Table 2. Baseline characteristics of patients.

Features	Total (n=234)
Demographic data	
Age (years)	37 (12–74)
Men (%)	161 (69)
Biolochemical markers	
Albumin (g/dl)	4.1 (2.5–5.0)
Platelets ($\times 10^3/mm^3$)	179 (43–338)
ALT (IU/l)	141 (13–2644)
AFP (ng/ml)	7 (0–1863)
IP-10 (ng/ml)	214 (66–3253)
Virological markers	
HBV genotypes: A/B/C (%)	1/2/231 (0/1/ 99)
HBsAg (IU/ml)	8039 (2–261647)
HBeAg (PEIU/ml)	245.3 (0.01–3179.7)
HBV DNA (log copies/ml)	7.7 (3.6–8.9)
HBcrAg (log U/ml)	7.8 (5.4–9.2)
PC mutations: wild/mix/ mutant (%)	132/100/2 (56/43/1)
CP mutations: wild/mix/ mutant/others (%)	55/50/126/3 (24/21/54/1)
Pathological features	
Fibrosis stages: 0/1/2/3/4 (%)	15/73/54/38/54 (7/31/23/16/ 23)
Lymphocytic aggregation: 0/1/2/3/4 (%)	6/65/107/45/11 (2/28/46/19/5)
Piecemeal necrosis: 0/1/2/3/4 (%)	59/52/57/58/8 (25/22/24/25/4)
Lobular inflammation: 0/1/2/3/4 (%)	4/91/104/32/3 (2/39/44/14/1)
Antiviral treatments	
Within 1 year of biopsy (%)	91 (39)
Antiviral agents: 1/2/3/4* (%)	44/33/13/1 (49/36/14/1)
Duration of follow up (months)	86.5 (12.0–213.0)

Qualitative variables are expressed in the number with percentage in parentheses, and quantitative variables are expressed in the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

piecemeal necrosis in the liver, as well as treatments within 1 year after the entry and type of antiviral agents, were not associated with early HBeAg seroconversion (Table 3).

Evaluation of HBV markers for predicting early HBeAg seroconversion

HBV markers were compared for sensitivity and specificity in predicting early HBeAg seroconversion by the receiver operating characteristic analysis (Figure 1). HBeAg at the time of liver biopsy was the best predictor of early HBeAg seroconversion, with the widest area under the curve of 0.750; it was larger than those of HBcrAg (0.708), HBV DNA (0.650) and HBsAg (0.630). Hence, HBeAg was selected as the best HBV marker predictive of early seroconversion. Based on the receiver operating characteristic curve, HBeAg titers were dichotomized by 100 PEIU/ml in the immunoassay.

Independent predictors for early HBeAg seroconversion

A multivariate logistic regression analysis was performed to select independent predictors of early HBeAg seroconversion from among variables significant in the univariate analysis (Table 4). Of all factors, including histological characteristics, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation remained as independent factors predictive of early HBeAg seroconversion (Table 4A). Of factors exclusive of histological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/ml remained as independent factors for early HBeAg seroconversion (Table 4B).

Combinations of two independent factors for predicting early HBeAg seroconversion

Two combinations of independent factors were evaluated for the performance in predicting early HBeAg seroconversion. The patients who had two predictors in combination, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation, achieved early HBeAg seroconversion in the highest frequency at 66.0% (31/47). In a remarkable contrast, merely 6.9% (4/58) of the patients without either of these predictors achieved early HBeAg seroconversion (Figure 2A).

Likewise, early seroconversion was achieved by 18 of the 30 (60.0%) patients with the other combination of independent factors, exclusive of pathological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/l. By contrast, only 6 of the 99 (6.1%) patients without either of them achieved early HBeAg seroconversion (Figure 2B).

Sensitivity, specificity, positive predictive value and negative predictive value of predicting early HBeAg seroconversion are: 74.5% (31/58), 90.9% (160/176), 66.0% (31/47) and 85.6% (160/187), respectively, for the combination of HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation; and 31.0% (18/58), 93.2% (164/176), 60.0% (18/30) and 80.4% (164/204), respectively, for the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l.

Long-term clinical outcomes

Besides the 58 patients with early HBeAg seroconversion, an additional 97 patients achieved HBeAg seroconversion during a median follow-up period of 86.5 months. Cumulative

Table 3. Univariate analysis of risk factors for early HBeAg seroconversion.

Variables	Early HBeAg seroconversion		p value
	Achieved (n=58)	Not achieved (n=176)	
Demographic data			
Age (years)	36 (17–69)	37 (12–74)	0.303
Men (%)	41 (71)	120 (68)	0.721
Biolochemical markers			
Albumin (g/dl)	4.1 (2.8–4.8)	4.1 (2.5–5.0)	0.877
Platelets ($\times 10^3/mm^3$)	171 (43–291)	186 (57–338)	0.487
ALT (IU/l)	227 (18–2072)	121 (13–2644)	0.002
AFP (ng/ml)	12 (1–1863)	6 (0–683)	0.070
IP-10 (ng/ml)	259 (77–1743)	204 (66–3253)	0.029
Virological markers			
HBV genotypes A/B/C (%)	0/0/58 (0/0/100)	1/2/173 (1/1/98)	1
HBsAg (IU/ml)	5127 (8–261647)	9033 (2–128511)	0.003
HBeAg (PEIU/ml)	20.9 (0.01–1985.0)	377.1 (0.01–3179.7)	<0.001
HBV DNA (log copies/ml)	7.2 (3.7–8.7)	7.8 (3.6–8.9)	0.001
HBcrAg (log U/ml)	7.2 (5.7–9.2)	8.0 (5.4–9.1)	<0.001
PC mutations: wild/mix/mutant (%)	26/31/1 (45/53/2)	106/69/1 (60/39/1)	0.075
CP mutations: wild/mix/mutant/others (%)	8/9/40/1 (14/15/69/2)	47/41/86/2 (27/23/49/1)	0.040
Pathological features			
Fibrosis stage: 0/1/2/3/4 (%)	1/12/18/14/13 (2/21/31/24/22)	14/61/36/24/ 41 (8/35/20/14/23)	0.033
Lymphocytic aggregation: 0/1/2/3/4 (%)	0/11/27/17/3 (0/19/47/29/5)	6/54/80/28/8 (3/31/45/16/5)	0.087
Piecemeal necrosis: 0/1/2/3/4 (%)	7/12/18/19/2 (12/21/31/33/3)	52/40/39/39/6 (30/23/22/22/3)	0.068
Lobular inflammation: 0/1/2/3/4 (%)	0/13/29/15/1 (0/22/50/26/2)	4/78/75/17/2 (2/44/43/10/1)	0.002
Antiviral treatments within 1 year after biopsy (%)	28 (48)	63 (36)	0.091
Antiviral agents: 1/2/3/4* (%)	18/5/5/0 (64/18/18/0)	26/28/8/1 (41/44/13/2)	0.051

Qualitative variables are expressed by the number of patients with percentage in parentheses, and quantitative variables are expressed by the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up >10 years after liver biopsies (Figure 3). Of note, HCC developed in 18 of the 234 (7.7%) patients during the follow-up.

Figure 4A compares cumulative HBeAg seroconversion rates stratified by HBeAg titers and grades of lobular inflammation. The patients, who had the combination of HBeAg <100 PEIU/ml and lobular inflammation grades ≥2, gained an HBeAg seroconversion rate higher than those having 3 other combinations. Likewise, cumulative HBeAg seroconversion rates stratified by HBeAg titers and ALT levels are compared in Figure 4B. HBeAg seroconversion rate of the patients, who had the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l, was higher than those with 3

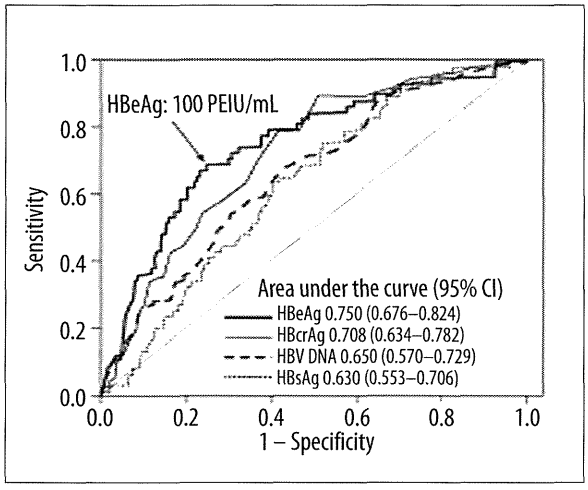


Figure 1. Receiver operating characteristic curves for evaluation of the power of predicting early HBeAg seroconversion.

other combinations, with definitive (p=0.003 and p<0.001) or marginal (p=0.061) significance.

Discussion

HBeAg seroconversion is important as a clinical target in the management of chronic hepatitis B. In the absence of therapeutic interventions, HBeAg seroconversion occurs spontaneously at a rate of 0.8–15% per year [28]. To date, many factors have been found in association with HBeAg seroconversion, including older age, high ALT levels, genotype B (compared with C), the Knodell's index of histologic activities, the amount of HBV core antigen in the liver, high serum AFP levels, increased immunoglobulin-M anti-HBc titers, increased serum β_2-microglobulin concentrations, enhanced expression of HLA-antigens on the membrane of hepatocytes, non-vertical transmission modes, low HBV DNA levels, and high serum levels of IL-10 as well as IL-12 [7–19].

It would be clinically useful to predict early HBeAg seroconversion, because antiviral treatments can be withheld in the patients in whom HBeAg disappears and anti-HBe develops within a certain time limit, perhaps 1 year. In the present study, the majority of patients (99% of the 234 examined) were infected with HBV of genotype C. Patients with persistent HBV infection in Japan are infected with HBV of either genotype B or C, with an increasing gradient of C toward the south [29,30]. All

Table 4. Multivariate analysis for the risk of early HBeAg seroconversion.

Variables	Odds ratio	95% confidence interval	p value
(A) All factors including histological characteristics			
HBeAg (<100 PEIU/ml)	8.430	4.173–17.032	<0.001
Lobular inflammation (≥2)	4.330	2.009–9.331	<0.001
(B) Factors exclusive of histological characteristics			
HBeAg (<100 PEIU/ml)	7.327	3.703–14.497	<0.001
ALT (≥200 IU/l)	3.093	1.562–6.127	0.001

HBeAg – hepatitis B e antigen; ALT – alanine aminotransferase.

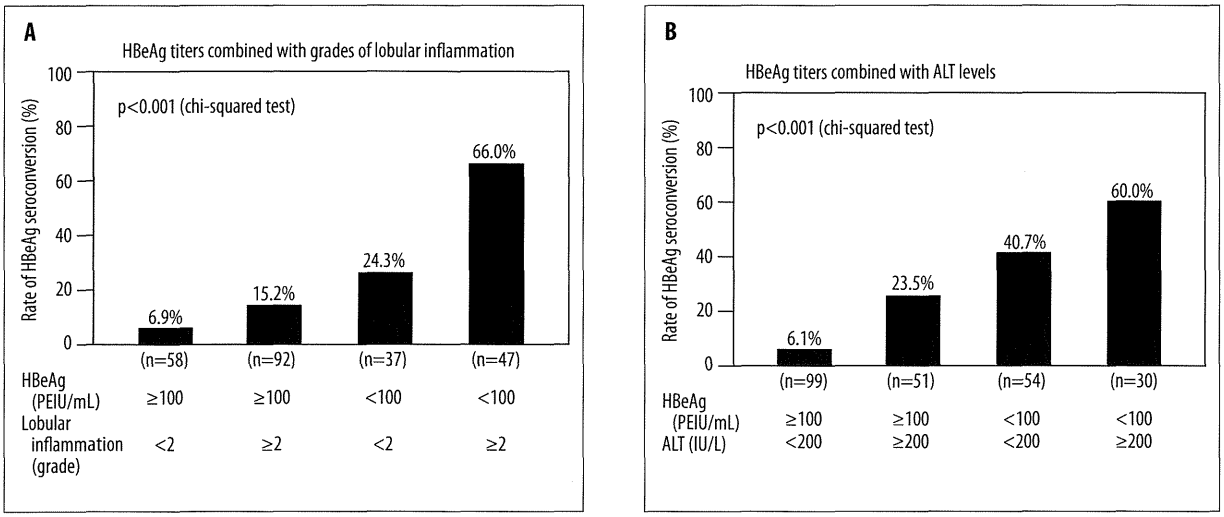


Figure 2. Probability of early HBeAg seroconversion. (**A**) The rate of early HBeAg seroconversion assessed by HBeAg titers and grades of lobular inflammation. (**B**) The rate of early HBeAg seroconversion assessed by HBeAg titers and ALT levels.

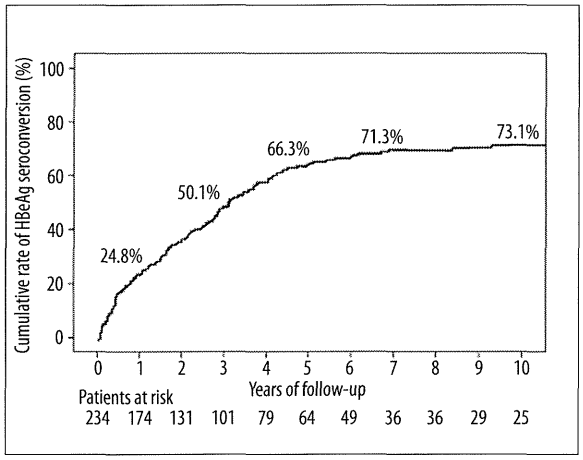


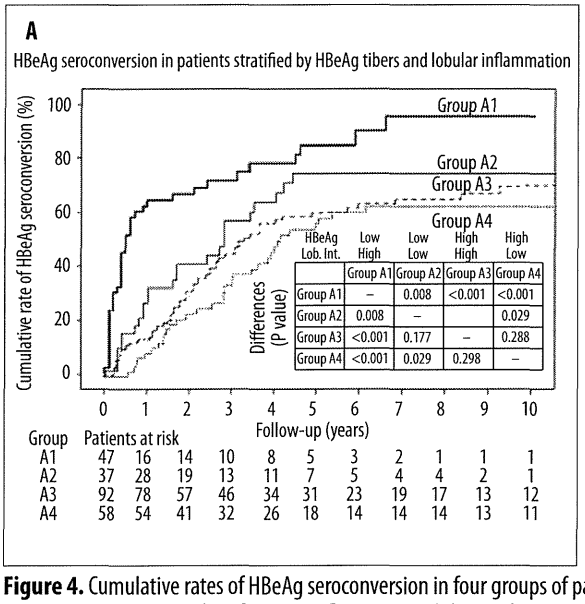
Figure 3. Cumulative rates of HBeAg seroconversion in the 234 patients during 10 years. Cumulative rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up.

the 234 patients had received liver biopsies before they were started to be followed for HBeAg seroconversion. The present study is unique in that, not only serological variables, but also histological parameters were evaluated for the association with early HBeAg seroconversion within 1 year. By univariate analysis, many factors that have been reported in association with HBeAg seroconversion predicted early HBeAg seroconversion. Among them, only HBeAg (<100 PEIU/ml) and lobular inflammation (grades ≥2) remained as independent factors for early HBeAg seroconversion by multivariate analysis.

Previous clinical studies have indicated that serial monitoring of HBsAg, HBeAg and HBV DNA levels during antiviral treatments is useful for predicting HBeAg seroconversion [20–23]. Although the determination of HBV DNA in sera remains as an important tool for monitoring outcomes of patients with chronic hepatitis B, it is technically challenging, costly, and subject to inconsistency. Hence, three serological markers of HBV replication, HBsAg, HBeAg and HBcrAg, were quantitated for evaluating the performance in predicting early HBeAg seroconversion, in comparison with HBV DNA levels. In the receiver operating characteristic analysis, HBeAg levels performed the best amongst these four replication markers, with an area under curve wider than those of the other three. Since the quantitation of HBeAg is relatively easy, fast, and inexpensive, HBeAg would be qualified as a sensitive and practical predictor of early HBeAg seroconversion [20–23].

The histological activity has been reported to predict early HBeAg seroconversion in previous studies [14,31]. Therefore, pathological parameters including the stage of fibrosis, as well as grades of portal inflammation, piecemeal necrosis and lobular inflammation, were evaluated in this study. By multivariate analysis, lobular inflammation of grades ≥2, represented by focal necrosis or acidophil bodies, was identified as an independent factor for early seroconversion. Hence, portal inflammation without necrosis would not be enough, but instead, severe lobular inflammation may be required for predicting early seroconversion.

Many previous studies have identified a variety of factors associated with HBeAg seroconversion [7–19], but a combination of serum markers of HBV with pathological parameters was evaluated rarely. Therefore, the combination of HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation was evaluated for the predictability of early HBeAg seroconversion. Patients with neither HBeAg <100 PEIU/ml nor grades ≥2 lobular inflammation had a minimal chance for early HBeAg seroconversion (6.9% [4/58]), whereas a high proportion of patients with both of these predictors did accomplish early seroconversion (66.0% [31/47]) (Figure 2A). Thus, the combination of histologic activity and serum HBV marker would be very useful for predicting early HBeAg seroconversion, and serve in decision making whether or not



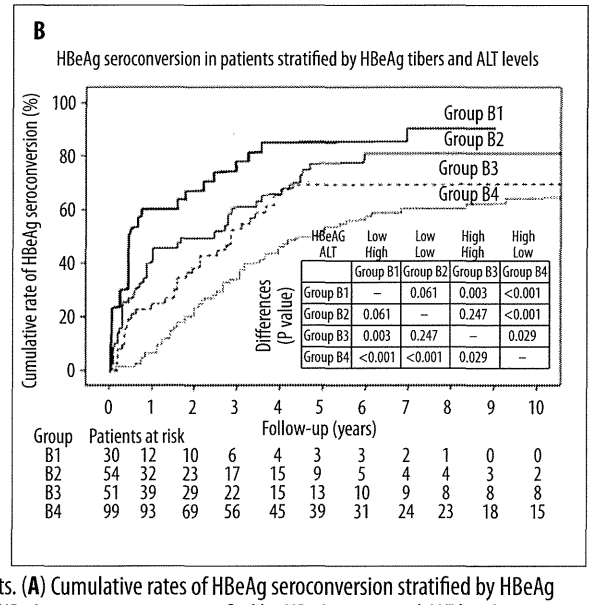


Figure 4. Cumulative rates of HBeAg seroconversion in four groups of patients. (**A**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and grades of lobular inflammation. (**B**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and ALT levels. HBeAg titers were dichotomized into low (<100 PEIU/ml) or high (≥100 PEIU/ml); lobular inflammation grades into low (<2) or high (≥2); and ALT levels into low (<200 IU/l) or high (≥200 IU/l).

to commence antiviral treatments in HBeAg-positive patients with chronic hepatitis B. Although some patients received antiviral treatments, they would not have influenced the evaluation to any serious extent. Within the first 1 year of follow-up, antiviral treatments were given comparably frequently to patients with and without early HBeAg seroconversion (48% *vs.* 36%, p=0.091). In addition, HBeAg seroconversion is achieved by at most 12–27% of patients who had received antiviral treatments during the first year [28].

Although liver biopsy is essential for defining the stage of disease progression, it has some limitations, in that it is invasive and accompanies the risk of complications. By multivariate analysis, exclusive of pathological factors, ALT ≥200 IU/l remained as an independent factor (Table 4). ALT ≥200 (IU/l), corresponding to 5 × the upper limit of normal [ULN], coincided with the cut-off point recognized by the receiver operating characteristic curve (data not shown). In previous studies, also, ALT levels ≥5 × ULN were predictive of early HBeAg seroconversion [19,32–33]. Present results are in line with these observations, and point to the capability of ALT ≥200 IU/l to replace lobular inflammation of grades ≥2 in the patients in whom liver biopsy is not feasible.

Conclusions

The results of this study indicate that the combination of low HBeAg titers and high grades of lobular inflammation is clinically useful for predicting early HBeAg seroconversion in patients with chronic hepatitis B. When and if liver biopsy is not to be performed, ALT can substitute for lobular inflammation. The combination of low HBeAg titers, with either high grades of lobular inflammation or elevated ALT levels, predicted not only early, but also long-term HBeAg seroconversion.

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GENETIC POLYMORPHISM-DISEASE ASSOCIATION

HLA-DP gene polymorphisms and hepatitis B infection in the Japanese population

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OMURA AND NAGASAKI, JAPAN

The mechanisms underlying the different outcomes of hepatitis B virus (HBV) infection are not fully understood.[1] Kamatani et al[2] identified an association of the single nucleotide polymorphisms (SNPs) human leukocyte antigen (*HLA*)-*DPA1* (rs3077) and *HLA-DPB1* (rs9277535) with chronic HBV infection in a genome-wide association study (GWAS). Additional studies confirmed that rs3077 and rs9277535 were associated with chronic HBV infection in the Han-Chinese population and strengthened the findings from previous GWAS.[3-6] Furthermore, Hu et al[7] reported that SNPs in *HLA-DP* (rs3077 and rs9277535) were associated with both HBV clearance and hepatocellular carcinoma (HCC) development. To investigate the association of these *HLA-DP* variants with the disease progression of HBV infection, we genotyped the 2 SNPs (rs3077 and rs9277535) in different clinical stages of liver disease in Japanese HBV carriers.

CLINICAL SUMMARY

A total of 241 HBV carriers (positive for hepatitis B surface antigen) who visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between 1999 and 2007 were enrolled. As controls, 143 healthy Japanese volunteers (56 men and 87 women aged 16–63 years, with a mean age of 31.3 ± 8.9 years) without any history of liver disease were enrolled. All patients did not have any other types of liver diseases, such as chronic hepatitis C, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The study protocol was approved by the Ethics Committees of National Nagasaki Medical Center, and informed consent was obtained from each individual. Of the 241 HBV carriers, 69 were considered to be asymptomatic carriers on the basis of sustained normalization of the serum alanine aminotransferase (ALT) levels together with seropositivity for anti-hepatitis Be antigen throughout the study. On the other hand, 172 of the 241 HBV carriers were considered to have chronic liver disease, such as chronic hepatitis (57), cirrhosis (65), or HCC (50) manifested by elevated ALT levels and by clinical or histologic findings on examination of liver tissue during the follow-up period. Of the 50 patients with HCC, 6 (12%) were found to have chronic hepatitis and 44 (88%) had cirrhosis. All patients were regularly followed with measurements of serum ALT and HBV markers, such as hepatitis B surface antigen, hepatitis Be antigen, anti-hepatitis Be antibody, and HBV-DNA. A total of 79 patients had undergone liver biopsy during the study to assess the degree of liver fibrosis. However, liver biopsy was not performed in patients who had apparent biochemical, endoscopic, and ultrasound features of liver cancer. Tumor markers such as alpha-fetoprotein and des-γ-carboxy-prothrombin were measured with ultrasonography of the liver every 6 months to detect HCC in an early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle tumor biopsy specimens. The genotype of rs3077 (*HLA-DPA1*) and rs9277535 (*HLA-DPB1*) was determined by direct sequencing. The apolipoprotein B mRNA-editing enzyme catalytic peptide 3G (*APOBEC3G* H186R) genotyping was performed on the basis of the report by An et al.[8]

The frequencies of the 2 SNPs of *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) are listed in Table I. There was a significant difference in the frequencies between these 2 SNPs between Japanese HBV carriers and healthy subjects, as described previously.[3] We divided HBV carriers into 2 groups: a nonadvanced group (asymptomatic carriers or chronic hepatitis, n = 115) and an advanced group (liver cirrhosis or HCC, n = 126). The frequencies of CC (rs3077) or GG (rs9277535) genotypes were higher in the advanced group compared with those in the nonadvanced group; however, the difference was not significant (Table I). Next, we stratified the HBV carriers for the presence or absence of the *APOBEC3G* H186R variant and examined the effects of *HLA-DP* polymorphisms on the progression of HBV-related liver disease. Both C and G alleles of rs3077 and rs9277535 significantly increased the risk for advanced liver disease in HBV carriers lacking the H186R variant (Table II).

A 2-stage GWAS identified SNPs including rs3077 and rs9277535 located in *HLA-DPA1* and *HLA-DPB1*, which were associated with a susceptibility to chronic HBV infection.[2] After the first Japanese GWAS, 5 studies replicated the association of these 2 *HLA-DP* SNPs (rs3077 and rs9277535) and chronic HBV infection in the Han-Chinese population.[3-7] Among these studies, an association between HBV-related HCC and rs9277535 or rs3077 was demonstrated.[7] In this study, we examined whether these 2 SNPs (rs3077 and rs9277535) in *HLA-DP* genes were associated with the disease progression and susceptibility to HBV infection in a Japanese population. As demonstrated previously, we reconfirmed that rs3077 and rs9277535 in the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with HBV infection. Although some differences in the frequencies of rs3077 and rs9277535 genotypes between HBV carriers with advanced liver disease (liver cirrhosis and HCC) and those without advanced liver disease were observed, these differences were not statistically significant.

Recent evidence suggests that APOBEC3G inhibits HBV production by interfering with HBV replication through hypermutation of the majority of the HBV genome.[8] Because of the *APOBEC3G* gene's ability to regulate HBV replication, mutations of the gene may cause a deleterious variation that may affect the outcome of HBV infection. Among the SNPs identified in the *APOBEC3G* gene, H186R variant was strongly associated with a decline in $CD4^+$ T-cell numbers and accelerated progression to acquired immune deficiency syndrome–defining conditions in human immunodeficiency virus–infected individuals.[9,10] Viral disease outcome is influenced by host variability in immune response genes and genes that control viral replication or mutation rate.[11] *APOBEC3G* coding region variant might influence the progression of HBV infection by inducing the replication of HBV.[12] Therefore, genetic diversity of immune response genes, such as *HLA*, and genes that control viral replication, such as *APOBEC3G*, could contribute to the variability in outcome of HBV infection. To minimize the effects

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Table I. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and HBV infection

SNP ID	HBV carrier n = 241 (%)	Healthy subjects n = 143 (%)	*P* value*	OR (95% CI)	Advanced HBV carrier n = 115 (%)	Nonadvanced HBV carrier n = 126 (%)	*P* value*	OR (95% CI)
rs3077								
C/C	148 (61.4)	47 (32.9)			77 (67.0)	71 (56.3)		
C/T	79 (32.8)	72 (50.3)			33 (28.7)	46 (36.5)		
T/T	14 (5.8)	24 (16.8)			5 (4.3)	9 (7.1)		
C allele (allele frequencies)	375 (77.8)	166 (58.0)	<0.0001	2.533 (1.843–3.483)	187 (81.3)	188 (74.6)	0.077	1.480 (0.957–2.290)
rs9277535								
G/G	143 (59.3)	45 (31.5)			73 (63.5)	70 (55.6)		
A/G	82 (34.0)	72 (50.3)			36 (31.3)	46 (36.5)		
A/A	16 (6.6)	26 (18.2)			6 (5.2)	10 (7.9)		
G allele (allele frequencies)	368 (76.3)	162 (56.6)	<0.0001	2.471 (1.804–3384)	182 (79.1)	186 (73.8)	0.170	1.345 (0.880–2.056)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were calculated using the chi-square test.

Table II. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and the outcome of HBV infection in HBV carrier without H186R variant

SNP ID	Advanced HBV carrier n = 90 (%)	Nonadvanced HBV carrier n = 108 (%)	*P* value*	OR (95% CI)
rs3077				
C/C	64 (71.1)	60 (55.6)		
C/T	22 (24.4)	40 (37.0)		
T/T	4 (4.4)	8 (7.4)		
C allele (allele frequencies)	150 (83.3)	160 (74.1)	0.026	1.750 (1.065–2.874)
rs9277535				
G/G	5 (5.6)	10 (9.3)		
A/G	24 (26.7)	39 (36.1)		
A/A	61 (67.8)	59 (54.6)		
G allele (allele frequencies)	146 (81.1)	157 (72.7)	0.049	1.614 (1.000–2.604)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were culculated using the chi-square test.

of viral factors, such as APOBEC3G-mediated HBV editing, and evaluate the effect of *HLA-DP* more precisely, we focused on the subjects without the H186R variant. Because the *APOBEC3G* coding region variant might influence the progression of HBV infection,[11] we investigated the effect of *HLA-DP* polymorphisms on the outcome of HBV infection in HBV carriers lacking the H186R variant.

Our results showed that *HLA-DP* polymorphisms were associated with the progression of HBV infection and that this association was significant in Japanese HBV carriers lacking H186R variants. Our data demonstrated that *HLA-DP* polymorphisms are important in determining the susceptibility and the progression of HBV infection in the Japanese population.

One limitation of our study is the lack of information of HBV genotypes in the patients studied. Another limitation is that the number of HBV carriers (n = 241) is relatively small. Larger studies are needed to confirm the results of our study.

CONCLUSIONS

We confirmed that rs3077 and rs9277535 SNPs in the *HLA-DP* locus are associated with the susceptibility and progression of HBV infection in the Japanese population. Further functional analyses are warranted to validate the biological plausibility of these SNPs in chronic HBV infection.

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ORIGINAL ARTICLE

Evaluation of long-term entecavir treatment in stable chronic hepatitis B patients switched from lamivudine therapy

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Abstract
Purpose Current Japanese guidelines recommend that patients should be switched from lamivudine to entecavir when they meet certain criteria. This analysis examines the efficacy and safety of long-term entecavir therapy in patients who were switched to entecavir after 24 weeks' lamivudine therapy in Japanese studies ETV-047 and ETV-060.
Methods The Phase II Japanese study ETV-047 assessed the efficacy of different entecavir doses when compared with lamivudine. A total of 33 Japanese patients who received lamivudine 100 mg daily in ETV-047 entered the open-label rollover study ETV-060 and subsequently received treatment with entecavir 0.5 mg daily. Hepatitis B virus (HBV) DNA suppression, alanine aminotransferase (ALT) normalization, hepatitis B e antigen (HBeAg) seroconversion, and resistance were evaluated among patients with available samples for up to 96 weeks. Safety was assessed throughout the treatment period.
Results After 96 weeks of entecavir therapy in ETV-060, 90% of patients achieved HBV DNA <400 copies/mL as compared to 21% of patients who completed 24 weeks of lamivudine therapy in ETV-047. Increasing proportions of patients achieved ALT normalization and HBeAg seroconversion following long-term entecavir treatment. No patients experienced virologic breakthrough, and substitutions associated with entecavir resistance were not

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observed in patients with detectable HBV DNA. Entecavir was well tolerated during long-term treatment.

Conclusions Switching lamivudine-treated patients with chronic hepatitis B to entecavir results in increased virologic suppression with no evidence of resistance through 2 years of entecavir therapy. These findings support recommendations in the current Japanese treatment guidelines that stable lamivudine patients should be switched to entecavir.

Keywords Japanese · Chronic hepatitis B · Entecavir · Lamivudine · Switch

Introduction

Chronic hepatitis B virus (HBV) infection affects more than 350 million people worldwide, and is a leading cause of liver-related mortality [1]. Although Japan has one of the lowest prevalence rates for chronic hepatitis B (CHB) (0.8%) among Asian countries, it is still estimated that over 1 million people are chronically infected with HBV [2]. These individuals are at an increased risk of developing cirrhosis, liver failure or hepatocellular carcinoma (HCC) [3].

Lamivudine was the first nucleoside analog introduced for the treatment of CHB. In clinical trials, it demonstrated superior efficacy to placebo for HBV DNA suppression, alanine aminotransferase (ALT) normalization and hepatitis B e antigen (HBeAg) seroconversion [4, 5]. However, a major limitation of lamivudine therapy is the development of resistance, which occurs in up to 70% of patients through 4 years of therapy [6]. Entecavir is a potent inhibitor of HBV replication [7]. In global Phase III studies, entecavir demonstrated superior histologic, virologic and biochemical responses when compared with lamivudine in nucleoside-naïve patients and lamivudine-refractory patients at 48 weeks [8–10]. In the Japanese Phase II study ETV-047, treatment with entecavir resulted in a superior reduction in HBV DNA as compared to lamivudine [11]. In contrast to lamivudine, entecavir has been shown to have a high genetic barrier to resistance; the cumulative probability of resistance through 5 years of treatment has been reported to be 1.2% [12]. The genetic barrier is lower in patients who are infected with lamivudine-resistant HBV and consequently higher resistance rates are observed in this population with long-term treatment [12].

Current Japanese treatment guidelines recommend that all treatment-naïve CHB patients with ALT levels $\geq$31 IU/L should be treated, dependent on their viral load. The thresholds for treatment are HBV DNA ≥ 5 $\log_{10}$ copies/mL in HBeAg-positive patients, ≥ 4 $\log_{10}$ copies/mL in HBeAg-negative patients, and ≥ 3 $\log_{10}$ copies/mL in cirrhotic patients [13]. Lamivudine, adefovir, and entecavir are currently approved for the treatment of CHB in Japan. Entecavir 0.5 mg once daily is the first choice therapy for treatment-naïve HBeAg-positive and negative patients aged 35 years or older. In treatment-naïve patients <35 years, the guidelines recommend treating first with interferon for HBeAg-positive patients, and treating HBeAg-negative patients with HBV DNA ≥ 7 $\log_{10}$ copies/mL with entecavir until undetectable HBV DNA is achieved, followed by a combination of entecavir and interferon for 4 weeks, and finally interferon monotherapy for 20 weeks. HBeAg-negative patients with HBV DNA <7 $\log_{10}$ copies/mL should be monitored or can receive interferon therapy. For patients who are lamivudine experienced, but not necessarily resistant, the guidelines also recommend that patients can be switched to entecavir 0.5 mg daily if they have received lamivudine therapy, and have HBV DNA <2.1 $\log_{10}$ copies/mL. Patients with HBV DNA ≥ 2.1 $\log_{10}$ copies/mL can also be switched to entecavir 0.5 mg once daily if they do not have viral breakthrough. Limited data on the efficacy of entecavir in this patient population are available; however, the design of the Japanese study ETV-047 and the rollover study ETV-060 presents an opportunity to assess the efficacy of this treatment option. This report examines the long-term efficacy, safety and resistance of entecavir 0.5 mg daily among patients who were directly switched from lamivudine following 24 weeks' treatment in ETV-047.

Materials and methods

Study population

Study ETV-047 was a Phase II, randomized, double-blind study conducted to evaluate the dose–response relationship of entecavir and compare the antiviral activity and safety of entecavir to lamivudine in Japanese patients with CHB. In ETV-047, 137 patients were randomized to receive one of three entecavir doses [0.01 mg ($n = 35$), 0.1 mg ($n = 34$) or 0.5 mg ($n = 34$), once daily] or lamivudine [100 mg ($n = 34$), once daily] for 24 weeks. The study design and complete inclusion criteria have been described previously [11]. Briefly, eligible patients had HBeAg-positive or -negative CHB with compensated liver disease, HBV DNA ≥ 7.6 $\log_{10}$ copies/mL by PCR assay, <12 weeks' prior therapy with anti-HBV nucleoside analogs and ALT levels 1.25–10 × upper limit of normal (ULN). After completion of treatment in ETV-047, all patients were eligible to enroll immediately in the rollover study ETV-060, with no gap in dosing.

The rollover study ETV-060 was designed to provide open-label entecavir for patients who had completed therapy in the Japanese Phase II program. Patients who completed 24 weeks of treatment in ETV-047 enrolled in ETV-060 and received 0.5 mg entecavir once daily. After 96 weeks of treatment in study ETV-060, patients could complete the study and were eligible to receive commercially available entecavir, which was approved by Japanese health authorities while study ETV-060 was ongoing.

The current analysis describes results for a subset of 33 patients who received lamivudine for 24 weeks in ETV-047 and entecavir 0.5 mg once daily for up to 96 weeks in ETV-060.

Efficacy analyses

Efficacy assessments evaluated the proportions of patients who had available samples (non-completer = missing) every 24 weeks through 120 weeks' treatment. Efficacy end points assessed included HBV DNA <400 copies/mL by PCR assay, ALT normalization ($\leq 1.0 \times$ ULN), HBeAg seroconversion among patients who were HBeAg-positive at baseline, and hepatitis B surface antigen (HBsAg) loss. Serum HBV DNA was determined by Roche Amplicor® PCR assay (Roche Diagnostics K.K., Tokyo, Japan; limit of quantification = 400 copies/mL) in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serologic tests for HBV were performed at SRL, Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. On-treatment testing for resistance was carried out using a direct-sequencing PCR method.

Safety analyses

Safety analyses include the incidence of adverse events, serious adverse events, laboratory abnormalities, and discontinuations due to adverse events on-treatment throughout treatment in study ETV-060. On-treatment ALT flares were defined as ALT $>2 \times$ baseline and $>10 \times$ ULN.

Resistance analysis

Resistance testing was performed using a direct-sequencing PCR method. Paired samples from all patients with HBV DNA $\geq$400 copies/mL were analyzed for substitutions associated with entecavir or lamivudine resistance at week 96 (72 weeks of entecavir therapy) or week 120 (96 weeks of entecavir therapy). Patients who discontinued therapy prior to week 120 had their last on-treatment sample analyzed. All patients with virologic breakthrough (≥ 1 $\log_{10}$ increase from nadir on two consecutive measurements) were also tested for resistance.

Results

Study population

Of the 34 patients in ETV-047 who received treatment with lamivudine 100 mg once daily for 24 weeks, 33 entered ETV-060 and received treatment with entecavir 0.5 mg once daily. Two patients discontinued treatment during ETV-060: one due to an adverse event (depression) and the other due to insufficient effect. In addition, one patient completed treatment at week 76 (52 weeks of entecavir therapy) after meeting the criteria for protocol-defined complete response (undetectable HBV DNA by PCR assay, undetectable HBeAg and normal serum ALT).

Baseline demographic and disease characteristics for the switch cohort are presented in Table 1. The majority of patients (82%) in the cohort were male with a mean age of 43 years. The mean duration of entecavir therapy was 105.9 weeks (range 25–141 weeks). Baseline mean HBV DNA and ALT levels were 7.9 $\log_{10}$ copies/mL and 184 IU/L, respectively. Ninety-one percent of patients were HBeAg-positive and 88% had HBV genotype C infection.

Virologic end points

After completion of 24 weeks of lamivudine treatment in ETV-047, 21% (7/33) of patients in the switch cohort had achieved HBV DNA <400 copies/mL (Fig. 1). Following the switch to entecavir, the proportion of patients achieving HBV DNA <400 copies/mL increased to 82% (27/33) by week 48 (24 weeks of entecavir therapy). Viral suppression

Table 1 Baseline (pretreatment) demographics and disease characteristics: switch cohort

Characteristic	ETV-047/-60 lamivudine to entecavir switch cohort ($n = 33$)
Age, mean (years)	42.7
Male, *n* (%)	27 (82)
Ethnicity Japanese, *n* (%)	33 (100)
Entecavir treatment periods, mean (range) (weeks)	105.9 (25–141)
HbeAg-positive, *n* (%)	30 (91)
HBV DNA by PCR, mean $\log_{10}$ copies/mL (SD)	7.9 (0.80)
ALT (IU/L), mean (SD)	184.8 (132.9)
HBV genotype, *n* (%)	
A	2 (6)
B	2 (6)
C	29 (88)
Others	0

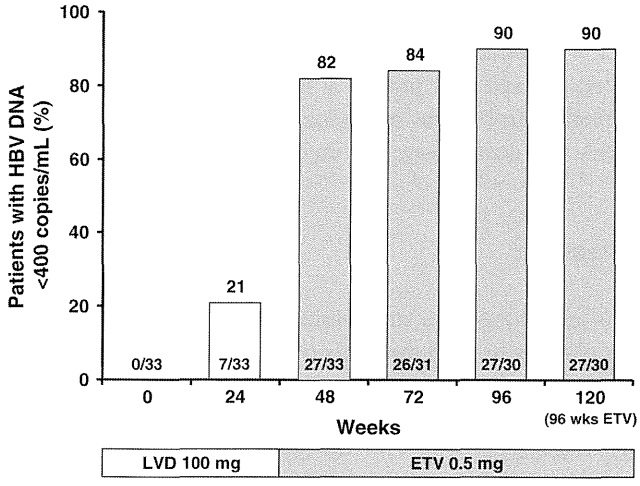

Fig. 1 Proportion of patients with HBV DNA <400 copies/mL through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine

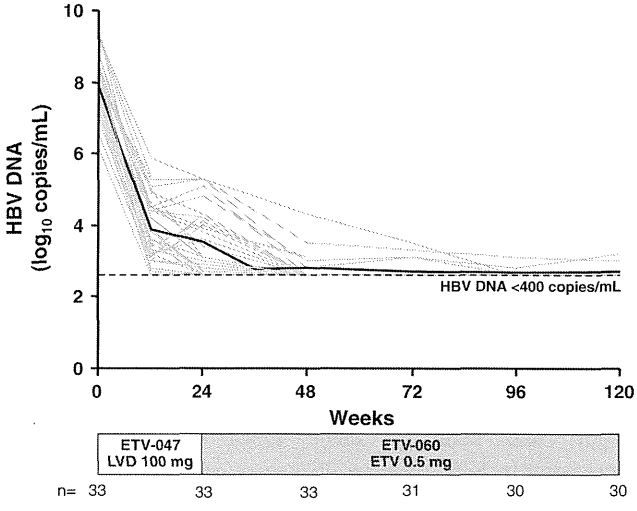

Fig. 2 HBV DNA suppression through week 120 (96 weeks of entecavir therapy). Individual patient HBV DNA profiles are plotted in *gray*. Mean HBV DNA levels are represented by the *solid black line*. *ETV* entecavir, *HBV* hepatitis B virus, *LVD* lamivudine

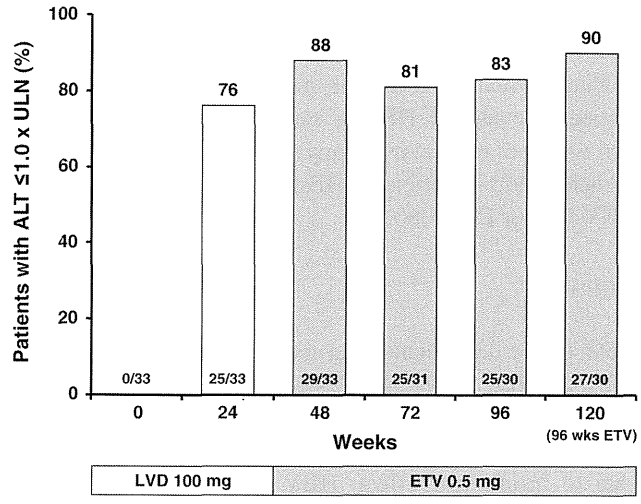

Fig. 3 Proportion of patients with ALT normalization (≤1.0 × ULN) through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples. *ALT* alanine aminotransferase, *ETV* entecavir; *LVD* lamivudine, *ULN* upper limit of normal

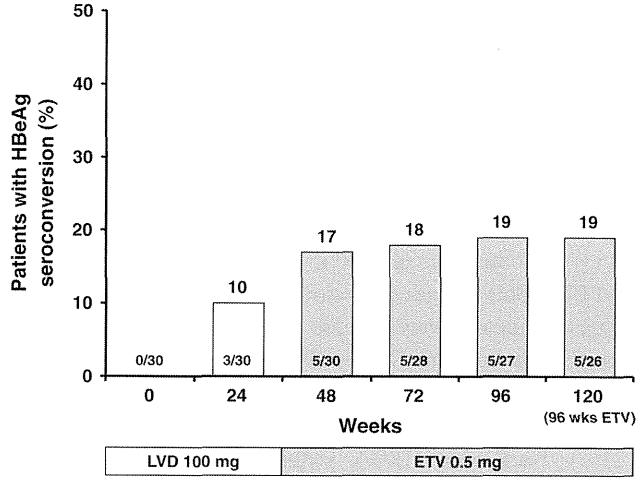

Fig. 4 Proportion of patients with HBeAg seroconversion through 120 weeks of therapy (ETV-047 to ETV-060). *Denominators* represent patients with available samples among the 30 patients HBeAg-positive at baseline. *ETV* entecavir, *HBeAg* hepatitis B e antigen, *LVD* lamivudine

was maintained with longer entecavir treatment, with 84% (26/31) and 90% (27/30) achieving HBV DNA <400 copies/mL at weeks 72 and 120, respectively (48 and 96 weeks of entecavir therapy). Mean HBV DNA levels decreased from a baseline of 7.90 to 3.52 $\log_{10}$ copies/mL after 24 weeks of lamivudine therapy in ETV-047, and reached 2.69 $\log_{10}$ copies/mL after 96 weeks of entecavir therapy in ETV-060 (week 120; Fig. 2). No viral breakthrough was observed during entecavir therapy.

Biochemical end points

ALT normalization (≤1.0 × ULN) was demonstrated in 76% (25/33) of patients after 24 weeks of lamivudine therapy in ETV-047 (Fig. 3). Following treatment with entecavir in ETV-060, ALT normalization was maintained in 90% (27/30) of patients achieving this end point by week 120. Minor fluctuations in the proportion of patients achieving ALT normalization were attributed to patients discontinuing entecavir therapy during the course of study ETV-060.

Serologic end points

HBeAg seroconversion was assessed among the 30 patients in the switch cohort who were HBeAg-positive at baseline in ETV-047 (Table 1; Fig. 4). Three patients (10%)

achieved HBeAg seroconversion during the initial 24-week lamivudine treatment period in ETV-047 (Fig. 4). Following switch to entecavir in ETV-060, two additional patients developed HBeAg seroconversion by week 120 (96 weeks of entecavir therapy). None of the patients in the switch cohort experienced HBsAg loss during treatment in ETV-047 or ETV-060.

Resistance

Four of the 33 patients who received entecavir therapy in ETV-060 had HBV DNA ≥400 copies/mL either at treatment discontinuation or at week 120. One patient discontinued therapy at week 68 (44 weeks of entecavir therapy) due to insufficient effect. HBV DNA prior to treatment discontinuation was 3.1 $\log_{10}$ copies/mL, however, resistance testing revealed no substitutions associated with entecavir resistance. The remaining three patients had HBV DNA ≥400 copies/mL at weeks 96 and 120; however, only two patients had samples available for testing. Neither patient's samples had substitutions associated with entecavir or lamivudine resistance either at weeks 96 or 120.

Safety

Entecavir was well tolerated during long-term treatment and the safety profile of patients in the switch cohort was consistent with that previously reported for patients who received continuous entecavir therapy in studies ETV-047 and ETV-060 (Table 2). Serious adverse events (Meniere's disease, subcutaneous abscess and ALT flare) were reported in three patients (9.1%). The most frequently reported adverse events during treatment in ETV-060, occurring in ≥10% of patients, were nasopharyngitis (76%), diarrhea (21%), back pain (18%), influenza (18%), and allergic rhinitis (15%). One patient discontinued entecavir therapy due to depression, which the investigator considered was possibly related to entecavir therapy. An ALT flare (ALT >2 × baseline and >10 × ULN) occurred in one patient at week 18, and was judged a serious adverse event by the investigator, but was not associated with a change in HBV DNA. No deaths were reported during the study.

Table 2 Summary of safety in ETV-060: switch cohort

On-treatment in ETV-060	Patients, *n* (%)
Any adverse events	33 (100)
Clinical adverse events	33 (100)
Laboratory adverse events	33 (100)
Grade 3/4 clinical adverse event	1 (3)
Grade 3/4 laboratory adverse event	5 (15)
Clinical serious adverse event[a]	3 (9)
Discontinuations due to adverse events	1 (3)
Deaths	0
ALT flares[b]	1 (3)

[a] Including ALT flares

[b] ALT >2 × baseline and >10 × ULN

Discussion

Profound long-term suppression of HBV DNA is required for patients to meet the goals of CHB therapy, which are to prevent cirrhosis, hepatic failure, HCC and liver-related death [14–16]. A major concern with long-term therapy is the increasing risk of selecting resistance mutations, especially for therapies with a low-genetic barrier to resistance, such as lamivudine. The current analysis presents results for a cohort of Japanese patients who were switched directly from lamivudine to long-term entecavir therapy. The results show that this switch cohort achieved additional HBV DNA suppression after the switch to entecavir. The proportion of patients with HBV DNA <400 copies/mL increased from 21% after 24 weeks of lamivudine treatment to 82% following an additional 24 weeks of entecavir treatment. Mean HBV DNA decreased from 3.52 $\log_{10}$ copies/mL at week 24 to 2.80 $\log_{10}$ copies/mL at week 48. Rates of HBV DNA suppression were maintained in this cohort, with 90% of patients achieving HBV DNA <400 copies/mL through 96 weeks of entecavir therapy (week 120). These results are comparable to those achieved by the cohort of patients who received entecavir 0.5 mg once daily in the Japanese Phase II studies and the rollover study ETV-060 [17]. At baseline in ETV-060, 56% of this cohort had achieved HBV DNA <400 copies/mL, increasing to 83% through 96 weeks of entecavir therapy. Among patients with abnormal ALT levels at ETV-060 baseline, 88% of patients in the entecavir 0.5 mg cohort achieved normalized ALT levels at week 96 as compared to 90% of patients in the switch cohort. Rates of HBeAg seroconversion at week 96 in ETV-060 were also similar (20 vs. 19%, respectively). These rates of viral suppression also show comparison favorably to those reported for the global nucleoside-naïve cohorts treated for a similar period of time [18, 19]. The potent antiviral activity of entecavir and its high genetic barrier to resistance is expected to minimize the potential for resistance in the switch cohort, allowing long-term therapy for patients. Liver biopsies were not obtained from patients in the switch cohort; however, the histologic benefits of long-term entecavir therapy have been recently reported for a cohort of naïve Japanese patients in the ETV-060 rollover study [20]. Following treatment with entecavir 0.5 mg daily for 3 years, all patients experienced histologic improvement and 57% experienced improvement in fibrosis score. In

addition, the results from a separate global study have confirmed the histologic benefits of long-term entecavir treatment [21].

Previous Japanese (ETV-052/-060) and global (ETV-026) studies have examined the efficacy of entecavir in lamivudine-refractory patients. In these studies, entecavir demonstrated efficacy, with 54% of Japanese patients achieving HBV DNA <400 copies/mL through 3 years' treatment [10, 22]. However, as a result of the lower genetic barrier in these patients, a major drawback of entecavir therapy in this population is the development of resistance. The cumulative probabilities of genotypic entecavir resistance among lamivudine-refractory patients were 33% through 3 years' treatment in Japanese patients and 51% through 5 years' treatment among patients in the global cohort [12, 22]. In the current study, no entecavir- or lamivudine-associated resistance substitutions were detected after 96 weeks of entecavir treatment. However, in contrast to the previous studies where the majority of patients had high baseline HBV DNA and documented lamivudine resistance [10, 23], patients in the switch cohort received entecavir after achieving variable degrees of HBV DNA suppression with 24 weeks of lamivudine therapy. Therefore, the fact that no resistance has been observed in this cohort to date is not unexpected. This observation is consistent with an analysis of lamivudine-refractory patients enrolled in the worldwide lamivudine-refractory study ETV-026. Patients with baseline HBV DNA <7 $\log_{10}$ copies/mL had a higher probability of achieving HBV DNA <300 copies/mL as compared to those who had baseline HBV DNA ≥ 7 $\log_{10}$ copies/mL (73 vs. 16%) [24]. Furthermore, among the 42 entecavir-treated patients in ETV-026 who achieved HBV DNA <300 copies/mL through 96 weeks of therapy, only one patient subsequently developed entecavir resistance.

Current recommendations on the treatment of patients with documented lamivudine resistance suggest that patients should receive a second drug without cross resistance. The combination of lamivudine and adefovir has been shown to be superior to adefovir monotherapy for the treatment of lamivudine resistance, especially in preventing the selection of adefovir resistance [25–27]. Although only short-term clinical data are available for tenofovir, rates of viral suppression among lamivudine-experienced or -resistant patients who received tenofovir monotherapy do not differ significantly from those of treatment-naïve patients [28, 29]. Small studies have also shown pegylated interferon alpha-2a to be a safe and beneficial treatment option for lamivudine-experienced patients [30]. However, the treatment options for Japanese lamivudine-resistant patients are more limited, since neither tenofovir nor pegylated interferon alpha-2a are currently approved in Japan.

The Japanese guidelines recommend that patients with detectable YMDD mutations should receive treatment with a combination of lamivudine and adefovir [13]. However, the guidelines also allow patients who have received <3 years of lamivudine therapy, have HBV DNA <400 copies/mL, and no breakthrough hepatitis or YMDD mutations to switch directly to entecavir. The results presented in this analysis suggest that the strategy of switching to entecavir is an effective one that may avoid the additional cost and potential toxicity of combination treatment with lamivudine and adefovir. Among patients in the switch cohort, 96 weeks of entecavir treatment was well tolerated and the safety profile was comparable with previous experience in Japanese patients. One patient experienced an ALT flare (ALT $>2 \times$ baseline and $>10 \times$ ULN) 18 weeks after initiating entecavir, which was not associated with a change in HBV DNA. This low rate of ALT flares is consistent with previous findings and demonstrates that lamivudine-treated patients can be switched safely to entecavir with a minimal risk of such flares [10, 22].

In summary, the data from the switch cohort presented in this analysis demonstrate that CHB patients can be switched from lamivudine to long-term entecavir. The treatment with entecavir resulted in increased rates of virologic suppression with no evidence of resistance through 2 years of therapy. These findings support recommendations in the current Japanese treatment guidelines that patients on stable lamivudine therapy with no YMDD mutations should be switched to entecavir.

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Semi-quantitative discrimination of HBV mutants using allele-specific oligonucleotide hybridization with Handy Bio-Strand

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The analysis of hepatitis B virus (HBV) mutations is important for understanding HBV progression and for deciding on appropriate clinical treatments. However, it is difficult to determine the quantitative abundance of various mutants in heterogeneous mixtures by conventional methods such as direct sequencing or the TaqMan assay. In this study, we investigated the possibility of using both allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) with the Handy Bio-Strand system for the quantitative identification of three well-defined HBV variants: the basal core promoter (BCP) mutations (nt1762 and nt1764), the pre-core (PC) mutation (nt1896), and variance at nt1858. Using standardized mixtures of wild-type and mutant DNA, optimal hybridization conditions for ASOH and ASOCH were determined. Next, the performance of these methods was evaluated using actual serum DNAs from HBV patients. Excellent reproducibility was obtained both in the analysis of internal positive controls and in the semi-quantitative categorization of heterogeneous viral mixtures into five abundance groups (0%, 25%, 50%, 75%, and 100% mutant virus). Combined with real-time PCR to determine the HBV viral load, this hybridization method offers a new tool with applications both in HBV clinical research and treatment.

[**Key words:** ASOH (allele-specific oligonucleotide hybridization); ASOCH (allele-specific oligonucleotide competitive hybridization); Basal core promoter; HBV (hepatitis virus type B); Pre-core; Handy Bio-Strand]

Hepatitis B virus (HBV) causes transient and chronic infection of the liver, and is one of the most widespread infectious diseases in the world. Due to frequent mutations, HBVs in patients are not uniform, and this diverse mutant population can affect the progression of HBV. Thus, the analysis of HBV mutations is important for understanding HBV progression and determining the appropriate clinical treatment (1–4). Understanding HBV mutations, HBV loads and other biomarkers will provide new approaches for predicting HBV progression.

A variety of HBV mutations that affect HBV virus activity and disease progression have been reported (5). Among these, the basal core promoter (BCP) mutation (6, 7), the pre-core mutation (PC) (8–11), and nt1858 variance (12) are the most well-defined. The PC mutation (G to A, nt1896) introduces a stop codon (TAG) into the ORF and aborts the translation of the precursor of HBeAg (13), resulting in seroconversion from HBeAg (+) to Anti-HBe Ab (+). Stem-loop RNA structures around nt1896 often pair with nt1858. Six HBV genotypes (A–F) have been defined for nt1858. Genotypes A and F have the base C at nt1858, which forms a stable bond with the base G at position 1896 in wild-type PC, and maintains the wild-type phenotype. The other genotypes (B, C, D and E) have T at position 1858. A double mutation in the BCP (nt1762 and nt1764) frequently occurs in chronic HBV patients. These BCP mutations increase viral replication and enhance disease activity.

A number of methods have been proposed to detect HBV mutations, including direct sequencing, RFLP (14), point mutation assays (15), INNO-LiPA Line Probe Assays (16), reverse dot blots (17), genotype-specific probe assays (GSPA) (12), DNA arrays (18), mass spectrometric assays (19), Molecular-beacon (20), and real-time PCR (21). Except for Molecular-beacon and real-time PCR, all of these are qualitative methods and do not provide quantitative assessments of heterogeneous mutant mixtures.

Previously, we developed a three-dimensional DNA array (Bio-Strand), as well as a total three-dimensional DNA array system (Handy Bio-Strand). This system consists of a DNA array (the Bio-Strand Tip), a spotter, a scanner, and analysis software (Hy-soft) (22). The per-

Abbreviations: ASOH, allele-specific oligonucleotide hybridization; ASOCH, allele-specific oligonucleotide competitive hybridization; HBV, Hepatitis B virus; BCP, basal core promoter; PC, pre-core; GSPA, genotype-specific probe assay; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; FI, fluorescence intensity.

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formance of Bio-Strand was first demonstrated with SNP genotyping (22–25). In this study, we developed a Handy Bio-Strand method that uses allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) to quantitatively discriminate HBV mutants. Our preliminary assessment of this method's ability to quantify specific HBV mutations in heterogeneous viral mixtures is described below.

MATERIALS AND METHODS

Materials and DNA extraction All PCR primers, Cy5 probes, and competitors (Table 1) were synthesized by SIGMA Genosys (Ishikari, Japan). Oligo5 software (Molecular Biology Insights, Cascade, USA) was used to design PCR primers and to estimate the free energy of internal stability of each probe. Positive control DNAs (D, E, F) and sample DNAs for blind test (C1–C10 and G1–G10) were purified from whole blood sera obtained from HBV patients using the SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Total nucleic acids were precipitated with 99% ethanol, dried, and resuspended in 50 μl sterile water. Using AMPLICOR HBV monitor (F. Hoffmann-La Roche, Basel, Switzerland), HBV loads of G3, G4, G5, C4, C5, and C6 were estimated to be $10^{3.0}$, $10^{3.0}$, $10^{7.0}$, $10^{4.9}$, $10^{8.5}$, and $10^{7.3}$ copies/ml, respectively. The Ethics Committee of the associated institution approved this study, and written informed consent was obtained from each patient.

Amplification of the target fragment The target DNA fragment (304 bps) containing the BCP and PC mutation sites and nt1858 was amplified by nested PCR. Amplification reaction mixtures (50 μl) containing 3 μl of total nucleic acid solution, 1× TAKARA Ex Taq buffer, 10 nmol dNTPs, 10 pmol HBV 1601-S, 10 pmol HBV 1974-AS, and 2.5 U TAKARA Ex Taq (TAKARA BIO, Shiga, Japan) were prepared, heated to 94 °C for 3 min, subjected to 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and then kept at 4 °C in a Thermal Cycler Dice (TAKARA BIO). An aliquot of the first PCR solution (1 μl) was further amplified using the primers HBV 1653-S and HBV 1959-AS under the same conditions. The PCR products were resolved on a 3% agarose gel and purified using a PCR clean-up kit and the Magtration System 12GC (Precision System Science, Chiba, Japan). The concentrations of the PCR products were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then diluted to 100 ng/μl with sterile water.

Preparation of standards The standard DNA fragments (Table 2) were amplified from total DNAs prepared from patient sera by nested PCR, as described above. The amplified DNA fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, USA) and their DNA sequences were determined using PCR primers by an ABI3730XL sequencer (Invitrogen). After sequence confirmation, three PCR-amplified HBV fragments were selected and denoted as D, E, and F. The PCR fragments were diluted to 100 ng/μl with sterile pure water and used to prepare standardized mixtures: D only, D/E = 75%/25%, D/E = 50%/50%, D/E = 25%/75%, E only, and F only, which were denoted P1 to P6, respectively (Table 3). These mixtures were spotted and fixed on the Bio-Strand and were used to determine optimal hybridization conditions.

Immobilization of DNA on Bio-Strand A three-dimensional DNA array (Bio-Strand Tip, Precision System Science), was prepared as described previously (22). A 5-μl aliquot of purified DNA solution (100 ng/μl) was mixed with an equal volume of 2 M NaOH and the mixture was spotted onto a thread using a spotting tool. After drying for a few minutes, the Bio-Strand was prepared by wrapping the thread around a cylindrical core (core pin). After fixing the denatured DNA onto the Bio-Strand by ultraviolet irradiation (wavelength 280 nm, 120 mJ), the Bio-Strand was inserted into a transparent plastic tip (Bio-Strand Tip).

ASOH and ASOCH Oligonucleotide hybridization and washing of the Bio-Strand Tip for ASOH and ASOCH were carried out using the Magtration System 12GC as described previously (22). The Bio-Strand Tip was immersed twice in 450 μl of the hybridization buffer [2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) with 200 μg/ml salmon sperm DNA (Invitrogen)], left for 10 min, and then incubated for 5 min in 450 μl of the hybridization buffer containing 10 nM of the Cy5 probes listed in Table 1. For ASOCH, non-labeled opposing oligonucleotides (10–100 nM) were added as competitors. After hybridization, the tip was subjected to successive washings in 450 μl of wash buffer (2× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, and 0.1× SSC with 0.1% SDS) for 2 min, and then soaked in 450 μl of 2× SSC. The Cy5 fluorescent hybridization signals were detected using a Handy Bio-Strand scanner (Precision System Science). To determine signal intensity, the average value was calculated from eight different spots. The fluorescence intensities (FIs) were calculated using Hy-soft software (Precision system science).

Blind test for BCP, PC, and nt1858 variance using patient DNA Blind tests for BCP, PC, and nt1858 variance were carried out using DNAs obtained from patients (C1 to C10 and G1 to G10). All samples and positive controls (P1–P6) were spotted and fixed on eight different areas of the same Bio-Strand. Each Bio-strand Tip was hybridized using either the ASOH or ASOCH method. After washing, the Handy Bio-Strand scanner was used to detect the Cy5 fluorescence signals on the Bio-Strand Tips. The signal intensity was calculated as the average value from the eight different spots. To determine the ratios of wild-type to mutant in the samples, the fluorescence intensities from the wild-type and mutant probes were compared with those of the standard mixture.

After the blind test, SNP types of patient DNA sequences were also determined by direct sequencing. Target DNA fragments were amplified using the primers HBV 1601-S and HBV 1974-AS PCR. After treatment with ExoSAP-IT (GE HealthCare Bioscience, Buckinghamshire, UK), they were applied for sequencing at Macrogen (Rockville, USA) with the primers HBV 1653-S or HBV 1959-AS PCR (22). The percent abundance or ratio at target site was determined by visual inspection of the electropherogram by two trained investigators with no knowledge of the HBV classifications of the samples.

TABLE 1. Sequences of PCR primers, Cy5 probes, and non-labeled competitors.

Name	Target	Use	DNA sequence (5′→3′)
HBV 1601-S	External	1st PCR	acgtcgcatggagaccaccg
HBV 1974-AS			ggaaagaagtcagaaggcaaa
HBV 1653-S	Internal	2nd PCR	cataagaagactcttggact
HBV 1959-AS			ggcaaaaaagagagtaactc
Cy5-CPR1	BCP	Cy5 Probes	Cy5-ggttaa**a**ggtctttg
Cy5-CPR2			Cy5-ggtta**atg**atctttg
Cy5-PC2	PC		Cy5-gctttg**g**ggca
Cy5-PC3			Cy5-ggctttag**g**gca
Cy5-GA3	nt1858		Cy5-atgtcc**c**actgtt
Cy5-GA5			Cy5-tgtcc**t**actgt
CP-GA2		Competitors	catgtcc**t**actgtt
CP-GA3			atgtcc**c**actgtt

The target sites of the HBV genomic sequence were denoted as bold characters.

TABLE 2. DNA Sequences at each mutation site in the standard PCR DNA fragments (D, E and F).

Standard PCR DNA Fragment	BCP		PC		nt1858
	nt1762/nt1764	Type	nt1896	Type	nt1858
D	A/G	wild	G	wild	C
E	T/A	mutant	A	mutant	T
F	A/G	wild	G	wild	T

RESULTS

Optimization of ASOH and ASOCH conditions for determining the relative abundance of the mutants BCP, PC and T-1858 Fig. 1 shows a schematic of the proposed method for determining the relative abundance of the targeted HBV species using the Handy Bio-Strand system. Viral DNA was isolated from patient sera by conventional methods, and the targeted DNA fragments were amplified by nested PCR. After purifying the DNA and determining its concentration, amplicons were fixed on the surface of microporous nylon thread (Bio-Strand) by ultraviolet irradiation. The ASOH and ASOCH reactions were carried out at room temperature with Cy5-labeled oligonucleotide probes that were designed to detect the clinically important HBV mutations at BCP and PC and the base at nt1858. Fluorescence signals were detected using a Handy Bio-Strand scanner, and the abundance of each mutant was determined.

TABLE 3. Percent abundance of each species in the standard mixture.

STD	Mixing (%)			Percent abundance (%)					
				BCP		PC		nt1858	
	D	E	F	Wild	Mutant	Wild	Mutant	C	T
P1	100	0	0	100	0	100	0	100	0
P2	75	25	0	75	25	75	25	75	25
P3	50	50	0	50	50	50	50	50	50
P4	25	75	0	25	75	25	75	25	75
P5	0	100	0	0	100	0	100	0	100
P6	0	0	100	100	0	100	0	0	100

STD shows the name of the standard mixture which was prepared by mixing three standard PCR DNA fragment (D, E and F).

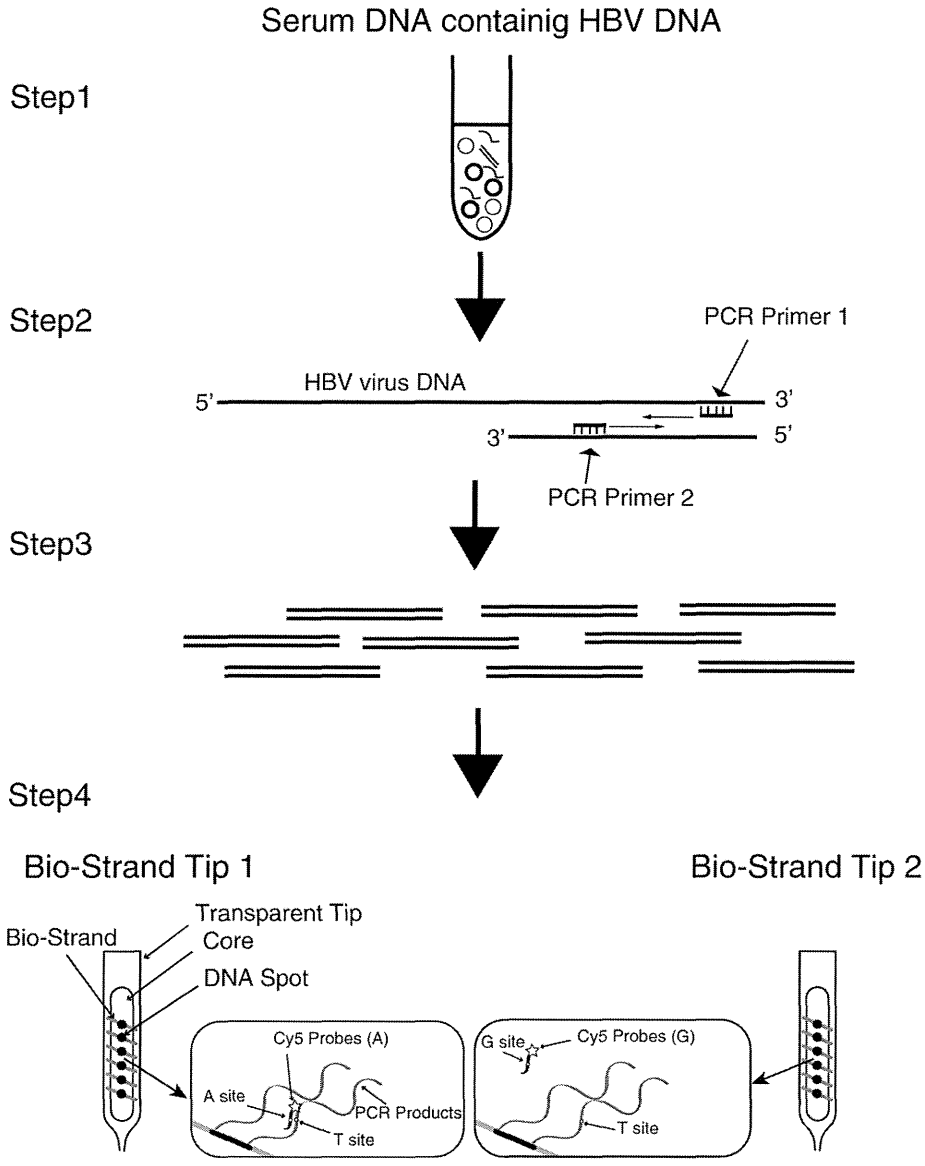


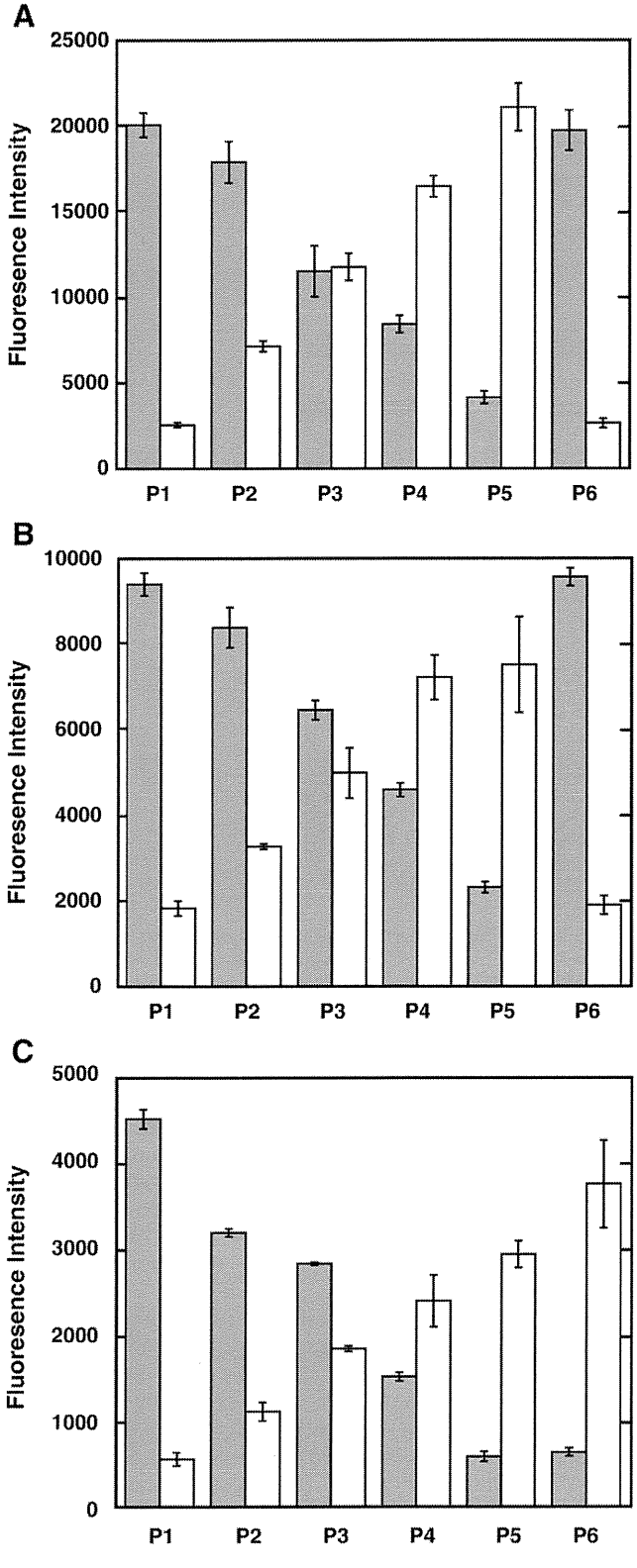
FIG. 1. Schematics of the ASOH and ASOCH assays using the Handy Bio-Strand system. Step1: Virus DNAs and RNAs are prepared from patient serum. Step2: HBV fragments (304 bps) are amplified using nested PCR. Step 3: The HBV fragments are purified, denatured, spotted and then fixed on Bio-Strand. Step 4: Two automatic hybridizations are separately carried out using two Bio-Strand Tips for a target site. Each Bio-Strand Tip contains different Cy5 probes. The stars and small circles show the Cy5 molecules and the target sites, respectively. The perfect-matching Cy5 probes bind to the SNP sites, but mismatching ones don't.

Fig. 2A shows the results of ASOH for the quantitative discrimination of the BCP mutation using the Cy5-CPR1 (wild-type) and Cy5-CPR2 (mutant) probes. The fluorescence intensities (FIs) of P1, P2, P3, P4, and P5 accurately reflected the abundance of the BCP mutants as 0%, 25%, 50%, 75%, and 100%, respectively. P1 gave a result similar to that of P6, demonstrating the high reproducibility of this assay. Unlike the previous results for SNP genotyping (22), accurate results were obtained without using competitors (non-labeled probes containing opposing sites). Double mutations (nt1762 and nt1764) in the center of the Cy5 probes are likely to be the reason for the high specificity and also the low background signals.

Fig. 2B shows the results obtained using ASOH with the Cy5-PC2 (wild-type) and Cy5-PC3 (mutant) probes to detect the PC mutation. Each FI increased in proportion to the increase in the amount of the PC mutant. The FIs of the standard solutions (P1, P2, P3, P4, and P5) allowed samples to be categorized into five groups according to the mutant content. In contrast to the results for the BCP mutant, the Cy5-PC2 and Cy5-PC3 probes did not give equal FIs for P3, which contained equal amounts of wild-type and mutant DNA. The FI for the wild-type DNA was about 27% higher than for the mutant DNA. This is probably due to non-specific binding of the Cy5-PC2 probe (wild-type, base G) to the mutant amplicon. To improve the specificity of Cy5-PC2, we added competitive unlabeled probe (CP-PC3) to the hybridization solution. Despite our expectations, this approach (ASOCH) did not improve the specificity of the Cy5 probes, and the total signal intensity was lower than that of ASOH (data not shown).

Concerning nt1858, it was difficult to distinguish between fragments E or F (T-1858) and fragment D (C-1858) by ASOH, due to the non-specific binding of the Cy5 probes (data not shown). We applied ASOCH using two different sets of probes, Cy5-GA3/CP-GA2 and Cy5-GA5/CP-GA3. Fig. 2C shows the ASOCH results for nt1858 with Cy5 probes and their unlabeled competitors (base C: 10 nM Cy5-GA3 and 100 nM CP-GA2; base T: 10 nM Cy5-GA5 and 100 nM CP-GA3). The background was moderately high, but the standard mixtures (P1–P6) were clearly distinguishable.

Our previous study estimated that the Handy Bio-Strand system hybridization signal error, the coefficient of variation (CV), was 4.6–11.3% (22). To obtain reliable classifications, standard mixtures were prepared within 2–3 fold of the previously determined CV, at 25% intervals and used as internal controls (Table 3). We also confirmed that standard mixtures prepared at 20% intervals worked well as for internal control (data not shown).



Determination of the relative abundance of BCP and PC mutants and nt1858T in patient serum DNA in a blind test To determine the accuracy for quantification of mutations by ASOH and ASOCH, blind tests were carried out using DNAs from patient sera.

Fig. 3A shows the results of using ASOH to detect BCP sequences in ten patients (C1–C10). The patient samples were classified into three groups. The first group (C1, C2, C3, C8, and C9) showed the same pattern as P1 (0% mutant BCP). The second group (C4, C6, C7, and C10) showed the same pattern as P5 (100% mutant BCP). The last group, consisting only of patient C5, was similar to P3 (50% mutant BCP). All of the results, except for that of C5, were consistent with those obtained by direct sequencing. Direct sequencing of C5 exhibited that it contained 25% mutant BCP, which is lower that that observed by our method (Table 4).

Fig. 3B shows the results of classification of the same ten patient serum DNA samples on PC mutant using ASOH. The ten samples were classified into three groups. The first group (C1, C2, C4, C5, and C8) resembled P1 (0% mutant PC). The second group (C3, C6, C9, and C10) showed a pattern similar to P5 (100% mutant PC). The FI pattern of C7 was similar to that of P2 (25% mutant PC). All the results were consistent with the results from direct sequencing.

Fig. 3C shows the percent abundance of T-1858 in the ten patient samples (G1–G10) determined by the ASOCH method. The patient samples were classified into four groups. The first group (G4, G5, G6, G7, G9, G10) exhibited a similar pattern to P5 and P6 (100% T-1858). This group belonged to HBV genotypes B, C, D, and E. The second group included G2 and G8, and had FI patterns similar to that of P1 (0% T-1858). The second group belonged to HBV genotypes A and F. The third group (only G1) showed a similar FI pattern to P2 (25% T-1858). The last group (only G3) showed an FI pattern similar to P4 (75% T-1858). All the results, except for those of G1 and G3, were consistent with direct sequencing. Minor T base peak was not recognized at the direct sequencing for G1. G3 showed the different ratio between our method and direct sequencing. These inconsistencies should be due to the difficulty in recognizing a minor peak derived from nt1858 at our direct sequences.

Estimation of the internal stability of the probes Under optimized hybridization conditions, competitive hybridization (ASOCH) was not effective as a PC mutation (nt1896) assay. To determine the reason for this unexpected result, we estimated the free energy of internal stability (ΔG) of each probe by the neighbor method using the Oligo5 program (Molecular Biology Insights, Cascade, USA), because a difference in internal stability at this site might have a significant impact on the ASOCH reaction. Figs. 4A–C show the calculated ΔG of the probes for BCP, PC, and nt1858, respectively. The ΔG of the PC mutation probe was determined to be very low (-12 kcal/mol), whereas the ΔGs of the BCP probe and the nt1858 probe had moderate values (-6 kcal/mol). The internal stability of the PC mutation probes was approximately twice as high as that of the BCP or nt1858 probes. Thus, it appears that the high internal stability of the PC mutation probe may interfere with the exchange between the Cy5 probes and their non-labeled competitors, providing a possible explanation for the inability of ASOCH to increase probe specificity.

FIG. 2. Optimization of the ASOH and ASOCH assays using positive controls containing each mutation site. (A) Each BCP mutant sample was hybridized using ASOH with 10 nM Cy5-CPR1 (wild-type, A/G) or 10 nM Cy5-CPR2 (mutant, T/A). Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) Each PC mutation was hybridized using ASOH with 10 nM Cy5 PC2 (wild-type, G) or 10 nM Cy5-PC3 (mutant, A). Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 was hybridized using ASOCH with 10 nM Cy5-GA3 (C) and 100 nM CP-GA2 (T) or 10 nM Cy5-GA5 (T) and 100 nM CP-GA3 (C). Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (mixtures of amplicons D, E, or F) were fixed onto the Bio-Strand. All data shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

DISCUSSION

Using the Handy Bio-Strand system, we have demonstrated that ASOH and ASOCH can semi-quantitatively determine population differences of HBV mutants. Our method is very reliable and applicable for determining the relative abundance of mutant species in sera from actual HBV patients. As shown in Table 4, while there were no differences between two methods used in the classification of major species, but contradictory results were observed for C5 (BCP mutation), G1 (nt1858), and G3 (nt1858). This inconsistency is likely caused by inaccurate quantification of minor peaks by direct sequence. It was speculated that our method was able to estimate the actual percent abundance of mixture species roughly (~25%). That is because the internal controls and the same patient samples always show similar hybridization patterns at the repeated experiments, and it was demonstrated that our method shows more reliable ratio than direct sequencing at the previous SNP genotyping study for heterotype samples (22) (Data not shown).

Compared with other methods, the Handy Bio-Strand method has some advantages. It may have higher compatibility than other methods with regards to the quantification of various mutations, because the design of the Cy5 probes and their competitors are much simpler than the design of TaqMan and Molecular-beacon probes. Conventional methods such as PCR-RFLP (14, 15) and direct sequencing are very simple, but they pose difficulties as quantitative assays. Direct sequencing can only detect major HBV species and is unlikely to identify minor mutants. Waltz et al. also reported that direct sequencing could not detect minor populations (<20%) of HBV

TABLE 4. Abundance determined by Handy Bio-Strand or direct sequencing.

Target site	Sample	Handy Bio-Strand	Direct sequencing
BCP	C1	W	W
	C2	W	W
	C3	W	W
	C4	M	M
	C5	W = M (1:1)	W > M (3:1)
	C6	M	M
	C7	M	M
	C8	W	W
	C9	W	W
	C10	M	M
PC	C1	W	W
	C2	W	W
	C3	M	M
	C4	W	W
	C5	W	W
	C6	M	M
	C7	W > M (3:1)	W > M (3:1)
	C8	W	W
	C9	M	M
	C10	M	M
nt1858	G1	C > T (3:1)	C
	G2	C	C
	G3	C < T (1:3)	C = T (1:1)
	G4	T	T
	G5	T	T
	G6	T	T
	G7	T	T
	G8	C	C
	G9	T	T
	G10	T	T

W and M represent wild type and mutant type, respectively. Concerning to species mixtures, the ratios of wild type to mutant type (or C to T at nt1858) are shown under the abundance results.

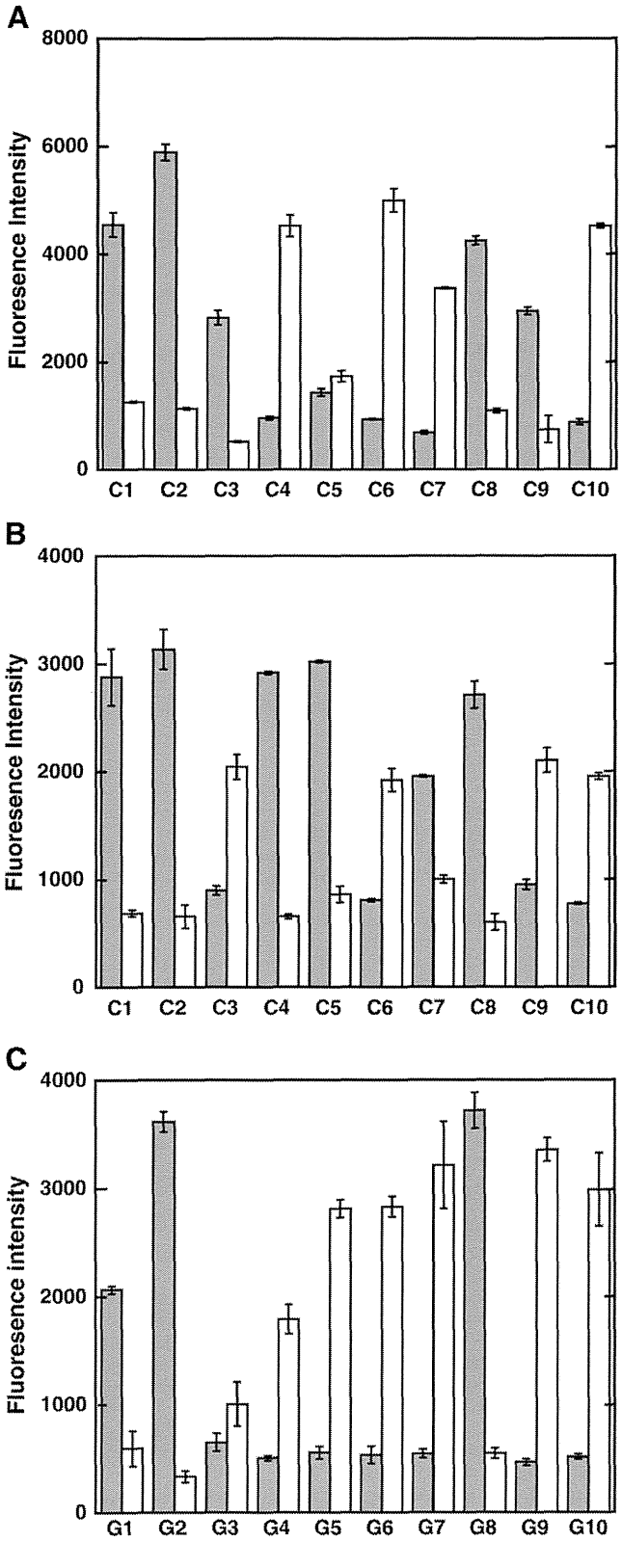


FIG. 3. Blind quantification of the BCP and PC mutations and nt1858T in DNA from patient serum. (A) The BCP mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) The PC mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 site was analyzed by ASOCH. Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (P1–P6) and patient samples (C1–C10 or G1–G10) were separately fixed on each Bio-Strand. Conditions for both hybridization and internal positive controls were the same as those in Fig. 2. All data are shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

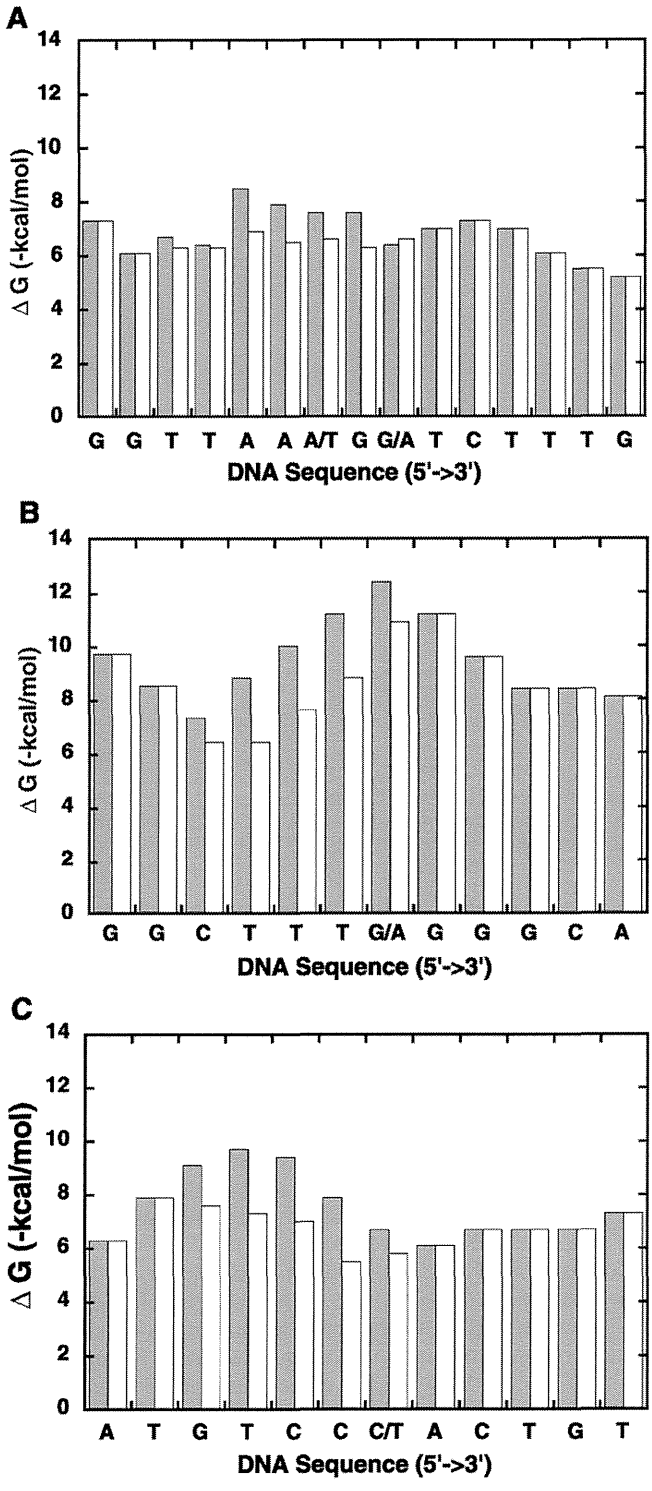


FIG. 4. Free energy of each probe around the mutation site. (A) The free energy of each BCP probe. Black bars and white bars show the free energy values of the Cy5-CPR1 probe (wild-type) and the Cy5-CPR2 probe (mutant), respectively. (B) The free energy of each PC probe. Black bars and white bars show the free energy values for the Cy5-PC2 probe (wild-type) and the Cy5-PC3 probe (mutant), respectively. (C) The free energy of each nt1858 probe. Black bars and white bars show the free energy values for the Cy5-GA5 probe (T-1858) and the Cy5-GA3 probe (C-1858), respectively.

mutants in a heterogeneous mixture (20). Real-time PCR, like that used in the TaqMan assay, is a very sensitive and reliable method for quantifying genes, but there may be some difficulties with sequences containing only a single base mutation. Shin et al. reported that annealing curve analysis was necessary for detecting YMDD mutants after real-time PCR (26). Our method is robust enough to detect a variety of mutations, and little time is needed to adjust the experimental conditions because of the simple probe designs. We have also determined the percent abundance of other HBV mutation sites (including L528M and YMDD (5)) using ASOCH (manuscript in preparation).

The Handy Bio-Strand system can simultaneously analyze 17–34 patient DNA samples using Cy5-labeled oligonucleotide probes. As shown in our previous report (22), Bio-Strands can be reused 2–3 times by washing out the bound Cy5 probes with hot water. Since the three targeted sites (BCP, PC and nt1858) used in this study are located on the same DNA fragments (304 bp) (Supplementary data), repeated automatic hybridization can semi-quantitatively determine the percent abundance of all three species, thereby reducing the time needed to prepare the Bio-Strand Tip.

Amplification bias during nested PCR either does not occur or does not pose a significant problem as repeated experiments were carried out changing the template amount and PCR cycles without effect on the hybridization patterns.

Since real-time PCR is the best method for estimating HBV load in copies/ml, we propose the following method. First, the HBV load in a patient's serum should be precisely determined by real-time PCR. Second, the percent abundance of each HBV mutant site should be determined using ASOH or ASOCH with the Handy Bio-Strand system. Semi-quantitative mutant populations may then be calculated from these two parameters. We believe that these data provide an important new approach to the diagnosis of HBV and the design of HBV-specific treatments in future clinical studies.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiosc.2009.06.023.

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