Here, although the pre-C mutant became predominant in the HBeAg seroconversion group during follow-up, the proportion of the BCP mutation did not change. Hepatitis B virus (HBV) viral markers were significantly higher in patients without the mutations in an HBeAg positive status. HBV DNA and hepatitis B surface antigen levels were higher in patients with the pre-C mutation in an anti-HBe positive status. Taken together, the association of the pre-C mutation on viral load appears to be opposite before and after HBeAg seroconversion in patients with HBV infection.

Kamijo N, Matsumoto A, Umemura T, Shibata S, Ichikawa Y, Kimura T, Komatsu M, Tanaka E. Mutations of pre-core and basal core promoter before and after hepatitis B e antigen seroconversion. *World J Gastroenterol* 2015; 21(2): 541-548 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i2/541.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i2.541

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma[1-4].

In the natural history of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and the remission of hepatitis[5-7]. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion[8,9].

Several mutations in the HBV genome have been reported to associate with HBeAg seroconversion. When the pre-core (pre-C) and core genes in the HBV genome are transcribed and translated in tandem, HBeAg is produced and secreted into the circulation[10,11]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of detectable HBeAg[12,13]. The double mutations of A1762T and G1764A in the basal core promoter (BCP) of the HBV genome have also been shown to reduce HBeAg synthesis by suppressing the transcription of pre-C mRNA[14-16]. However, the detailed mechanisms of HBeAg seroconversion, including the involvement of mutations that decrease the production of HBeAg, have not been fully clarified. Orito *et al*[17] reported that a predominance of the pre-C mutation was correlated with anti-HBe, while BCP mutations were not associated with either anti-HBe or HBeAg. We previously uncovered that the pre-C and BCP mutations were frequently seen in patients with active replication after HBeAg seroconversion, but not in those with inactive replication[18], which suggested that HBeAg seroconversion was not associated with either mutation in such patients. Since the follow-up duration of these previous reports was limited, this study analyzed the changes in pre-C and BCP mutations among patients who were followed over a longer time course. Furthermore, we assessed the mutations not only in patients who seroconverted from HBeAg to anti-HBe, but also in those whose HBeAg or anti-HBe positive status did not change during follow-up.

MATERIALS AND METHODS

Patients

Three groups of patients with chronic hepatitis B who were categorized according to HBeAg/anti-HBe positive status were enrolled between 1985 and 2000. The subjects were selected retrospectively from a database of patients who had been followed for at least two years, had not received anti-viral therapy, such as nucleos(t)ide analogues, and whose stored serum samples were available from both the start and end of follow-up. We recruited only patients with HBV genotype C since this genotype is predominant in Japan and because the clinical significance of pre-C and BCP mutations differs among genotypes. The first group consisted of 18 patients whose HBeAg was persistently positive throughout the study period. The second group contained 25 patients in whom HBeAg seroconverted to anti-HBe. The third group was made up of 43 patients whose anti-HBe was persistently positive.

Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions a minimum of 6 mo apart in all patients before the start of follow-up. Tests for hepatitis C and human immunodeficiency virus antibodies were negative in all subjects. Patients who demonstrated accompanying hepatocellular carcinoma or signs of hepatic failure at the initial follow-up were excluded from the study.

Stored serum samples were kept frozen at -20 ℃ or below until assayed. This study was approved by the Ethics Committee of Shinshu University School of Medicine.

Conventional hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and anti-HBe, were tested using commercially available enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan)[19]. HBsAg was quantified[20] using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 8.9 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal and no signal detection was considered to be a negative signal. Six HBV genotypes (A-F) were

Table 1 Clinical and virological backgrounds among 3 groups of patients classified according to changes in hepatitis B e antigen/anti-hepatitis B e

Characteristic	HBeAg/anti-HBe status			*P* value
	Continuously +/- (*n* = 18)	From +/- to -/+ (*n* = 25)	Continuously -/+ (*n* = 43)	
Age (yr)[1]	44 (24-63)	37 (18-53)	51 (25-77)	< 0.001
Gender (M:F)	11:7	14:11	24:19	> 0.2
Follow-up period (yr)[1]	6.3 (2.1-14.6)	10.8 (2.0-23.7)	8.5 (2.2-16.6)	0.006
Genotype C[2]	18 (100)	25 (100)	43 (100)	1
Viral markers at first follow-up				
HBV DNA (log copies/mL)[1]	8.6 (5.7-> 8.9)	6.1 (< 2.1-> 8.9)	< 2.1 (< 2.1-8.2)	< 0.001
HBsAg (log IU/mL)[1]	4.6 (1.6-5.5)	3.6 (-0.9-4.6)	2.6 (< 0.05-4.3)	< 0.001
HBcrAg (log U/mL)[1]	> 6.8 (5.5->6.8)	6.8 (3.1-> 6.8)	3.0 (< 3.0-6.8)	< 0.001
Viral markers at final follow-up				
HBV DNA (log copies/mL)[1]	7.1 (< 2.1-> 8.9)	3.3 (neg.-6.2)	< 2.1 (neg.-7.0)	< 0.001
HBsAg (log IU/mL)[1]	3.3 (1.0-5.1)	2.8 (< 0.05-2.8)	1.3 (< 0.05-4.2)	< 0.001
HBcrAg (log U/mL)[1]	6.7 (4.4-> 6.8)	< 3.0 (< 3.0-6.2)	< 3.0 (< 3.0-5.3)	< 0.001

[1]Data are expressed as the median (range); [2]Data are expressed as a positive number (%). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis B core-related antigen.

evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al*[22]. Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc.) as described previously[23,24]. The HBcrAg assay simultaneously measured all antigens (e, core, and p22cr) encoded by the pre-C/core genes of HBV. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL with a quantitative range of 3.0 to 6.8 log U/mL.

Determination of pre-C and BCP mutations

The pre-C and BCP mutations were determined using nucleic acid samples extracted from 100 μL of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in wild type HBV and A1896 in the mutant were determined by mini-sequence reactions using labeled nucleotides that were complementary to either the wild type or mutant[25]. The results were expressed as percent mutation rates according to the definition by Aritomi *et al*[26] Samples were judged as positive for the pre-C mutation when the mutation rate exceeded 50% in the present study since the mutation rate was found to steadily increase to 100% once surpassing 50%[25].

The double mutation in the BCP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories)[25,26]. This kit detected T1762 and/or A1764 using the polymerase chain reaction (PCR) with primers specific for either wild type or mutant BCP. Results were recorded as wild, mixed, or mutant type. The pre-C and BCP mutations were tested at the start and end of follow-up with kits having manufacturer-established detection limits of 1000 copies/mL.

Full HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously[27]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 3.1; Foster City, CA) with an automated ABI DNA sequencer (Model 3100, Applied Biosystems Carlsbad, CA).

Statistical analyses

The proportions of clinical factors were compared among groups using the χ^2 and Fisher's exact probability tests. Group medians were compared by means of the Mann-Whitney *U* test and Kruskal-Wallis test. The changes in proportions of the pre-C and BCP mutations between the study start and end points were compared using McNemar's test. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Patients

The clinical and virological backgrounds of the 3 groups are summarized in Table 1. Median age was lowest in patients with seroconversion, intermediate in those with persistent HBeAg, and highest in those with persistent anti-HBe. Gender ratio was similar among the 3 groups. Following our study design, all patients had HBV ge-

notype C.

Changes in pre-C and BCP mutations

The presence of the pre-C mutation could be evaluated in 60 (98%) of 61 HBeAg positive samples and 94 (85%) of 111 HBeAg negative samples. We were able to assess the existence of the BCP mutation in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples.

The changes in the proportion of the pre-C mutation between the start and end of follow-up are shown in Figure 1A. Wild type pre-C accounted for 94% of patients whose HBeAg was continuously positive at study onset and remained constant. Wild type pre-C was also predominant at the start of follow-up (76%, 19/25) in patients who experienced HBeAg seroconversion, but the mutant type had become predominant ($P = 0.022$) by the end of follow-up (65%, 15/23); 11 of 19 wild type pre-C patients converted to mutant type, while 2 of 6 patients with mutant type pre-C reverted to wild type. Mutant type pre-C accounted for 62% of the patients who were continuously positive for anti-HBe at study onset. Such patients with wild type pre-C at the start of follow-up tended to maintain this status (78%), although 22% of initially mutant type pre-C subjects had changed to wild type by the study end point ($P = 0.687$).

Of the 143 samples with determined BCP mutations, 34 (24%) were wild, 11 (8%) were mixed, and 98 (69%) were mutant types. Because few patients with mixed type BCP reverted to wild type in the present and past studies[18], samples were considered to be positive for the BCP mutation when they were either mixed or mutant type.

The changes in the proportion of the BCP mutation between the start and end of follow-up are shown in Figure 1B. Mutant type BCP accounted for 61% of patients whose HBeAg was continuously positive at study onset and remained constant. In patients who experienced HBeAg seroconversion, mutant type BCP was predominant at the start of follow-up (84%, 21/25) and remained so (80%, 16/20) until final follow-up; 3 of 4 patients with wild type BCP and 15 of 16 patients with mutant type BCP maintained their status throughout the study period. Mutant type BCP initially accounted for 82% of patients who were continuously positive for anti-HBe. Both wild (60%) and mutant (84%) types tended to remain constant until the study end point. When all points of measurement were counted for which both pre-C and BCP mutations were evaluated, the prevalence of the pre-C mutation (18%, 9/57) was significantly lower than that of the BCP mutation (82%, 42/57) in patients with persistent HBeAg ($P < 0.001$), as well as in subjects with persistent anti-HBe [62% (53/86) *vs* 78% (67/86), $P = 0.030$], albeit to a lesser degree.

Comparison of viral loads according to pre-C/BCP mutation and HBeAg/anti-HBe positive status

We next compared the serum levels of HBV DNA, HBsAg, and HBcrAg according to pre-C and BCP mutation and HBeAg and anti-HBe positive status (Figure 2). Both pre-C and BCP mutations could be evaluated in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples. HBV DNA levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status ($P < 0.001$) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status ($P < 0.01$). On the other hand, HBV DNA levels were significantly lower in patients without the pre-C mutation than in those with it in an anti-HBe positive status ($P = 0.012$).

A similar tendency to HBV DNA levels was observed for HBsAg levels. HBsAg levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status ($P < 0.001$) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status ($P < 0.001$). HBsAg levels were significantly higher in patients with the pre-C mutation than in those without it irrespectively of the existence of the BCP mutation ($P = 0.041$).

HBcrAg levels were significantly lower with presence of pre-C and/or BCP mutations in an HBeAg positive status ($P < 0.05$, respectively). HBcrAg levels were uniformly low regardless of the presence of mutations in anti-HBe positive status subjects.

Full genome sequences in patients with and without appearance of the pre-C mutation

Full HBV genome sequences were determined after HBeAg seroconversion in 6 patients who seroconverted without the appearance of the pre-C mutation. All patients were positive for BCP mutations: 1 subject had T1753G and C1766T mutations, although the other mutations reported by Okamoto *et al*[14] were not identified.

DISCUSSION

Although both pre-C and BCP mutations have been associated with HBeAg seroconversion by reducing the production of HBeAg[13-15], their manifestation patterns appear to be different[17]. In the present study, the BCP mutation was already prevalent during the HBeAg positive chronic hepatitis phase and approached 80% around the time of HBeAg seroconversion. On the other hand, the pre-C mutation clearly manifested following the time of seroconversion. These results indicate that the appearance of the pre-C mutation, but not the BCP mutation, is directly associated with seroconversion. It is noteworthy that a considerable number of patients experienced HBeAg seroconversion without evidence of the pre-C G1896A mutation. Furthermore, wild type pre-C remained unchanged in almost all patients whose anti-HBe was continuously positive. Thus, two types of HBeAg seroconversion may exist for chronic HBV in terms of the appearance or absence of the G1896A pre-C mutation. We previously speculated on the possible

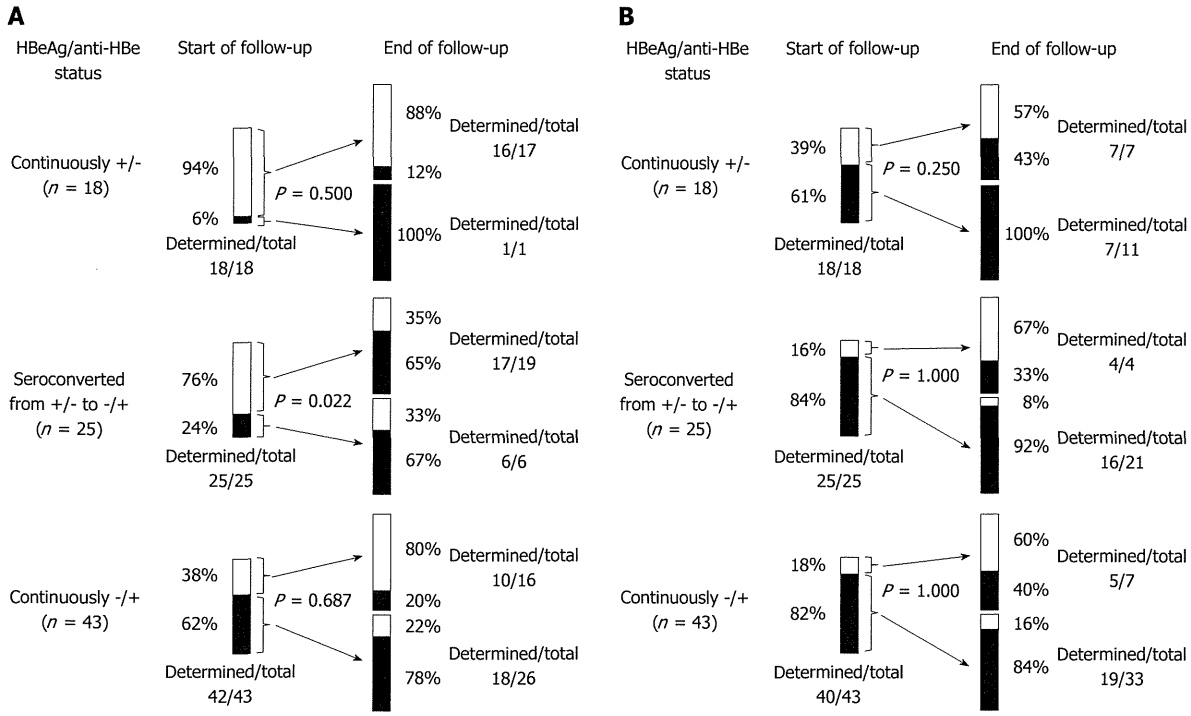

Figure 1 Comparison of changes in pre-core (A) and basal core promoter (B) mutation type among 3 groups of patients classified according to hepatitis B e antigen /anti-hepatitis B e positive status. A: A significant difference was seen in patients with hepatitis B e antigen (HBeAg) seroconversion ($P = 0.022$). One patient whose pre-core (pre-C) mutation was undetermined at the start of follow-up was wild type at the end point; B: Of the 3 patients whose basal core promoter (BCP) mutation was undetermined at the start of follow-up, 2 were wild type and 1 was undetermined at the end point. HBeAg: Hepatitis B e antigen.

existence of two seroconversion types in an analysis of HBV patients who experienced seroconversion[18]. Here, we were able to strengthen this notion by including patients who maintained an HBeAg or anti-HBe positive status in a study of longer duration. It should be noted that the absence of the pre-C G1896A mutation does not necessarily indicate the absence of mutations that halt HBeAg production; several patterns of mutations apart from G1896A have been associated with an HBeAg negative phenotype, such as point mutations in the ATG initiation region and deletion/insertion of nucleotides leading to premature termination[13]. Accordingly, we analyzed full genome sequences in 6 patients who seroconverted without the appearance of the pre-C mutation and uncovered T1753G and C1766T mutations in one subject[14] that might be associated with seroconversion. We observed that several patients reverted from mutant pre-C to wild type in the present report. As this important finding has not been confirmed by sequence analysis, we are planning to determine and compare entire genomic sequences using paired samples before and after HBeAg seroconversion in a future study.

We witnessed that serum HBV DNA was significantly lower in patients with the pre-C and/or BCP mutation in an HBeAg positive phase, which indicated that immune processes from the host to eliminate HBV were stronger in individuals with the mutations than in those without. This also supported the generally held belief that pre-C and BCP mutations appear as a result of host immune pressure[14]. Contrary to the HBeAg positive phase, HBV DNA was significantly higher in subjects with the pre-C mutation in an anti-HBe positive phase. Kawabe *et al*[28] have reported that patients with wild type pre-C demonstrate significantly lower viral loads and ALT levels than those with mutant pre-C among HBeAg negative patients with HBV genotype C infection. Collectively, these results imply that patients with the pre-C mutant have a higher potential to progress to hepa-titis after HBeAg seroconversion. This is consistent with the fact that HBeAg negative hepatitis is usually caused by HBeAg non-producing mutant strains of HBV. Indeed, viral replication seems to be considerably suppressed in patients with wild type HBV after achieving HBeAg seroconversion since this strain has the ability to produce HBeAg when actively replicated.

We adopted serum levels of HBsAg, HBcrAg, and HBV DNA in the present study as markers to estimate HBV replication activity. HBsAg and HBcrAg levels have been reported to reflect HBV cccDNA levels in hepatocytes[20,24,29]. HBsAg has also attracted attention as a useful predictor of treatment outcome by interferon and others[30]. Furthermore, the loss of HBsAg is an important indicator in the treatment of HBV carriers. HBcrAg assays simultaneously measure all antigens encoded by the pre-C/core genome, which include the HB core, e, and p22cr antigens, and have been reported to predict the clinical outcome of patients treated with nucleotide or nucleoside analogues[31]. HBsAg patterns according

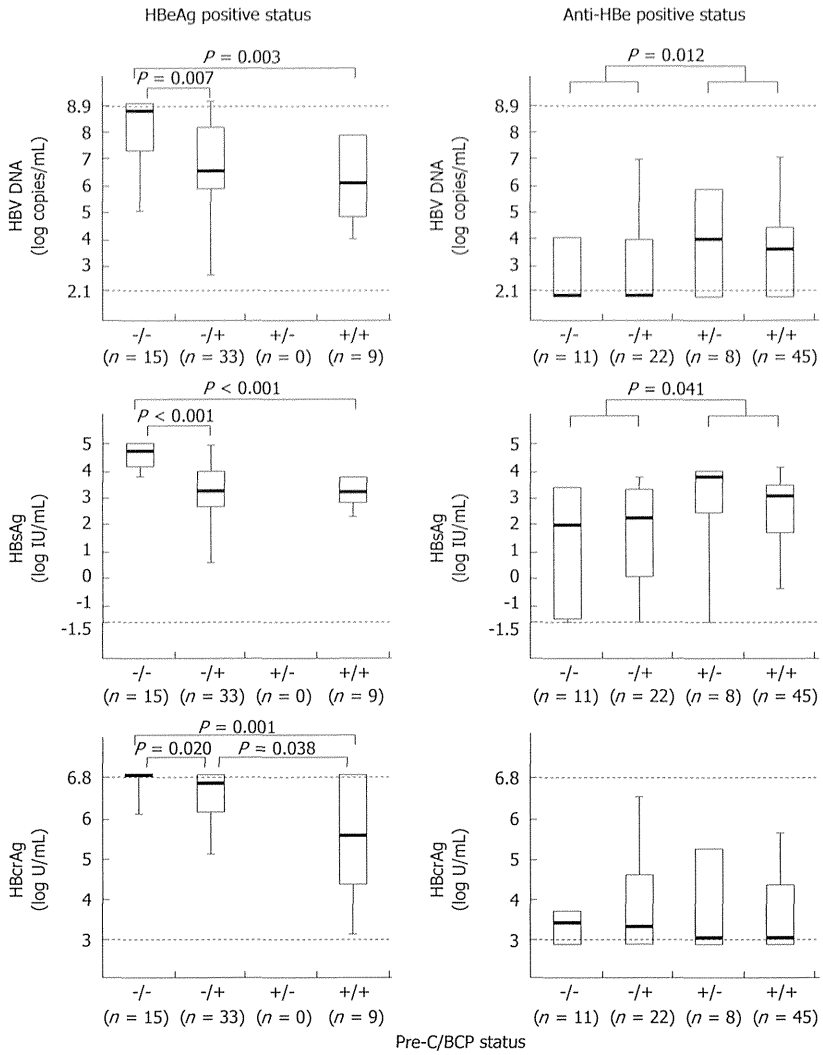

Figure 2 Comparison of serum hepatitis B virus DNA, hepatitis B surface antigen, and hepatitis core-related antigen levels among patients with wild (-/-) and mutant types of the pre-core and basal core promoter mutations. Fifty-seven of 61 samples obtained from HBeAg positive cases and 86 of 111 samples obtained from anti-HBeAg positive cases were eligible for analysis. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis core-related antigen; pre-C: Pre-core; BCP: Basal core promoter.

to HBeAg/anti-HBe and pre-C/BCP status were similar to HBV DNA patterns both in HBeAg and anti-HBe positive states; HBsAg was significantly lower in patients with pre-C and/or BCP mutations than in those with wild type pre-C but was significantly higher in patients with the pre-C mutation than in those without it in an anti-HBe positive state. These results confirmed that the pre-C mutation was oppositely associated with viral load in patients before and after HBeAg seroconversion. Since elevated levels of HBV DNA and HBsAg are related to a higher rate of hepatocarcinogenesis, pre-C mutation patterns appear to be clinically important, at least in the context of HBV genotype C patients. We witnessed that the patterns of HBcrAg were similar to those of HBV DNA in the HBeAg positive state but different in the anti-HBe positive state. This difference may reflect the fact that the main antigen measured by the HBcrAg assay is HBeAg.

In conclusion, our findings indicate that the association of the pre-C G1896A mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion. These observations may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

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COMMENTS

Background

Although pre-core (pre-C) and/or basal core promoter (BCP) mutations in the hepatitis B virus (HBV) genome have been reported to associate with hepatitis

B e antigen (HBeAg) seroconversion, the detailed mechanisms have not been fully clarified.

Research frontiers

In this study, the authors show that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion.

Innovations and breakthroughs

Recent reports have highlighted the importance of pre-C and BCP mutations of the HBV genome in association with HBeAg seroconversion. This study analyzed the changes in pre-C and BCP mutations in patients over a long follow-up period. The authors demonstrate that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection.

Applications

This study may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

Terminology

In the natural history of chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually accompanied by a decrease in HBV replication and the remission of hepatitis. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion.

Peer review

The authors investigated the pre-C and/or BCP mutations before and after HBeAg seroconversion. They found that the association of the pre-C mutation on viral load is opposite in patients before and after HBeAg seroconversion. It is an interesting report. However there are several concerns.

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Review Article

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B

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Nucleoside/nucleotide analogs (NUC) can lead to rapid reduction in hepatitis B virus (HBV) DNA levels in blood and normalization of alanine aminotransferase levels in many patients. They also provide histological improvement which results in a reduction in liver carcinogenesis. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment. One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period. For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently. We performed research to investigate characteristics of the course after discontinuation of treatment and definition of hepatitis relapse and estimate the relapse rate. "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the study results. Because the guidelines are written in Japanese, we explain them in English as a review article.

Key words: discontinuation of treatment, hepatitis B virus cccDNA, hepatitis B, hepatitis relapse, nucleoside/nucleotide analog

INTRODUCTION

BECAUSE NUCLEOSIDE/NUCLEOTIDE analogs (NUC) that have been recently introduced to treat hepatitis B strongly inhibit proliferation of hepatitis B virus (HBV), they can lead to rapid reduction in HBV DNA levels in blood and normalization of alanine aminotransferase (ALT) levels in many patients.[1] They also provide histological improvement which results in a reduction in liver carcinogenesis[2,3] and can be administrated p.o. with few side-effects, so they are widely used in clinical practice. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment.[4] One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period.[5]

For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses occur after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently.

We performed research funded by a Health and Labor Sciences Research Grant to investigate characteristics of the course after discontinuation of treatment, definition of hepatitis relapse and estimation of relapse rate.[6] "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the

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study results.[7] The guidelines do not always recommend discontinuation of NUC. We determined them to be referred to if it is necessary to consider discontinuation of NUC due to various reasons.

SERUM MARKERS REFLECTING AMOUNT OF HBV CCCDNA IN HEPATOCYTES

THE REPLICATION PROCESS of HBV in hepatocytes is shown in Figure 1. HBV is an enveloped DNA virus containing a relaxed circular DNA genome converted into a cccDNA episome in the nucleus of infected cells.[8–11] These cccDNA molecules serve as transcriptional templates for production of viral RNA that encode both viral structural and non-structural proteins. Hepatitis B surface antigen (HBsAg) is translated from 2.1-kb and 2.4-kb mRNA. On the other hand, hepatitis B core antigen (HBcAg), p22cr antigen (p22crAg)[12] and hepatitis B e-antigen (HBeAg) are translated from 3.5-kb mRNA which also serves as pregenome RNA. HBeAg is secreted into the blood stream as a secretion protein, and p22crAg forms genome negative core particles. HBcAg forms nucleocapsid particles by incorporating pregenome RNA. Once the pregenome RNA is reverse transcribed to DNA, the particles are enveloped with lipid layer containing HBsAg and then secreted into blood stream as virions.[9,10] When the reverse transcriptation is inhibited by NUC, virus particles with RNA genome are secreted instead of those with DNA genome.[13,14]

Hepatitis B virus cccDNA is a stable molecule like chromosomal DNA which can be barely destroyed by DNase in natural conditions. Because NUC are inhibitors of reverse transcriptase, they have no direct effect on reducing intrahepatic cccDNA levels. Therefore, reactivation of HBV replication which originates from HBV cccDNA and incidental hepatitis relapse occurs when NUC are discontinued.

It is generally considered that HBV cccDNA levels in hepatocytes are well correlated with the proliferative potential of HBV;[5] serum markers reflecting the cccDNA level are suggested to be useful as clinical indicators. Serum level of HBV DNA correlates well with intrahepatic level of HBV cccDNA in the natural course but not under NUC treatment. NUC reduce serum level of HBV DNA rapidly by inhibiting the reverse transcription, but this inhibition does not reduce the cccDNA level.[5] On the other hand, serum levels of HBsAg and hepatitis B core-related antigen (HBcrAg) have been reported as markers reflecting cccDNA levels in hepatocytes even under NUC treatment.[15–18] HBcrAg assay measures all antigens coded by precore/core genome simultaneously which include HBcAg, HBeAg and p22crAg, and has been reported to be useful for predicting clinical outcomes of patients who were treated with NUC.[6,18–23] HBsAg level has received attention recently as a new marker and has been reported to be efficient in prediction of treatment effects by interferon and others.[15,16]

AIMS OF THESE GUIDELINES

THESE GUIDELINES AIM to identify patients with a higher possibility of successful discontinuation or patients who should continue treatments and avoid

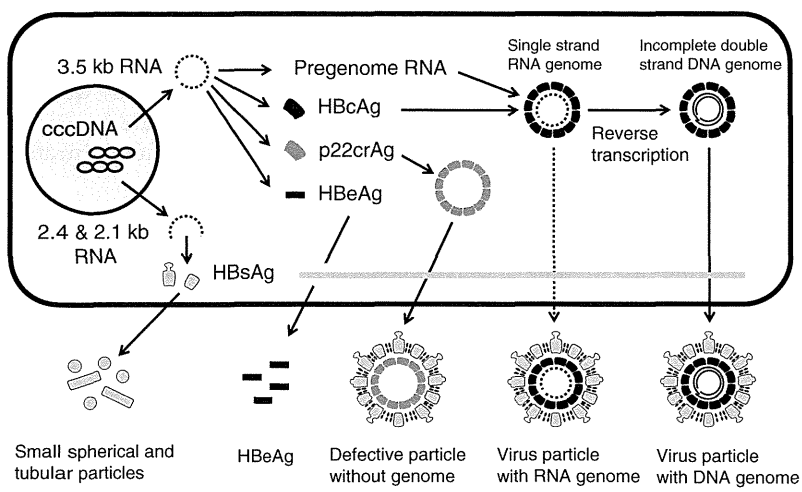

Figure 1 Replication process of hepatitis B virus (HBV) which originates from HBV cccDNA molecules pooled in nucleus of hepatocyte. HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; p22crAg, p22cr antigen.

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risks resulting from discontinuation of NUC as much as possible by establishing indicators for follow up after discontinuation (Appendix 1-I). Successful discontinuation in the guidelines is defined as final achievement of the inactive carrier state with ALT level of less than 30 IU/L and HBV DNA level in blood of less than 4.0 log copies/mL. These criteria were defined in compliance with the guidelines for treatment of chronic hepatitis B in Japan.[24] It is known that patients in the inactive carrier state show no progression of hepatic diseases and a reduction in the carcinogenic rate[25,26] and the criteria are considered to be appropriate.

REQUIREMENTS TO AVOID RISK OF DEVELOPING SEVERE HEPATITIS RESULTING FROM RELAPSE

WE ARE CURRENTLY unable to predict hepatitis relapse after discontinuation of NUC with sufficient accuracy. Therefore, we reviewed the risk of developing severe hepatitis and established requirements to prevent severe hepatitis (Appendix 1-II).[27] The presence of understanding the risks of hepatitis relapse and severe hepatitis by both doctors and patients as well as the availability of a follow-up system after discontinuation and appropriate treatment for relapse are the basic essential requirements. Considering that patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis can easily develop severe hepatitis and have higher risks of carcinogenesis in the future, we determined that those patients should not easily discontinue NUC.

ASSESSMENT OF PROLIFERATIVE POTENTIAL OF HBV AND CONDITIONS TO REDUCE THE RELAPSE RISK

IT HAS BEEN experienced that patients with insufficient reduction of HBV DNA level or with HBeAg positive at the time of discontinuation of NUC can develop hepatitis relapse at higher rates after discontinuation. The tendency was also confirmed scientifically in our study.[6] The cut-off value of HBV DNA level to predict hepatitis relapse was 3.0 log copies/mL by receiver operating characteristic (ROC) analysis. Almost all patients with higher HBV DNA levels or were HBeAg positive relapsed within a year while nearly 30% of patients with HBV DNA levels less than 3.0 log copies/mL and without HBeAg were in a stable condition for a long period (Fig. 2). Based on these results, we included sufficient reduction in HBV DNA levels and

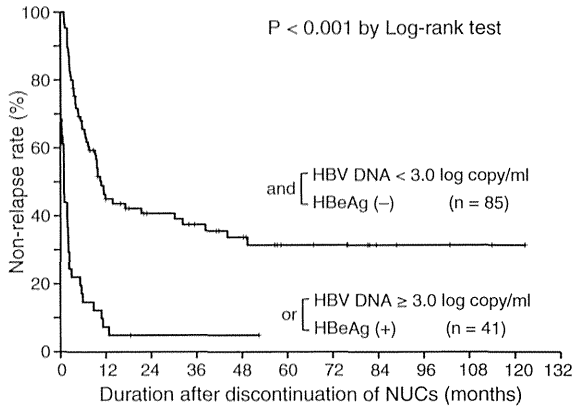

Figure 2 Comparison of non-relapse rates using Kaplan–Meier method between 41 patients with serum hepatitis B virus (HBV) DNA not lower than 3.0 log copies/mL or with hepatitis B e-antigen (HBeAg) and 85 patients with serum HBV DNA lower than 3.0 log copies and without HBeAg at the time of nucleoside/nucleotide analog (NUC) discontinuation.

HBeAg negativity in requirements for discontinuation. We determined the reference range of sufficient reduction in HBV DNA levels in the actual guidelines not to be less than 3.0 log copies/mL but to be negative by real-time polymerase chain reaction (PCR) in consideration of safety.

Factors relating to hepatitis relapse after discontinuation were analyzed in the population except for patients who were obviously predicted to relapse after discontinuation, or those with HBV DNA levels of not less than 3.0 log copies/mL or were HBeAg positive. The following factors were calculated to be significant: duration of treatment period of NUC; HBsAg levels at the time of discontinuation; and HBcrAg levels at the time of discontinuation. Because the cut-off value in duration of treatment period was calculated as 16 months, we overestimated and established that NUC should be discontinued more than 2 years after the initial administration in the guidelines.[6]

Two cut-off values were suggested from the results of the ROC analysis for the HBsAg and HBcrAg levels at the time of discontinuation (Fig. 3): 1.9 and 2.9 log IU/mL for the HBsAg level and 3.0 and 4.0 log U/mL for the HBcrAg level, respectively. Based on this, HBsAg and HBcrAg levels were scored as shown in Appendix 1-III and three groups – low-risk, medium-risk and high-risk – were determined. The percentage of prediction success was 80–90% in the low-risk group, approximately 50% in the medium-risk group and 10–20% in the high-risk

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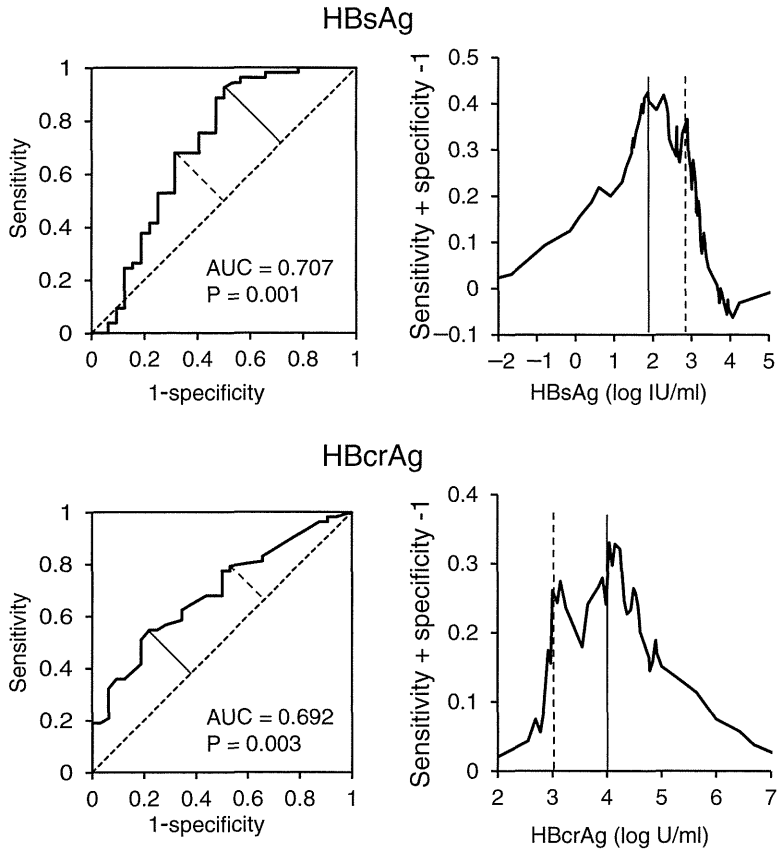

Figure 3 Receiver operating characteristic (ROC) analysis of hepatitis B surface antigen (HBsAg) and HB core-related antigen (HBcrAg) levels to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg levels. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points. AUC, area under the ROC.

group (Fig. 4). In further investigation of factors relating to hepatitis relapse in each group, no factors were newly found in the low- and medium-risk groups but age was a significant factor in the high-risk group. Although the percentage of prediction success rate is low in the high-risk group (10–20%), it resulted in slightly higher rates of 30–40% with those patients younger than 35 years old.[6] It was interesting to find that the combination of HBsAg and HBcrAg levels were useful in preparing these guidelines for discontinuation. Because productions of HBsAg and HBcrAg are regulated by different promoter and enhance systems of HBV genome, their clinical values vary.

FOLLOW-UP METHOD AFTER DISCONTINUATION AND CONDITIONS FOR RETREATMENT

FOLLOW-UP AFTER DISCONTINUATION of NUC includes periodical measurement of HBV DNA levels (real-time PCR) and ALT levels. This study revealed that

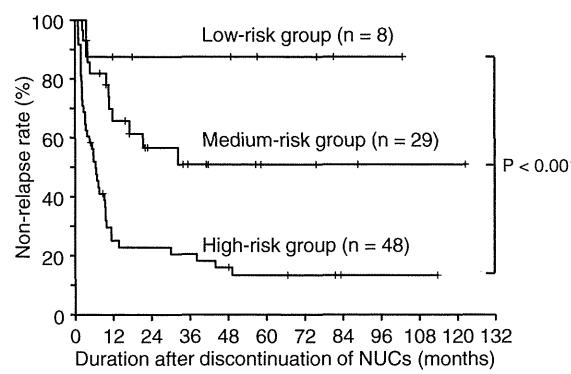

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen and HB core-related antigen levels at the time of nucleoside/nucleotide analog (NUC) discontinuation.

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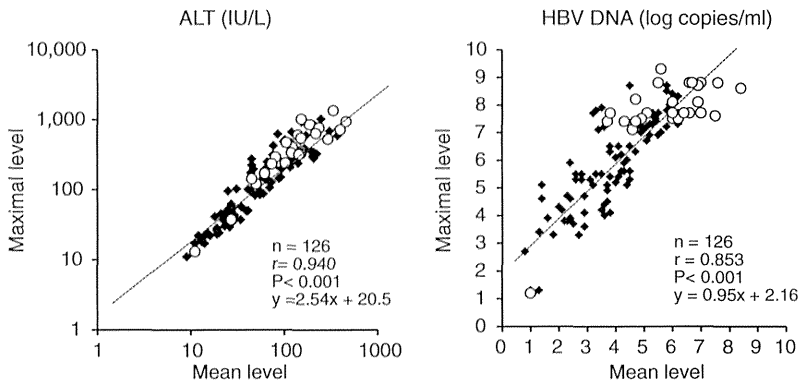

Figure 5 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (left) and hepatitis B virus (HBV) DNA (right) after discontinuation of nucleoside/nucleotide analog (NUC). Open circles indicate patients with detectable hepatitis B e-antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

relapse after discontinuation occurs mostly within 1 year, gradually decreases after 1 year and rarely occurs after the first 3 years of discontinuation.[6] Therefore, we determined it necessary to pay attention especially to relapse immediately after discontinuation. In particular, we determined that it is desirable to follow up patients by blood tests at every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.

One of the important points is what the definition of hepatitis relapse is and how to follow up after discontinuation. Transient abnormalities in the ALT level or the HBV DNA level may be observed in approximately two-thirds patients who would finally achieve the inactive carrier state. Therefore, even if the ALT or HBV DNA levels show mild elevations, it is possible to follow up without retreatment. However, no criteria have been identified about when to discontinue follow up and start retreatment. We assessed the transitions of ALT levels and HBV DNA levels after discontinuation of NUC by the mean and maximum values to identify the criteria. From this assessment, a strong correlation was shown between the mean and the maximum value in both (Fig. 5).[6] Results of the ROC analysis revealed that the mean ALT of 30 IU/L corresponded to the maximum ALT of 79 IU/L and the mean HBV DNA of 4.0 log copies/mL corresponded to the maximum HBV DNA of 5.7 log copies/mL. Patients with ALT values of not less than 80 IU/L after discontinuation are highly likely to show a mean value of more than 30 IU/L and not assumed to finally meet the criteria for successful discontinuation. Similarly, patients with HBV DNA value of not less than 5.8 log copies/mL after discontinuation are most likely to show a mean value of more than 4.0 log copies/mL and not assumed to meet the criteria for successful discontinuation. Based on these results, we established the condition that patients with ALT value of not less than 80 IU/L or HBV DNA level of not less than 5.8 log copies/mL are less likely to finally achieve the inactive carrier state and should be considered for retreatment with NUC. It is considered that NUC can be discontinued more efficiently and specifically in this condition. Physicians can use more severe criteria at their own discretion in consideration of safety. Less strict criteria also can be used, but it is recommended that the treatment should be done under a certain policy and do not follow the treatment without any aims.

KEY POINTS AND FUTURE ISSUES

THIS MAY BE the first guideline for discontinuation of NUC. Most of the data used in this guideline are retrospective and some points remain unsolved. Over 90% of the patients enrolled had genotype C and over 90% of cases were treated with lamivudine until discontinuation.[6] Therefore, key points and future issues are summarized in Appendix 1-V. This guideline provides information to support physicians to decide NUC discontinuation timing but physicians should actually consider for each patient whether NUC can be discontinued or not because long-term prognosis after NUC discontinuation is not yet clear enough and patients' wishes and physicians' decision need to be prioritized. When NUC cannot be successfully discontinued, one of the options is re-administration of NUC. However, it has not been investigated whether re-administration of NUC results in the emergence and development of resistant strains. Further, it is not resolved which NUC should be given when re-administration is required. The consent of patients will be necessary on these points.

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One of the issues to be investigated in the future is to improve accuracy in predicting hepatitis relapse after discontinuation. Investigations on the following approaches are suggested: higher sensitivity HBV DNA, HBV RNA,[13,14] HBV genotypes and HBV genetic mutations. Because these guidelines were prepared based on retrospective studies, it is necessary to validate them with prospective studies. In addition, how to actively discontinue NUC by sequential treatment with interferon also should be included as an important issue to be investigated.

Three kinds of NUC are available now in Japan. Lamivudine was the first NUC introduced into Japan in 2000. Adefovir dipivoxil is used mainly for patients with lamivudine resistance. Entecavir is now recommended as the first-choice NUC. Over 10 years have passed since the first NUC became available in Japan and this is the first full-scale guideline for NUC discontinuation. Although this guideline may not be completely sufficient and needs further investigations, this is the first step leading to a better one in the future.

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APPENDIX

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs 2012

I. Aims of these guidelines

IN TREATMENT WITH nucleoside/nucleotide analogs (NUC) in patients with chronic hepatitis B, it is an important treatment goal to aim at drug-free status by discontinuation of NUC. However, discontinuation of NUC often results in hepatitis relapse which may become severe. Sufficient consideration must be given to the risk in case of discontinuation.

Hepatitis B surface antigen (HBsAg) negativity is the goal of treatment with NUC, but it cannot be always achieved easily. Therefore, discontinuation may be considered even if HBsAg remains positive. These guidelines aim to discontinue NUC in such conditions and finally achieve the inactive carrier state (alanine aminotransferase [ALT] <30 IU/L and hepatitis B virus [HBV] DNA level in blood <4.0 log copies/mL).

It is currently unknown which of the two options for NUC, discontinuation or continuation, is effective on life prognosis or liver carcinogenesis. We established these guidelines to be referred in case of considering discontinuation due to various reasons. We aimed to identify patients with a high possibility of successful discontinuation or patients who should inversely continue the treatment and establish indicators for follow up after discontinuation to avoid risks resulting from discontinuation of NUC as much as possible.

II. Requirements to avoid risk of developing severe hepatitis resulting from relapse

The following requirements are determined for discontinuation to previously assume and avoid the risk of developing severe hepatitis.

1. Both the doctor and the patient fully understand the risk of a high frequency of hepatitis relapse that may become severe.
2. It is possible to follow up as well as to treat appropriately in case of relapse. (Involvement of a specialist is recommended.)
3. The patient has mild hepatic fibrosis with good hepatic functional reserve and will not easily develop severe hepatitis in relapse. (NUC should not be discontinued in patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis.)

III. Assessment of proliferative potential of HBV and conditions to reduce the relapse risk

1. Requirements for discontinuation of nucleoside/nucleotide analogs.
   Almost all patients with high proliferative potential of HBV will relapse after discontinuation. It is essential not to discontinue NUC in these patients and the requirements were determined as follows: (i) HBV DNA level in blood is negative (real-time PCR) at the time of discontinuation; and (ii) hepatitis B e-antigen (HBeAg) level in blood is negative at the time of discontinuation.
2. Condition for duration of treatment period of NUC.
   Because short-term treatment with NUC can easily result in relapse, it is recommended to meet the following condition: more than 2 years after the initial administration of NUC.
3. Assessment of relapse risk by scoring of viral antigen levels.
   For the patients who meet the requirements for discontinuation (HBV DNA negative and HBeAg negative at the time of discontinuation), the HBsAg level and the HBcrAg level at the time of discontinuation can be scored to predict the relapse risk by the following three groups based on the total score. This risk prediction aims to determine whether NUC should be discontinued or not by reference to it to reduce the relapse risk.

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HBsAg levels at the time of discontinuation	Scores	Hepatitis B core-related antigen (HBcrAg) levels at the time of discontinuation	Scores
<1.9 log IU/mL (<80 IU/mL)	0	<3.0 log U/mL	0
1.9–2.9 log IU/mL (80–800 IU/mL)	1	3.0–4.0 log U/mL	1
≥2.9 log IU/mL (≥800 IU/mL)	2	≥4.0 log U/mL	2

Relapse risk	Total scores	Percentage of prediction success	Assessment
Low-risk group	0	80–90%	Discontinuation can be considered. It is essential to pay attention to relapse because some patients of low risk may develop hepatitis relapse.
Medium-risk group	1–2	~50%	Discontinuation can be considered depending on the situation. Further consideration is needed about conditions and the way to discontinue in the future.
High-risk group	3–4	10–20%	Continuous treatment is recommended. However, patients under 35 years old show a relatively higher rate of successful discontinuation of 30–40%.

IV. Follow-up method after discontinuation and conditions for retreatment

1. HBV DNA levels (real-time PCR) and ALT levels must be periodically measured after discontinuation of NUC to pay attention to HBV proliferation and hepatitis relapse resulting from proliferation.
2. Relapse after discontinuation is mostly observed within 1 year and then gradually decreases. It is rare to relapse after the first 3 years. Therefore, it is necessary to pay attention to relapse immediately after discontinuation. In particular, patients should be followed up by blood tests every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.
3. Transient abnormalities in ALT levels or HBV DNA levels may be observed in approximately two-thirds of patients who successfully discontinued NUC and would finally achieve the inactive carrier state. Therefore, even if the ALT level or the HBV DNA level shows mild elevations, it is possible to keep following up without retreatment. However, patients who meet the following condition are less likely to finally achieve the inactive carrier state and should be considered for NUC retreatment.

Condition to consider retreatment with NUC
ALT ≥80 IU/L or HBV DNA ≥5.8 log copies/mL after discontinuation

V. Key points and future issues

1. The status differs in each patient. Objectives and significance also differ by patient. Thus, doctors must determine whether NUC should be discontinued or not in consideration of those conditions. In case of considering discontinuation, it is recommended to consult with a specialist of hepatic diseases.
2. In case of retreatment with NUC due to hepatitis relapse after discontinuation, it is unknown whether it results in higher emergence of strains resistant to NUC or not compared with patients without discontinuation.
3. Because HBV carriers rarely experience hepatitis relapse even in the inactive carrier state (HBV DNA <4.0 log copy/mL and ALT <30 IU/L), they must be followed up after successful discontinuation. Liver carcinogenesis also requires follow up.
4. The followings are included in future issues; improvement of accuracy in the criteria for discontinuation of NUC; investigation of the criteria used in these guidelines in a prospective study; and investigation of the way to actively discontinue NUC using sequential treatment with interferon.

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
* Resolved individuals were HBsAg negative and HBcAb positive.
** 419 of 467 healthy controls were de-identified, without information on age.
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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding