている。アジアでは HBV は主に母子感染によって感染し，慢性化，その後肝癌へ進展する症例も多く，肝炎が持続する患者においては，積極的な治療介入が必要である。現在，B 型慢性肝炎，肝硬変の治療法としては，インターフェロン，核酸アナログ製剤による抗ウイルス療法が中心となっている。

近年，HBs 抗原量と B 型肝炎の病態や治療効果の関連性が報告されており，スクリーニング目的だけでなく，HBV キャリアの治療モニター・効果予測のマーカーとしても注目されている。既にアーキテクト・HBsAgQT (アボットジャパン㈱) が臨床応用されている[1]。2007 年に化学発光免疫測定法 (CLEIA

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法)を測定原理とした，高感度かつ広い測定範囲で定量可能なHISCL HBsAg試薬を開発した。既に基礎的検討がいくつか行われており[2]，日常検査に有用であることが確認されている。我々は，今回HBs抗原定量値の臨床的有用性について知見を得たので報告する。

I. 対象および方法

A. 対　象

名古屋市立大学病院およびその関連施設に通院中のB型慢性肝疾患患者313例(無症候性キャリア[ASC] 81例，慢性肝炎[CH] 132例，肝硬変[LC] 30例，肝細胞癌[HCC] 33例，不明37例)を対象とした。患者背景については**Table 1**に示す。治療の有無については，過去あるいは検体採取時にインターフェロン治療，核酸アナログ製剤による治療歴を有するものを「抗ウイルス療法あり」とした。なお，本研究は，担当医より対象者に文書によって説明，同意を取得した後，実施した。

B. 方　法

HBs抗原定量値は，全自動免疫測定装置HISCL-2000i専用試薬であるHISCL HBsAg試薬(シスメックス㈱)にて測定した。本試薬は最小検出感度0.03 IU/mLと高く，測定範囲が0.03～2,500 IU/mLと広いことが特徴である。今回，0.03 IU/mL以上を陽性と判定し，測定範囲を超えた検体については，自動希釈機能により40倍希釈を行い，測定した。

また，HBV Genotypeはイムニス HBV ゲノタイプEIA(㈱特殊免疫研究所)，HBV-DNAはアンプリコア法(ロシュ・ダイアグノスティックス㈱)またはTMA法にて測定を行った。

II. 結　果

A. 年齢別のHBs抗原量

B型慢性肝疾患患者313例のうち，年齢，治療の有無についての情報を有する280例について，治療群および非治療群において年齢別にHBs抗原量を比較した。治療群，非治療群ともに40歳未満と50代，40歳未満と60歳以上，40代と60歳以上で有意差が見られ，高齢になるに従いHBs抗原量は低値となる傾向がみられた(**Fig. 1**)。

B. HBe抗原別のHBs抗原量

治療の有無についての情報を有するHBe抗原陽性例70例とHBe抗原陰性例205例について，HBs抗原量を比較したところ，治療の有無に拘らず，HBe抗原陰性群の方が有意にHBs抗原量が低い結果となった(**Fig. 2**)。

C. 病態別のHBs抗原量

病態(ASC，CH，LC，HCC)が把握できている276例においてHBs抗原量を比較したところ，有意差はみられなかった(**Fig. 3**)。

D. HBV-DNA量とHBs抗原量

治療の有無別に，HBV-DNA量(Log copies/mL)とHBs抗原量(Log IU/mL)の情報がある260例(治療群111例，未治療群149例)について，両者の相関関係を検討した。治療群は$y=0.130x+2.71$，$r=0.267$，未治療群は$y=0.302x+1.48$，$r=0.457$となり，未治療群にのみ弱い正の相関性がみられた(**Fig. 4**)。治療群においては抗ウイルス療法によりHBV-DNAは減少するがHBs抗原量の減少までは得られない症例が多いために相関がみられなかった。

E. Genotype別のHBs抗原量

Genotype(以下，GT)が判定可能であった症例は290例であり，内訳はGT-A 10例，GT-B 68例，

Table 1　Characteristics of chronic hepatitis B patients

Male : Female	181 : 132
Age	54±14
Genotype (A, B, C, D, unknown)	10, 68, 206, 6, 23
Disease Stage (ASC, CH, LC, HCC, unknown)	81, 132, 30, 33, 37
ALT	50±109
HBV-DNA (median, log copies/mL)	4.4
HBeAg　(+) : (−) : unknown	72 : 207 : 34
HBeAb　(−) : (+) : unknown	64 : 214 : 35
Anti-viral treatment : no treatment : unknown	118 : 177 : 18

※ They are only the known data.

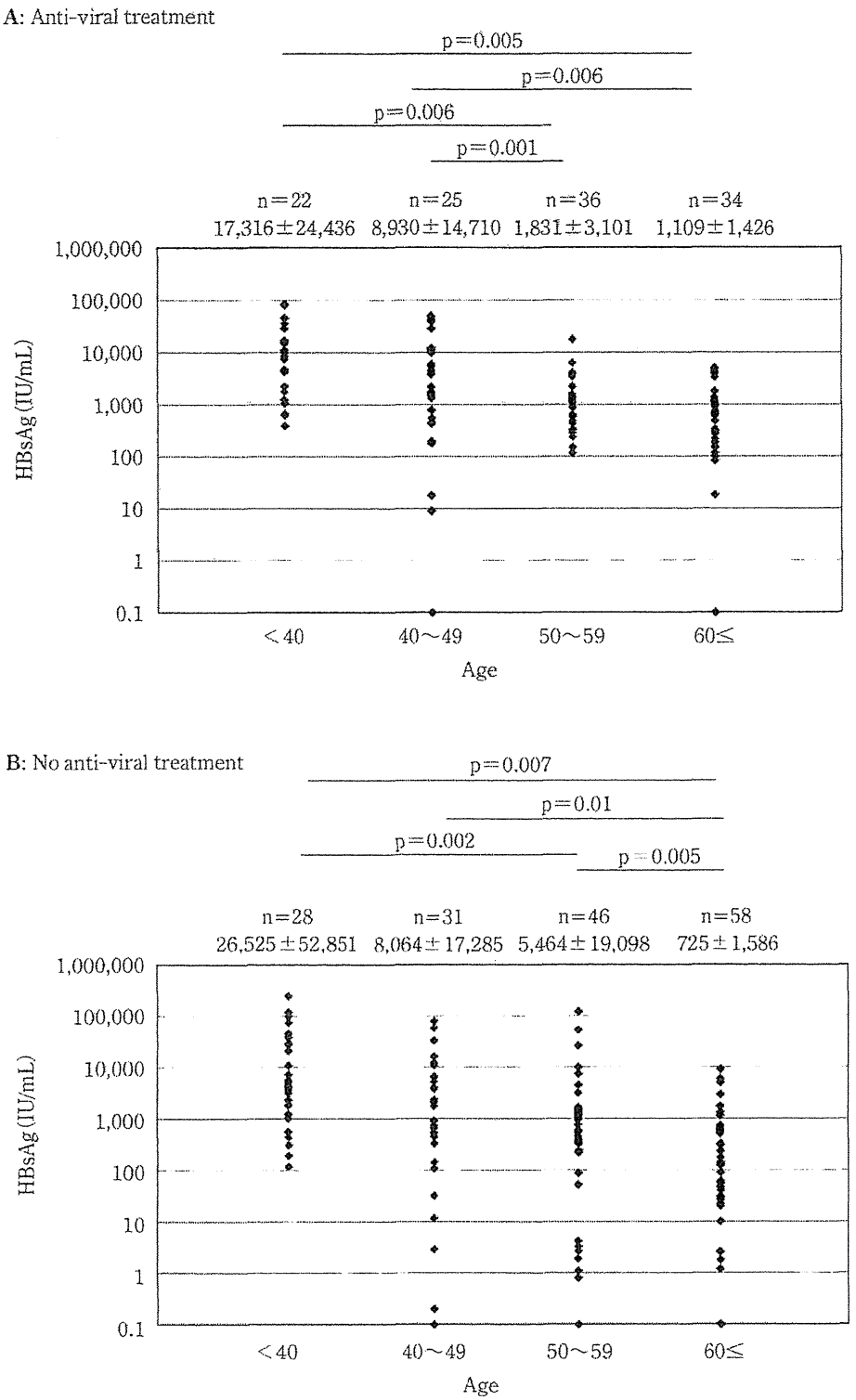


Figure 1　Quantity of HBsAg according to the age.

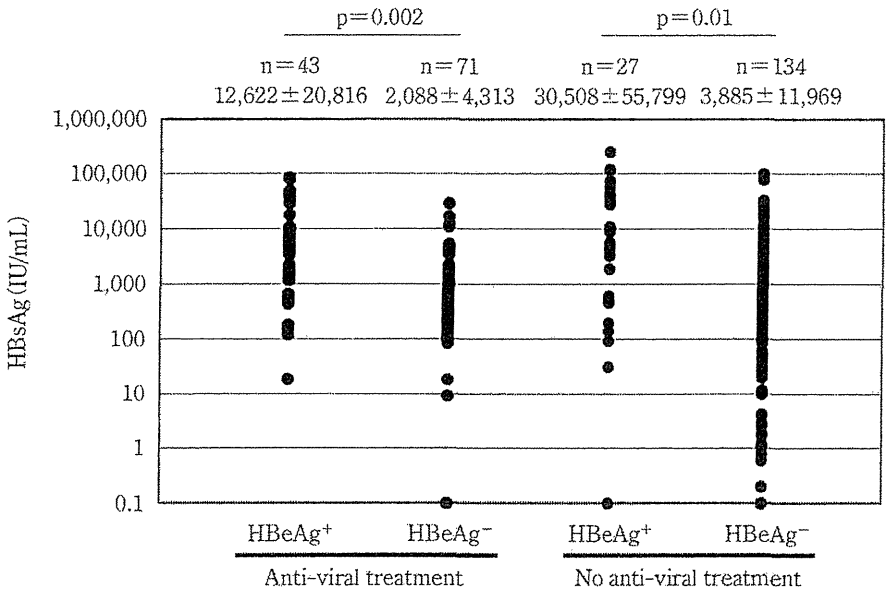


Figure 2 Quantity of HBsAg according to HBeAg status.

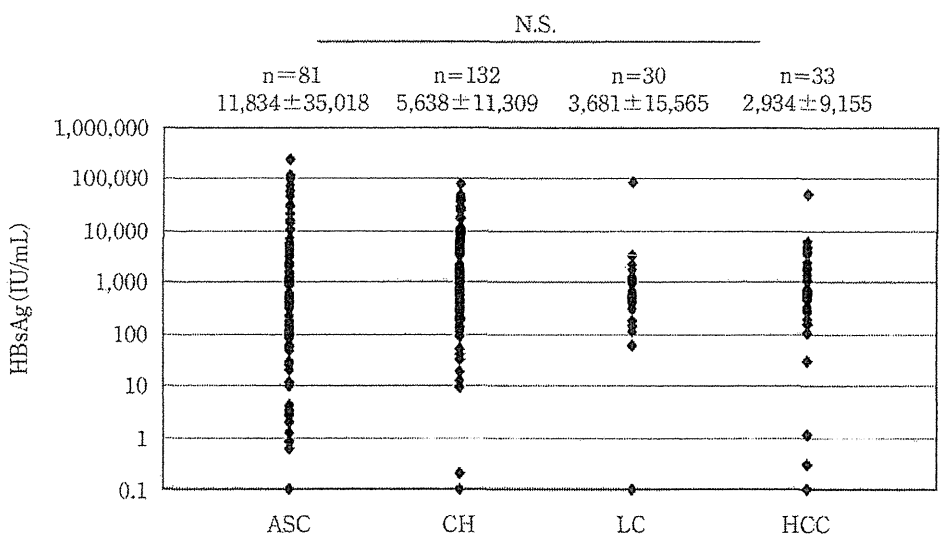


Figure 3 Quantity of HBsAg according to Disease Stage.

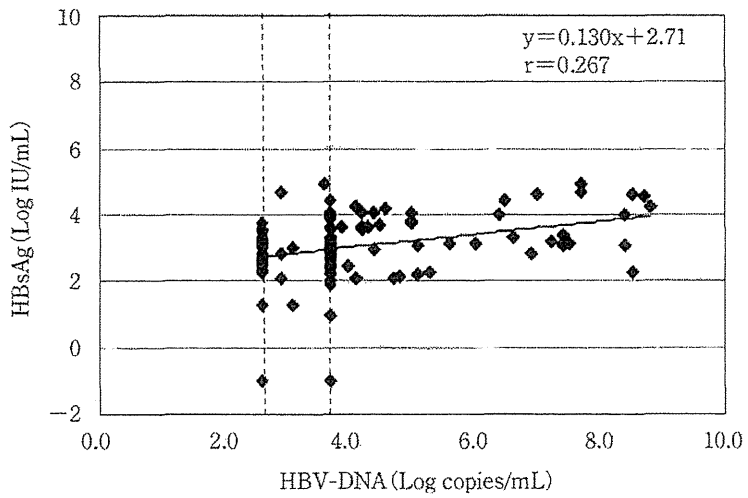
GT-C 206例，GT-D 6例であった。これら4群でHBs抗原量を比較したところ，GT-A＞GT-D＞GT-C＞GT-Bの順でHBs抗原量が高い結果となり，GT-AとC，CとBの間において有意差をみとめた(**Fig. 5**)。

III. 考　　察

現在，HBs抗原測定は定性検査として用いられることが多く，感染の有無を確認するスクリーニングが主な測定目的である。しかし，最近ではHBs抗原を定量検査として測定できる試薬がいくつか発売されており，B型肝炎の病態や治療効果との関連性も報告されている。これまでに，HBs抗原の定量測定が可能な試薬に関し，基礎的検討がいくつか報告され，日常検査において有用であることが確認されている[2)]。そこで，今回，HBs抗原定量値の臨床的有用性について検討した。

年齢別にHBs抗原量を比較したところ，治療の有無に拘らず高齢になるにつれてHBs抗原量は低値となる傾向がみられ，年齢との関連性が明らかとなった。Genotype別の比較検討では，GT-A＞D＞C＞Bの順で高値である傾向がみられた。近年，本邦において従来外来株であったGT-AによるB型急性肝炎が特に若年者において増加しており，慢性化率

A: Anti-viral treatment



B: No anti-viral treatment

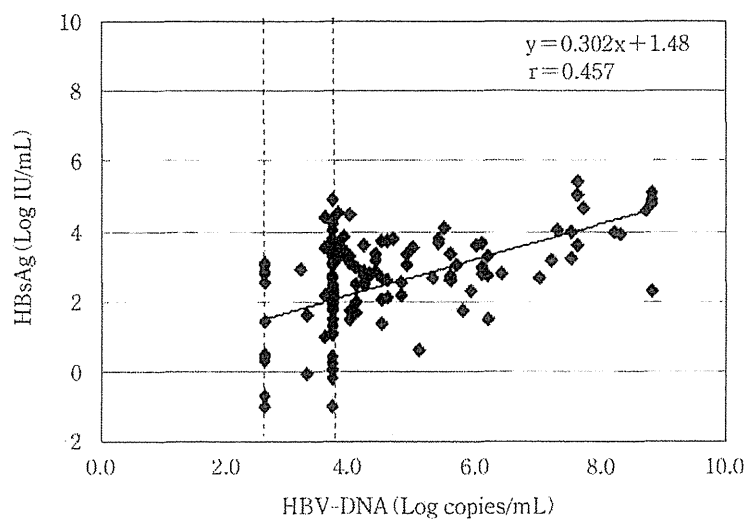


Figure 4　The correlation between HBsAg and HBV DNA.

が高いことが特徴で，実際にB型慢性肝疾患患者においてもGT-Aの割合が増加していることが報告されている[1]。したがって，GT-Aの患者は比較的若年である例が多く，HBs抗原量が高値であることについては年齢が影響している可能性が考えられる。今後，多数のGT-A例において，他のgenotypeと年齢をマッチさせHBs抗原量を比較検討する必要性がある。

HBe抗原別の検討では，治療の有無に拘らずHBe抗原陽性群が陰性群に比べてHBs抗原量が有意に高い結果となった。一般的にHBe抗原陽性例はHBVの増殖力が強いため血中のウイルス量が多いことが多く，HBs抗原量も高いということは妥当な結果であると考える。

HBV-DNA量とHBs抗原量の相関関係を検討したところ，未治療群にのみ弱い正の相関性が認められた。治療群ではHBV DNAが検出感度以下にも拘らずHBs抗原量が高い症例を多くみとめた。核酸アナログ製剤やインターフェロン投与により，HBV-DNAは速やかに減少する症例が多いが，HBs抗原量が短期間で減少する例は多くはない。そのため，HBV-DNAのモニタリングだけでは，治療効果判定が難しく，HBs抗原定量値を経時的に把握する重要性も報告されている[3]。また，インターフェロン治療中のHBs抗原定量値をモニタリングすることで治療効果の予測が可能であるとの報告もあり[4]，

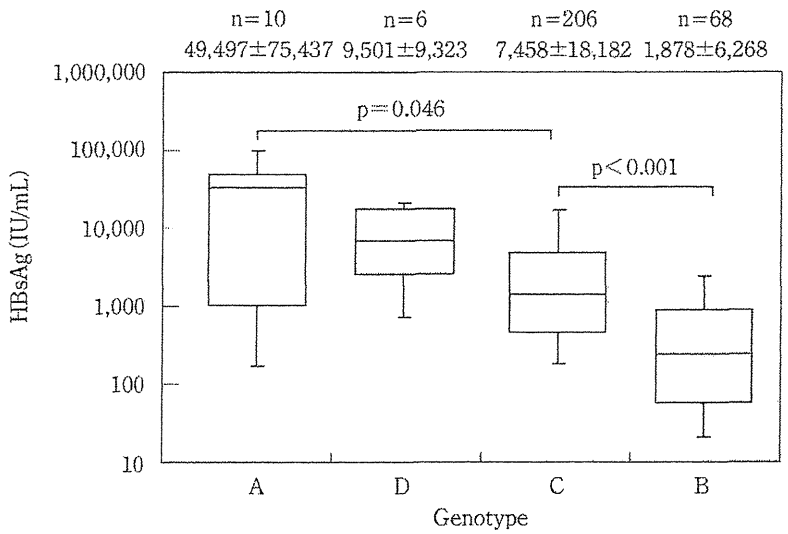


Figure 5 Quantity of HBsAg according to genotype.

国際的にはHBs抗原定量値が広く用いられている。今後，本邦でも広くHBs抗原定量値が測定されるようになることが予想される。

IV. ま と め

今回，HISCL HBsAg試薬を用い，HBs抗原定量値の臨床的有用性について検討した。病態別の検討では差が認められなかったものの，年齢，HBe抗原，Genotype別の検討ではHBs抗原定量値に有意差を認めた。今後，さらに多数例でHBs抗原定量値の臨床的意義，有用性について検討がなされ，抗ウイルス療法の効果判定，予後予測などに利用されていくものと考えられる。

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