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H. 知的所得権の出願・登録状況

なし

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書（平成24年度）

B型肝炎ウイルスｅ抗体陽性無症候性キャリアの長期予後に関する検討

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分担研究課題：B型肝炎ウイルスe抗体陽性無症候性キャリアとその再活性化に関わるB型肝炎ウイルス因子の解明-HBV の *in vitro* 実験系の構築とその予備的検討-

研究要旨： B型肝炎ウイルス(HBV)e抗体(HBe)陽性無症候性キャリアとその再活性化の要因として、活性化に関わるHBVゲノムの変異を解明するための*in vitro*の実験系を構築し、予備検討を行った。B型急性肝炎、HBVキャリアからの急性増悪およびB型劇症肝炎症例から分離したHBV株から、それぞれ1.3倍長のゲノムを作製し、さらにCore promoterおよびpreCoreに関して野生型と変異型のものを構築した。これらをヒト肝癌由来の細胞株に形質導入することにより産生されるHBVゲノムをリアルタイムPCR法で、HBs抗原をELISA法で測定し、さらに HBVゲノムの形状をSouthern blot法、mRNA をNorthern blot法によって解析した。その結果、HBVの株によって、また同一株でもCore promoterやpreCoreの変異によって増殖に差があることを観察した。本研究で構築した *in vitro* の評価系は、HBe 抗体陽性無症候性キャリア及び再活性化に於けるウイルス因子の解析に有用であると考えられた。

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A. 研究目的

B 型肝炎ウイルス(HBV)の再活性化の問題は、発症する B 型肝炎が増悪し、重症化・劇症化することがあること、また、肝機能障害によって原疾患の治療を中断あるいは中止せざるを得なくなることである。本研究は、HBe 抗体陽性無症候性キャリアからの再活性化の要因として、活性化に関わる HBV ゲノム側の因子を解明することを目的とし、本年度は *in vitro* の実験系を構築し、予備検討を行った。

B. 研究方法

1）1.3 倍長 HBV ゲノムの構築

B 型急性肝炎、HBV キャリアからの急性増悪および B 型劇症肝炎症例から分離した HBV 株(各 subgenotype A2, B1, C2)から 1.3 倍長のゲノム（nt 1073-3215/1-2067)を作製し、プラスミドベクターpBluescript II SK の KpnI-NotI 部位に挿入した。さらに、site-directed mutagenesis によって Core promoter (nt1762, nt1764)および preCore (nt 1896) の塩基が野生型と変異型のものを構築した(表)。

2）肝癌細胞株での発現と解析

前述の 1.3 倍長の HBV ゲノムを有するプラスミド DNA を各種ヒト肝癌由細胞株（HepG2, HepG2/C3A, HuH7）に TransIT-LS を用いて形質導入（トランスフェクション）し、培養上清中に産生される HBV ゲノムをリアルタイム PCR 法で、HBs 抗原を ELISA 法でそれぞれ測定し、さらに HBV ゲノムの形状を Southern blot 法、mRNA を Northern blot 法によって解析した。

（倫理面への配慮）

自治医科大学倫理委員会の承認を得て実施し、血清の採取に際して文書で同意を得

ている。検体提供者は匿名化されているため、個人のプライバシーを侵害することはなく、人権上の問題は生じない。

C. 研究結果

1）形質導入後、初期の発現

1.3 倍長ゲノムを HepG2/C3A 細胞に形質導入することによって産生された HBV 量は３つの株間で差がみられた(C2>B1>A2)。また、同一株でも[Core promoter/preCore]の塩基がそれぞれ野生型(W)か変異型(m)によっても違いが観察された([m/m]>[m/W]>[W/m]>[W/W])。HepG2/C3A 細胞の親株である HepG2 細胞でも同じ傾向がみられたが、HBV の産生量は若干低かった。HuH7 細胞では、株毎の産生量に HepG2/C3A 細胞との違いがみられた。

産生された HBV のゲノムの形状は relaxed circular (RC) 型を主として他に partially double-stranded (PDS) 型および single-stranded (SS) 型が観察された。

2）形質導入後、長期培養の発現

HepG2/C3A 細胞への形質導入後、HBV 量は 6-10 日をピークに漸減したが、40 日以降 100 日経過後も約 10^8 copies/ml の HBV の産生が認められた。

D. 考察

HBe 抗体陽性無症候性キャリア及び再活性化に於けるウイルス因子の解明を目的とする *in vitro* の評価系として、肝癌由来細胞株に形質導入した HBV の株毎および変異による増殖の違いを判別できる必要がある。本研究では、HBV 株として B 型急性肝炎、HBV キャリアからの急性増悪および B 型劇症肝炎から分離した、各 subgenotype A2、B1 および C2 の HBV３株とそれぞれの Core promoter および preCore の塩基に関して野生型と変異型のゲノムを HepG2/C3A 細胞に形質導入し、産生される HBV ゲノム量、HBs 抗原量、mRNA 等の解析を行った結果、３つの株間で、また Core promoter /preCore の変異によって増殖に違いがあることを観察することができた。今後、この評価系を用いて HBe 抗体陽性の無症候性キャリア及び再活性化症例から分離した HBV 株の解析を行い、再活性化に於けるウイルス因子の解明に向けた研究を行う予定である。また、長期培養した細胞核内の HBV ゲノムが cccDNA として存在するか否かを調べ、キャリアモデルとしての利用が可能性か検討する。

E. 結論

本研究で構築した *in vitro* の評価系は、HBe 抗体陽性無症候性キャリア及び再活性化に於けるウイルス因子の解析に有用であると考えられた。

F. 健康危険情報

該当無し

G. 研究発表

1. 論文発表：なし
2. 学会発表：なし

H. 知的所得権の出願・登録状況

1. 特許取得：なし
2. 実用新案登録：なし
3. その他：なし

検体名	診断	HBV DNA [copies/ml]	Original or mutated HBV construct	Core promoter		preC	P gene (premature termination)
				nt 1762	nt1764	nt1896	
A2	B型急性肝炎	$1.1x10^{10}$	A2_W/W	A	G	G	
			A2_m/W	T	A	G	
			A2_W/W (Pstop)	A	G	G	+
B1	HBVキャリアの急性増悪	$1.1x10^{11}$	B1_m/m	T	A	A	
			B1_W/m	A	G	A	
			B1_m/W	T	A	G	
			B1_W/W	A	G	G	
			B1_W/W (Pstop)	A	G	G	+
C2	B型劇症肝炎	$1.1x10^{11}$	C2_m/m	T	A	A	
			C2_W/m	A	G	A	
			C2_m/W	T	A	G	
			C2_W/W	A	G	G	
			C2_W/W (Pstop)	A	G	G	+

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ．研究成果の刊行物・別刷

Quantification of hepatitis B surface antigen can help predict spontaneous hepatitis B surface antigen seroclearance

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Background and aim **The clinical outcomes of hepatitis B virus (HBV) carriers are favorable following hepatitis B surface antigen (HBsAg) seroclearance. The aim of this study was to investigate the clinical course of spontaneous HBsAg seroclearance and the factors predicting it.**

Methods **A total of 423 patients who tested positive for HBsAg and were referred to Chiba University Hospital between January 1985 and April 2008 were included in the study and the following characteristics were analyzed: age, sex, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, number of platelets, HBV genotype, past treatment with interferon, and HBsAg level. When a nucleotide analog was used for treatment, we stopped follow-up. Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method and less than 0.03 IU/ml of HBsAg was designated as HBsAg seroclearance.**

Results **The study group included 239 men and 184 women and their average age was 40.5 ± 13.8 years. Twenty-five patients achieved HBsAg seroclearance during the follow-up period with an incidence rate of 0.97% per year. Multivariate analysis revealed that HBsAg titer (compared with patients with a low HBsAg level: odds ratio = 0.45, 95% confidence interval: 0.29–0.70) at baseline was the only predictive factor for HBsAg seroclearance.**

Conclusion **HBsAg seroclearance occurred at a frequency of 0.97% per year without the use of a nucleotide analog. HBsAg titer at baseline was the only predictive factor for HBsAg seroclearance. *Eur J Gastroenterol Hepatol* 00:000–000 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: chronic hepatitis B, hepatitis B antigen level, hepatitis B surface antigen seroclearance

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Introduction

An estimated 350 million people worldwide are chronically infected with the hepatitis B virus (HBV) [1]. The loss of hepatitis B surface antigen (HBsAg) usually results in normalized serum alanine aminotransferase (ALT) levels and decreased HBV DNA levels, which may lead to improved hepatic necroinflammation, and is thought to indicate clinical healing [2,3]. However, HBsAg seroclearance is a rare event in chronic hepatitis B (CHB) and its incidence is estimated to be approximately 2–3% per year [4]. Because of its rarity, the clinical course during HBsAg seroclearance remains largely unknown, although the clinical course during hepatitis e antigen (HBeAg) seroclearance has been well documented [5,6]. Historically, various factors have been reported to predict HBsAg seroclearance [7] and various studies have been carried out to distinguish the positive and negative prognostic factors for HBV carriers [8,9]. Recently, quantitative serology has been developed for HBsAg and is a promising candidate assay for determining an accurate prognosis for HBV carriers [10]. In this study, on the basis of a cohort of patients with CHB with long-term follow-up, we investigated the clinical course during HBsAg seroclearance.

Materials and methods

Patients

This was a retrospective and hospital-based analysis. Between January 1985 and April 2008, all patients visiting the Chiba University Hospital and who were HBsAg-positive carriers ($n = 676$) were approached for participation in the study. This study was reviewed and approved by the Institutional Review Board of the Chiba University School of Medicine. The patients' consent was obtained for the storage and use of serum. Patients who were positive for the hepatitis C virus antibody and those who had another potential cause for chronic liver disease (autoimmune hepatitis and primary biliary cirrhosis) were excluded from the study. To exclude patients with an acute infection of HBV, we confirmed the persistent infection of HBV before the first visit to our hospital or low titers of the IgM-HBc antibody at entry for all the patients. Those patients who were monitored for less than 1 year or who had been given antiviral drugs (lamivudine or entecavir) before entry were also excluded from the analysis. As a result, 423 patients were selected for further analysis. Study participants were followed up every 6–12 months, and the serum samples obtained from the patients

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each year were stored at −20°C. The earliest sample from each patient was used to define the level of HBsAg at entry. The level of HBsAg in the most recent sample from each of 423 patients was evaluated. When the level of HBsAg was below the cutoff (0.03 IU/ml), we designated this as HBsAg seroclearance. To clarify the relationship between HBsAg seroclearance and other factors, age, sex, HBeAg status, HBV genotype, the use of interferon, HBsAg, HBV DNA, ALT, and the number of platelets were analyzed.

Laboratory assays

Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method and the HISCL-2000i (Sysmex Corporation, Kobe, Japan). A positive linear correlation was observed between our method and Architect HBsAg QT (Abbott Laboratories, Abbott Park, Illinois, USA), which is commonly used. A dilution test showed a linear correlation curve in the range from 0.03 to 2360 IU/ml, and the samples that showed a high HBsAg level above this range could be quantified after diluting 40 or 1600 times. In addition, our method can be applied to quantify the HBsAg in serum samples with different HBV genotypes/subgenotypes, as well as in serum-contained HBV vaccine escape mutants (126S, 145R) [11,12]. HBeAg and anti-HBe levels were determined by an enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory). Anti-HCV was detected by ELISA (Ortho Diagnostics, Tokyo, Japan). The serum HBV DNA level was quantified by a polymerase chain reaction assay (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a linear range of quantification of 2.6–7.6 log copies/ml. The six major genotypes of HBV (A–F) were determined by ELISA (HBV Genotype EIA, Institute of Immunology Co. Ltd, Tokyo, Japan).

Serial changes in hepatitis B surface antigen in the patients with hepatitis B surface antigen seroclearance

To monitor the serial changes in HBsAg levels in patients with HBsAg seroclearance, the level of HBsAg was evaluated in all available samples from these patients. Changes in ALT, platelets, and HBsAg were evaluated before and after HBsAg seroclearance.

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of the clinical parameters between the two groups was analyzed using a paired *t*-test, an unpaired *t*-test, the Welch *t*-test, and the χ^2-test. All analyses were performed using the statistical program SPSS 16.1 (SPSS Inc., Chicago, Illinois, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Characteristics of patients with hepatitis B surface antigen seroclearance

The baseline clinical and virological characteristics of the 423 HBsAg-positive carriers are shown in Table 1. During the follow-up period, monitoring of those patients who received treatment for HBV with nucleotide analogs was discontinued. Twenty-five patients showed HBsAg seroclearance with an incidence rate of 0.97% per year. For these 25 patients, we confirmed the negative results of HBsAg quantification in at least two sequential samples. Two of the 25 patients had received interferon (IFN) therapy before the start of follow-up and HBsAg seroclearance in these patients occurred over 10 years after IFN treatment. First, we investigated the relationship between HBsAg seroclearance and other virological and clinical markers. In terms of HBeAg status, the level of HBV DNA and HBsAg, the number of platelet, and the period of follow-up, there were obvious difference between the patients with and without HBsAg seroclearance (Table 1). No patient suffered from liver failure. Among those with HBsAg seroclearance, hepatocellular carcinoma (HCC) occurred only in one patient (4.0%) after HBsAg seroclearance. This patient underwent a hepatectomy to remove HCC and the degree of liver fibrosis was moderate (F2), not cirrhosis. In the control group, HCC occurred in 20 patients (5.0%).

Table 1 Baseline characteristics of hepatitis B surface antigen-positive patients

Parameters	Total patients	HBsAg seroclearance	No HBsAg seroclearance	*P* value
Number	423	25	398	
Sex (male/female)	239/184	18/7	221/177	NS[a]
Age (years)	40.5±13.8	44.6±9.4	40.2±14.0	NS[d]
HBeAg status (+/−)	183/240	3/22	180/218	0.003[a]
HBV DNA (log copies/ml)	5.6±1.9	4.6±1.8	5.6±1.9	0.007[b]
ALT (IU/l)	72.7±90.4	116.3±206.1	69.9±76.8	NS[d]
Platelet (/μl)	206 000±65 000	178 000±63 000	208 000±65 000	0.026[b]
Genotype A/B/C/D/not determined	5/31/261/1/125	1/2/18/0/4	4/29/243/1/121	NS[c]
Past use of interferon	16/407	2/23	14/384	NS[c]
HBsAg ($\log_{10}$ IU/ml)	3.37±1.21	2.47±1.28	3.44±1.13	<0.001[d]
Follow-up period (days)	2217±1844	3109±2249	2159±1802	0.044[b]

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NS, not significant.
[a]χ^2-test.
[b]Unpaired *t*-test.
[c]Fischer's exact test.
[d]Mann–Whitney *U*-test.

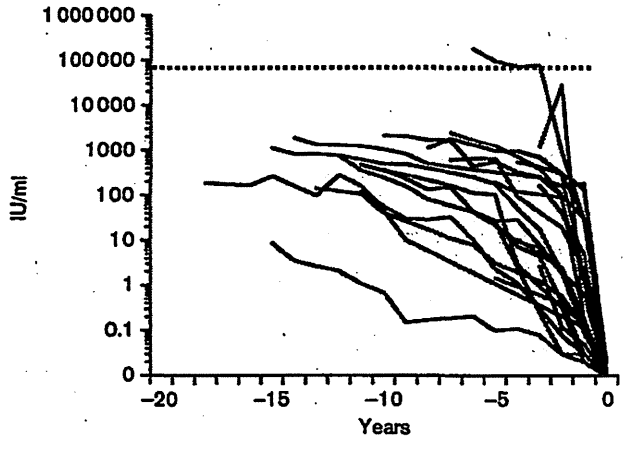
Serial changes in hepatitis B surface antigen, alanine aminotransferase level, and platelets before and after hepatitis B surface antigen seroclearance

The levels of HBsAg, ALT, and platelets in the patients with HBsAg seroclearance were evaluated annually (Figs 1, 2a and b). The average follow-up period after HBsAg seroclearance was 6.5 ± 5.7 years. HBsAg reappeared in three patients at 8, 10, and 11 years after HBsAg seroclearance. Two patients showed HBsAg seroclearance again within 2 and 3 years of the reappearance of HBsAg, but one patient could not be followed up after the reappearance of HBsAg. All 25 patients were negative for HBeAg and HBV DNA and had normal ALT levels. In addition, ALT levels did not fluctuate in these patients after HBsAg seroclearance. Platelets in the patients with HBsAg seroclearance did not show any difference between entry (180 000 ± 44 000/μl) and the end (179 000 ± 55 000/μl) of the follow-up period (paired *t*-test), although three of eight patients with less than 150 000/μl of platelets at HBsAg seroclearance showed an increase in platelets after HBsAg seroclearance.

Factors associated with the future seroclearance of hepatitis B surface antigen

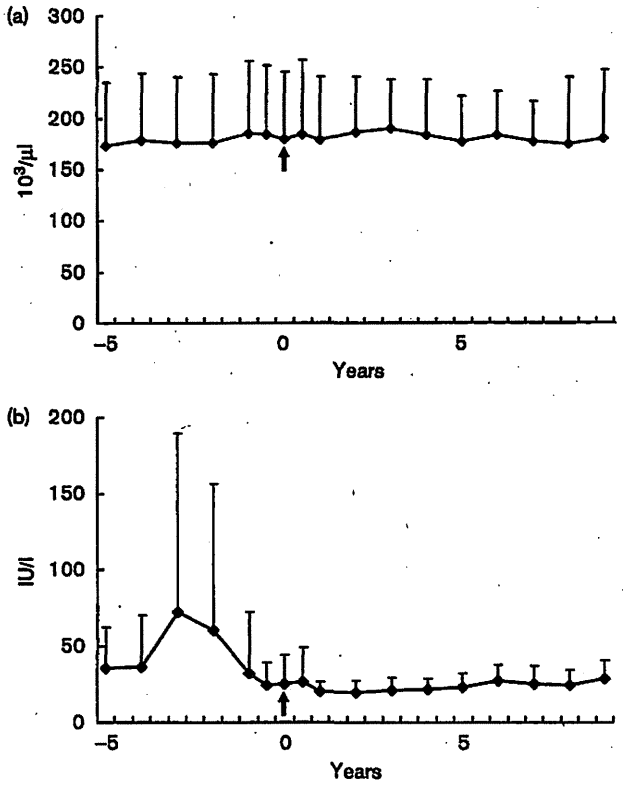
Next, we used the Cox proportional hazards model to investigate the factors associated with the future seroclearance of HBsAg (Table 2). Univariate analysis revealed that age [compared with younger patients: odds ratio (OR) = 1.06, 95% confidence interval (CI): 1.03–1.10], HBeAg negativity (compared with HBeAg positivity: OR = 7.88, 95% CI: 2.34–26.6), HBV DNA level (compared with patients with a low HBV DNA level: OR = 0.58, 95% CI: 0.46–0.75), and HBsAg titer (compared with patients with a low HBsAg level: OR = 0.39, 95% CI: 0.29–0.53) at baseline were predictive factors for HBsAg seroclearance. Multivariate analysis revealed that HBsAg titer (compared with patients with a low HBsAg level: OR = 0.45, 95% CI: 0.29–0.70) at baseline was a predictive factor for HBsAg seroclearance. Thus, these analyses revealed that a low HBsAg level was the most important factor associated with the future seroclearance of HBsAg. We performed the multivariate analysis again, changing the threshold of HBsAg from 1.0 to 5.0 log IU/ml in 1.0 log increments. We determined the threshold when the value of probability was the smallest. As a result, the threshold of HBsAg levels was determined to be 3.0 log IU/ml. The hazard ratio (95% CI) and the *P*-value were 5.32 (1.77–15.9) and 0.003, respectively. When the HBV carriers were divided into two groups, over 1000 IU/ml of HBsAg or not, HBsAg seroclearance occurred in HBV carriers with less than 1000 IU/ml of HBsAg at a higher rate and with a significant difference (log-rank test, $P < 0.01$; Fig. 3).

Fig. 1



Serial changes in hepatitis B surface antigen (HBsAg) levels in patients with HBsAg seroclearance. The average level of HBsAg at entry among all the patients was 16 994 IU/ml (dotted line), although the levels of most patients with HBsAg seroclearance were below the average. Twenty-five patients with HBsAg seroclearance showed a decline in the HBsAg level several years before HBsAg seroclearance.

Fig. 2



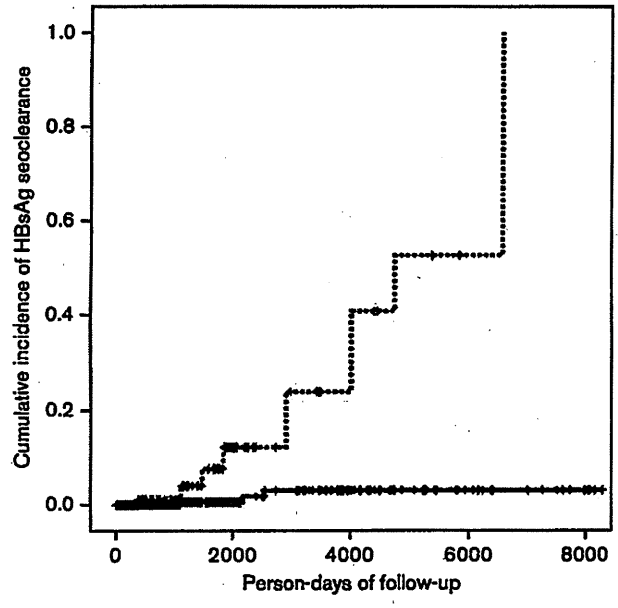
Serial changes in (a) the number of platelets and (b) alanine aminotransferase (ALT) levels before and after hepatitis B surface antigen (HBsAg) seroclearance. Platelets showed no change before and after HBsAg seroclearance. ALT levels fluctuated before HBsAg seroclearance, but did not fluctuate afterward. The arrows indicate the year of HBsAg seroclearance.

Table 2 Cox regression analysis for the predictive factors for hepatitis B surface antigen seroclearance

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age	1.06 (1.03–1.10)	0.001	1.03 (0.98–1.07)	NS
Sex male	2.35 (0.97–5.68)	NS		
HBeAg negative	7.88 (2.34–26.6)	0.001	2.62 (0.62–11.0)	NS
HBV-DNA	0.58 (0.46–0.75)	<0.001	0.94 (0.66–1.35)	NS
ALT	1.00 (1.00–1.00)	NS		
Platelet	1.00 (0.99–1.00)	NS		
Genotype A	1.92 (0.92–4.00)	NS		
Past use of interferon	1.47 (0.34–6.27)	NS		
HBsAg (log)	0.39 (0.29–0.53)	<0.001	0.45 (0.29–0.70)	<0.001

ALT, alanine aminotransferase; CI, confidence interval; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NS, not significant.

Fig. 3



Cumulative occurrence of seroclearance of hepatitis B surface antigen (HBsAg) based on the HBsAg levels over 1000 IU/ml of HBsAg or not by the Kaplan–Meier method. A significant difference was observed by the log-rank test ($P<0.01$). The dotted line indicates the group with a low HBsAg level.

Discussion

HBsAg is the fundamental diagnostic marker of HBV infection. HBsAg is a component of the Dane particle, which contains the viral genome, and of subviral particles. But the mechanisms that regulate the production of HBsAg, particularly the subviral particles, are largely unclear [13]. HBsAg seroclearance is a clinical goal for HBV carriers, because, after HBsAg seroclearance, clinical outcomes of HBV carriers are favorable and the incidence of liver failure and HCC in patients with HBsAg seroclearance is much lower than that in HBsAg-positive patients [2,3,14,15]. Individuals who become HBsAg negative can be considered to have resolved CHB. If we can predict the seroclearance of HBsAg among HBV carriers, this can help physicians manage CHB patients.

Spontaneous HBsAg seroclearance has been well documented and predictive factors for the seroclearance of HBsAg were also clarified. Liu *et al.* [4] reported that the level of HBV DNA was an important factor and Kim *et al.* [16] reported that old age and a normal ALT level were factors associated with HBsAg seroclearance. Tai *et al.* [7] reported that male sex, HBeAg negativity, older age, low maximal ALT level, and hepatic steatosis were factors associated with HBsAg seroclearance and that the estimated HBsAg seroclearance rates increased with age and reached a plateau after the age of 50 years. Our study clarified that the level of HBsAg, not the HBV DNA level, is a predictive factor for the clearance of HBsAg. In the previous reports [17,18] and ours [10], the level of HBV DNA showed a good correlation with the level of HBsAg, but there were quite a few outliers. In fact, nine (36.0%) of 25 patients with HBsAg seroclearance showed a high HBV DNA level (over 5.0 log copies/ml) at baseline. In contrast, only three (12.0%) of 25 patients showed a high HBsAg level (over 4.0 $\log_{10}$ IU/ml) at baseline. As far as HBsAg seroclearance is concerned, the HBsAg level is the most reliable predicting factor for it, and future analysis for the outliers between HBsAg and HBV DNA levels might provide a clue toward clarification of the mechanism of HBsAg seroclearance. In this study, the age at HBsAg clearance varied from 27 to 67 years and was scattered and showed no particular trend. This difference was attributed to the difference in the method of HBsAg quantification. Our study involved quantification of the HBsAg level using an assay with higher sensitivity (the cutoff level was 0.03 IU/ml) than traditional and qualitative analysis of HBsAg (the cutoff level was almost 1.0 IU/ml). In addition, most studies had not evaluated the quantitative HBsAg level as a prognostic factor for HBV carriers. In any case, to evaluate the HBsAg seroclearance precisely, HBsAg should be evaluated using a quantitative method.

Nine patients with HBsAg seroclearance showed ALT elevation within 5 years before HBsAg seroclearance. Five of nine patients showed a high HBV DNA level during ALT elevation, which might indicate a severe immune reaction for HBV. These results suggested that there exist two types of progress reaching to HBsAg seroclearance: one with a flare in the ALT level as a severe immune reaction for HBV and the other without it. We should clarify the difference between these two types in the future.

IFN therapy has antiviral and immunomodulatory effects and has been used in the treatment of CHB. In meta-analysis, IFN therapy could induce HBsAg seroclearance at the end of follow-up for at least 3 years [19,20]. In our