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### 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<sup>[18]</sup>, 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,

HBeAg, 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 ( $P = 0.041$ ).

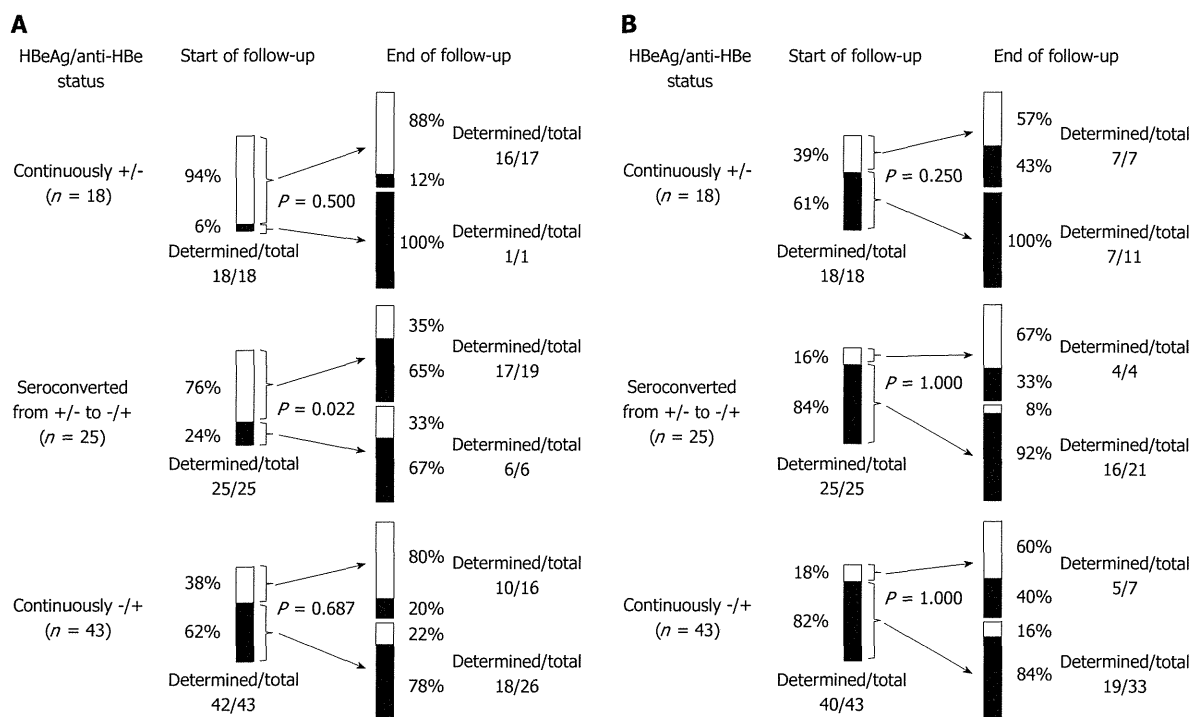
HBcrAg levels were significantly lower with presence of pre-C and/or BCP mutations in an HBeAg positive status ( $P < 0.05$ , respectively). HBcrAg levels were uniformly low regardless of the presence of mutations in anti-HBe positive status subjects.

### 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.*<sup>[14]</sup> were not identified.

## DISCUSSION

Although both pre-C and BCP mutations have been associated with HBeAg seroconversion by reducing the production of HBeAg<sup>[13-15]</sup>, their manifestation patterns appear to be different<sup>[17]</sup>. 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



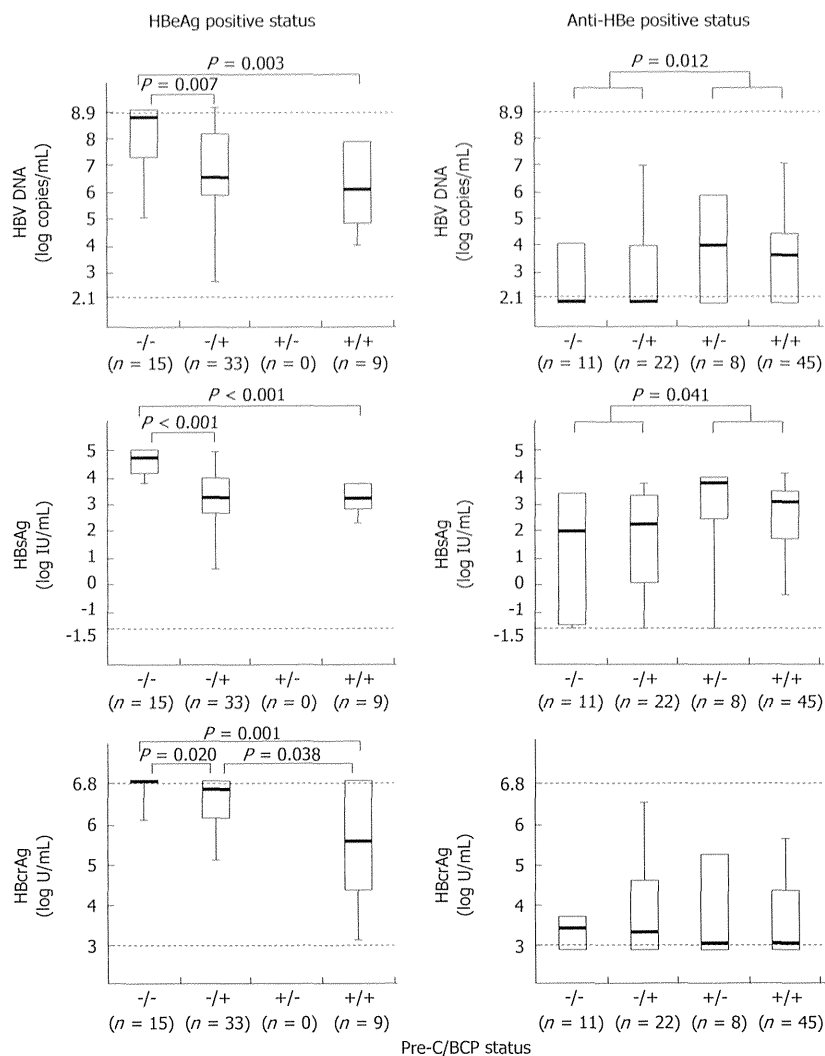
**Figure 1** Comparison of changes in pre-core (A) and basal core promoter (B) mutation type among 3 groups of patients classified according to hepatitis B e antigen /anti-hepatitis B e positive status. A: A significant difference was seen in patients with hepatitis B e antigen (HBeAg) seroconversion ( $P = 0.022$ ). 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<sup>[18]</sup>. 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<sup>[13]</sup>. 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<sup>[14]</sup> 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<sup>[14]</sup>. 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.*<sup>[28]</sup> 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 hepatitis 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<sup>[20,24,29]</sup>. HBsAg has also attracted attention as a useful predictor of treatment outcome by interferon and others<sup>[30]</sup>. 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<sup>[31]</sup>. 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.

## ACKNOWLEDGMENTS

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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.

## REFERENCES

- 1 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056-1075 [PMID: 17393513 DOI: 10.1002/hep.21627]
- 2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718 DOI: 10.1002/hep.21513]
- 3 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745 [PMID: 9392700 DOI: 10.1056/NEJM199712113372406]
- 4 Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; **44** Suppl 19: 102-107 [PMID: 19148802 DOI: 10.1007/s00535-008-2251-0]
- 5 Hoofnagle JH, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; **94**: 744-748 [PMID: 7235415 DOI: 10.7326/0003-4819-94-6-744]
- 6 Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS. Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. *Gastroenterology* 1983; **84**: 216-219 [PMID: 6848402]
- 7 Realdi G, Alberti A, Rugge M, Bortolotti F, Rigoli AM, Tremolada F, Ruol A. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. *Gastroenterology* 1980; **79**: 195-199 [PMID: 7399226]
- 8 Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. *Gastroenterology* 1986; **90**: 1268-1273 [PMID: 3956945]
- 9 Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522-1527 [PMID: 12029639 DOI: 10.1053/jhep.2002.33638]
- 10 Bruss V, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 1988; **163**: 268-275 [PMID: 3354197 DOI: 10.1016/0042-6822(88)90266-8]
- 11 Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; **106**: 1093-1104 [PMID: 3283145 DOI: 10.1083/jcb.106.4.1093]
- 12 Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588-591 [PMID: 2570285 DOI: 10.1016/S0140-6736(89)90713-7]
- 13 Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 1990; **64**: 1298-1303 [PMID: 2304145]
- 14 Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshida M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; **68**: 8102-8110 [PMID: 7966600]
- 15 Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 1996; **70**: 5845-5851 [PMID: 8709203]
- 16 Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishiro S. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 1995; **76** (Pt 12): 3159-3164 [PMID: 8847524 DOI: 10.1099/0022-1317-76-12-3159]
- 17 Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218-223 [PMID: 11124839 DOI: 10.1053/jhep.2001.20532]
- 18 Misawa N, Matsumoto A, Tanaka E, Rokuhara A, Yoshizawa K, Umemura T, Maki N, Kimura T, Kiyosawa K. Patients with and without loss of hepatitis B virus DNA after hepatitis B e antigen seroconversion have different virological characteristics. *J Med Virol* 2006; **78**: 68-73 [PMID: 16299733]
- 19 Umemura T, Tanaka E, Kiyosawa K, Kumada H. Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. *Clin Infect Dis* 2008; **47**: e52-e56 [PMID: 18643758 DOI: 10.1086/590968]
- 20 Matsumoto A, Tanaka E, Morita S, Yoshizawa K, Umemura T, Joshita S. Changes in the serum level of hepatitis B virus (HBV) surface antigen over the natural course of HBV infection. *J Gastroenterol* 2012; **47**: 1006-1013 [PMID: 22370816 DOI: 10.1007/s00535-012-0559-2]
- 21 Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; **44**: 1390-1399 [PMID: 16597867]
- 22 Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66-71 [PMID: 10350059]
- 23 Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to viral load. *J Clin Microbiol* 2002; **40**: 439-445 [PMID: 11825954]
- 24 Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and

- intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27-33 [PMID: 19031469 DOI: 10.1002/jmv.21339]
- 25 **Yamaura T**, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. *J Med Virol* 2003; **70**: 545-552 [PMID: 12794716 DOI: 10.1002/jmv.10429]
- 26 **Aritomi T**, Yatsushashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. *J Gastroenterol Hepatol* 1998; **13**: 1125-1132 [PMID: 9870800]
- 27 **Sugauchi F**, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; **82**: 883-892 [PMID: 11257194]
- 28 **Kawabe N**, Hashimoto S, Harata M, Nitta Y, Murao M, Nakano T, Shimazaki H, Arima Y, Komura N, Kobayashi K, Yoshioka K. The loss of HBeAg without precore mutation results in lower HBV DNA levels and ALT levels in chronic hepatitis B virus infection. *J Gastroenterol* 2009; **44**: 751-756 [PMID: 19430716 DOI: 10.1007/s00535-009-0061-7]
- 29 **Chan HL**, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, Sung JJ. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462-1468 [PMID: 18054753 DOI: 10.1016/j.cgh.2007.09.005]
- 30 **Li WC**, Wang MR, Kong LB, Ren WG, Zhang YG, Nan YM. Peginterferon alpha-based therapy for chronic hepatitis B focusing on HBsAg clearance or seroconversion: a meta-analysis of controlled clinical trials. *BMC Infect Dis* 2011; **11**: 165 [PMID: 21651820 DOI: 10.1186/1471-2334-11-165]
- 31 **Tanaka E**, Matsumoto A. Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B. *Hepatol Res* 2014; **44**: 1-8 [PMID: 23607862 DOI: 10.1111/hepr.12108]

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**Review Article**

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

Eiji Tanaka and Akihiro Matsumoto

Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

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 dis-

continuation. 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

**B**ECAUSE 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.<sup>1</sup> They also provide histological improvement which results in a reduction in liver carcinogenesis<sup>2,3</sup> and can be administered 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.<sup>4</sup>

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.<sup>5</sup>

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.<sup>6</sup> "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the

Correspondence: Eiji Tanaka, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan. Email: etanaka@shinshu-u.ac.jp  
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study results.<sup>7</sup> 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

**T**HE 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.<sup>8–11</sup> 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)<sup>12</sup> and hepatitis B e-antigen (HBeAg) are translated from 3.5-kb mRNA which also serves as pregenome RNA. HBcAg 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.<sup>9,10</sup> When the reverse transcription is inhibited by NUC, virus particles with RNA genome are secreted instead of those with DNA genome.<sup>13,14</sup>

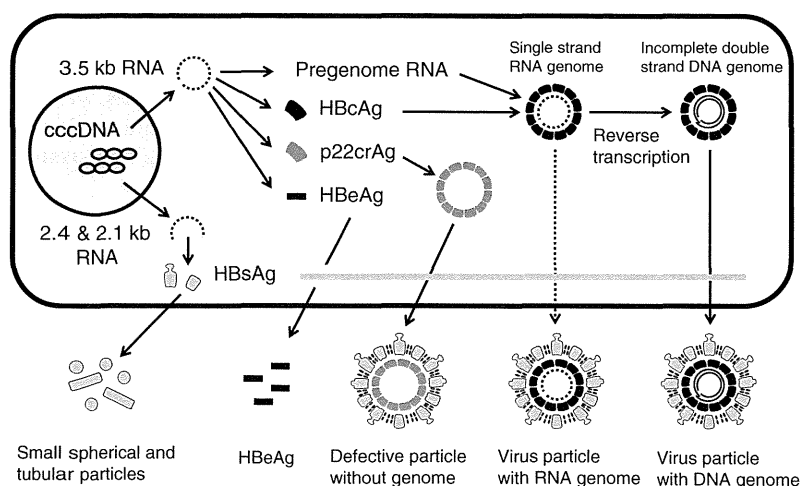
Hepatitis B virus cccDNA is a stable molecule like chromosomal DNA which can be barely destroyed by

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;<sup>5</sup> 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.<sup>5</sup> 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.<sup>15–18</sup> 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.<sup>6,18–23</sup> 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.<sup>15,16</sup>

### AIMS OF THESE GUIDELINES

**T**HESE 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.



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.<sup>24</sup> It is known that patients in the inactive carrier state show no progression of hepatic diseases and a reduction in the carcinogenic rate<sup>25,26</sup> 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).<sup>27</sup> 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.<sup>6</sup> 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

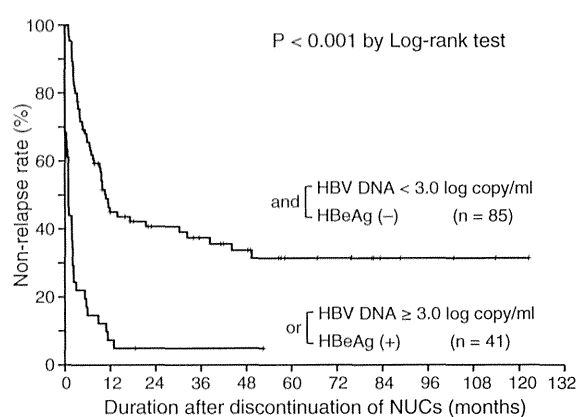
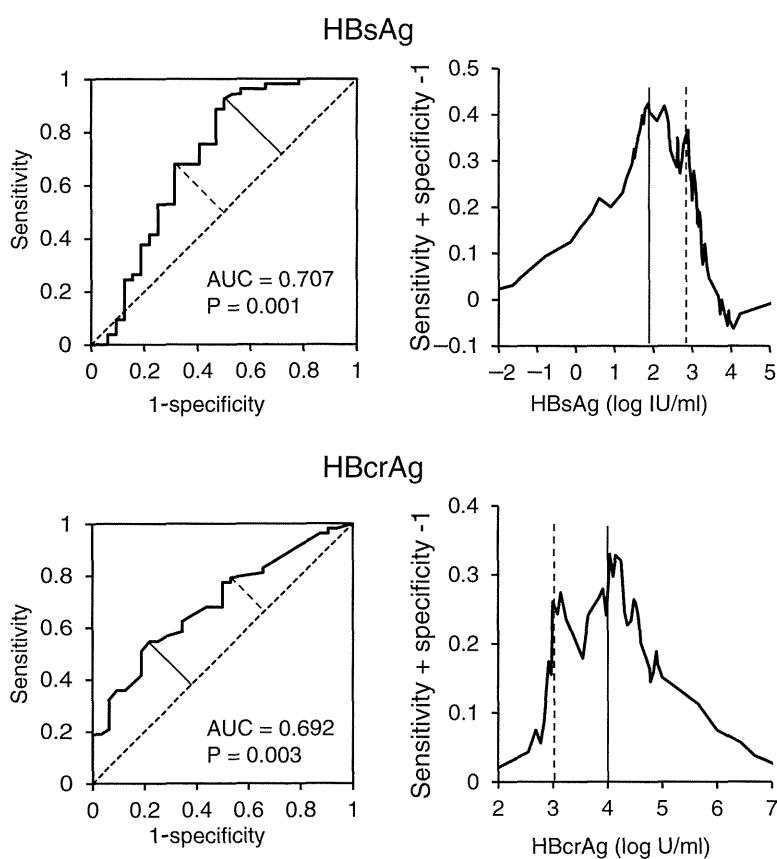


Figure 2 Comparison of non-relapse rates using Kaplan-Meier method between 41 patients with serum hepatitis B virus (HBV) DNA not lower than 3.0 log copies/mL or with hepatitis B e-antigen (HBeAg) and 85 patients with serum HBV DNA lower than 3.0 log copies and without HBeAg at the time of nucleoside/nucleotide 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.<sup>6</sup>

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

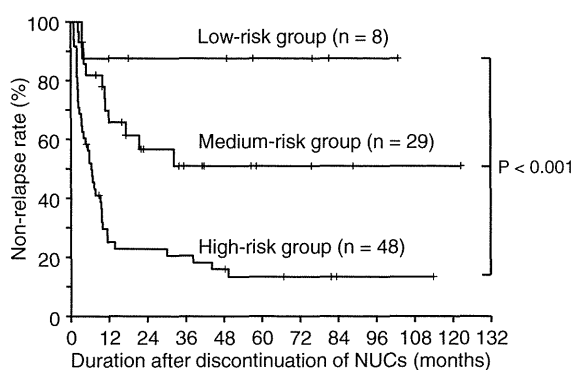


**Figure 3** Receiver operating characteristic (ROC) analysis of hepatitis B surface antigen (HBsAg) and HB core-related antigen (HBcrAg) levels to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg levels. Short 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.<sup>6</sup> 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.

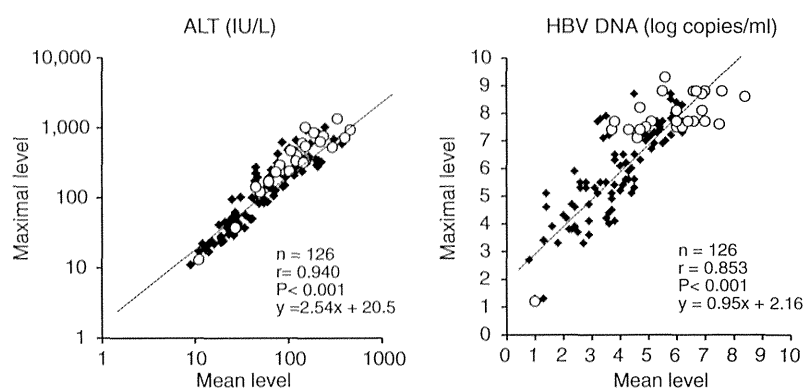
#### FOLLOW-UP METHOD AFTER DISCONTINUATION AND CONDITIONS FOR RETREATMENT

**F**OLLOW-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.

**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.<sup>6</sup> 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).<sup>6</sup> 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

**T**HIS 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.<sup>6</sup> 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.

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,<sup>13,14</sup> 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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## REFERENCES

- Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007; 132: 1574–85.
- Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 7 (351): 1521–31.
- Matsumoto A, Tanaka E, Rokuhara A *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatology Res* 2005; 32: 173–84.
- Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; 46: 254–65.
- Werle-Lapostolle B, Bowden S, Locarnini S *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; 126: 1750–8.
- Matsumoto A, Tanaka E, Suzuki Y *et al.* Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. *Hepatology Res* 2012; 42: 139–49.
- Tanaka E, Matsumoto A, Suzuki Y *et al.* Guidelines for avoiding risks resulting from discontinuation of nucleos(t)ide analogues in patients with chronic hepatitis B (2012). *Kanzo* 2012; 53: 237–242.
- Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 11 (337): 1733–45.
- Mason WS, Halpern MS, England JM *et al.* Experimental transmission of duck hepatitis B virus. *Virology* 1983; 131: 375–84.
- Summers J, Smith PM, Horwich AL. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; 64: 2819–24.
- Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; 7 (47): 451–60.
- Kimura T, Ohno N, Terada N *et al.* Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 2005; 280: 21713–9.
- Rokuhara A, Matsumoto A, Tanaka E *et al.* Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol* 2006; 41: 785–90.
- Hatakeyama T, Noguchi C, Hiraga N *et al.* Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007; 45: 1179–86.
- Chan HL, Wong VW, Tse AM *et al.* Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; 5: 1462–8.
- Moucari R, Lada O, Marcellin P. Chronic hepatitis B: back to the future with HBsAg. *Expert Rev Anti Infect Ther* 2009; 7: 633–6.
- Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; 81: 27–33.
- Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; 45: 3942–7.
- Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439–45.
- Tanaka E, Matsumoto A, Yoshizawa K, Maki N. Hepatitis B core-related antigen assay is useful for monitoring the antiviral effects of nucleoside analogue therapy. *Intervirology* 2008; 51 (Suppl 1): 3–6.

- 21 Hosaka T, Suzuki F, Kobayashi M *et al.* HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy. *Liver Int* 2010; 30: 1461–70.
- 22 Kumada T, Toyoda H, Tada T *et al.* Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: a propensity score analysis. *J Hepatol* 2013; 58: 427–33.
- 23 Shinkai N, Tanaka Y, Orito E *et al.* Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatol Res* 2006; 36: 272–6.
- 24 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; 40: 1–7.
- 25 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; 295: 65–73.
- 26 Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; 130: 678–86.
- 27 Lim SG, Wai CT, Rajnakova A, Kajiji T, Guan R. Fatal hepatitis B reactivation following discontinuation of nucleoside analogues for chronic hepatitis B. *Gut* 2002; 51: 597–9.

## 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.

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

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.

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

Nao Nishida<sup>1,2\*</sup>, Hiromi Sawai<sup>2,9</sup>, Koichi Kashiwase<sup>3</sup>, Mutsuhiko Minami<sup>3</sup>, Masaya Sugiyama<sup>1</sup>, Wai-Kay Seto<sup>4</sup>, Man-Fung Yuen<sup>4</sup>, Nawarat Posuwan<sup>5</sup>, Yong Poovorawan<sup>5</sup>, Sang Hoon Ahn<sup>6</sup>, Kwang-Hyub Han<sup>6</sup>, Kentaro Matsuura<sup>7</sup>, Yasuhito Tanaka<sup>7</sup>, Masayuki Kurosaki<sup>8</sup>, Yasuhiro Asahina<sup>9,10</sup>, Namiki Izumi<sup>8</sup>, Jong-Hon Kang<sup>11</sup>, Shuhei Hige<sup>12</sup>, Tatsuya Ide<sup>13</sup>, Kazuhide Yamamoto<sup>14</sup>, Isao Sakaida<sup>15</sup>, Yoshikazu Murawaki<sup>16</sup>, Yoshito Itoh<sup>17</sup>, Akihiro Tamori<sup>18</sup>, Etsuro Orito<sup>19</sup>, Yoichi Hiasa<sup>20</sup>, Masao Honda<sup>21</sup>, Shuichi Kaneko<sup>21</sup>, Eiji Mita<sup>22</sup>, Kazuyuki Suzuki<sup>23</sup>, Keisuke Hino<sup>24</sup>, Eiji Tanaka<sup>25</sup>, Satoshi Mochida<sup>26</sup>, Masaaki Watanabe<sup>27</sup>, Yuichiro Eguchi<sup>28</sup>, Naohiko Masaki<sup>1</sup>, Kazumoto Murata<sup>1</sup>, Masaaki Korenaga<sup>1</sup>, Yoriko Mawatari<sup>1</sup>, Jun Ohashi<sup>29</sup>, Minae Kawashima<sup>2</sup>, Katsushi Tokunaga<sup>2</sup>, Masashi Mizokami<sup>1\*</sup>

**1** The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan, **2** Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, **3** HLA Laboratory, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, Koutou-ku, Tokyo, Japan, **4** Department of Medicine, Queen Mary Hospital, Hong Kong, **5** Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, **6** Department of Internal Medicine, Yonsei University College of Medicine, Seoul, South Korea, **7** Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan, **8** Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Tokyo, Japan, **9** Department of Liver Disease Control, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan, **10** Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan, **11** Department of Internal Medicine, Teine Keijinkai Hospital, Sapporo, Hokkaido, Japan, **12** Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan, **13** Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka, Japan, **14** Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Okayama, Japan, **15** Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan, **16** Faculty of Medicine, Tottori University, Tottori, Tottori, Japan, **17** Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Kyoto, Japan, **18** Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, Osaka, Japan, **19** Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Aichi, Japan, **20** Department of Gastroenterology and Metabolism, Ehime University Graduate School of Medicine, Toon, Ehime, Japan, **21** Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Ishikawa, Japan, **22** Department of Gastroenterology and Hepatology, National Hospital Organization Osaka National Hospital, Osaka, Osaka, Japan, **23** Division of Gastroenterology and Hepatology, Department of Internal Medicine, Iwate Medical University, Morioka, Iwate, Japan, **24** Division of Hepatology and Pancreatology, Kawasaki Medical College, Kurashiki, Okayama, Japan, **25** Department of Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan, **26** Division of Gastroenterology and Hepatology, Saitama Medical University, Iruma, Saitama, Japan, **27** Department of Gastroenterology, Kitasato University School of Medicine, Sagami-hara, Kanagawa, Japan, **28** Department of Internal Medicine, Saga Medical School, Saga, Saga, Japan, **29** Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

## 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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\* E-mail: mmizokami@hospk.ncgm.go.jp (MM); nishida-75@umin.ac.jp (NN)

These authors contributed equally to this work.

## 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. doi:10.1371/journal.pone.0086449.t001



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-DPBI* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPBI\*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPBI\*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-DPBI\*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-DPBI\*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-DPBI\*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPBI\*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-DPBI\*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-DPBI\*02:01* and *HLA-DPBI\*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-DPBI\*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-DPBI* 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-DPBI\*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-DPBI\*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved

**Table 2.** Association of number of *DPBI\*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*	$1.55 \times 10^{-7}$	0.50 (0.39–0.65)

\*Population was adjusted using dummy variables.  
doi:10.1371/journal.pone.0086449.t002

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).

As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPBI\*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 *DPBI\*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 *DPBI\*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-DPBI* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPBI* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1\*02:02-DPBI\*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-DPBI* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1\*02:01-DPBI\*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-DPBI\*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-DPBI\*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-DPBI\*13:01*: *DPA1\*02:01-DPBI\*13:01*, *DPA1\*02:02-DPBI\*13:01*, and *DPA1\*04:01-DPBI\*13:01*, indicating that the association of *HLA-DPBI\*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPBI* 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, 0.34–0.64), 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

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1\*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1\*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1\*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1\*09:01* and *DPB1\*05:01*) and three protective alleles (*DPB1\*04:01*, *DPB1\*04:02* and *DPB1\*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

## Materials and Methods

### Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

### Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15  $\mu$ mol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm<sup>3</sup>, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100  $\mu$ l of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at  $-20^{\circ}$ C until use.

### Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in accordance with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

### Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for  $\alpha$  were set at  $\alpha=0.05/3$  in Japanese,  $\alpha=0.05/4$  in Korean,  $\alpha=0.05/5$  in Hong Kong, and  $\alpha=0.05/5$  in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were  $\alpha=0.05/10$ ,  $0.05/11$ ,  $0.05/12$ , and  $0.05/16$ , respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1\*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1\*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e.,  $(c1, c2, c3) = (0, 0, 0)$  for Japanese,  $(1, 0, 0)$  for Korean,  $(0, 1, 0)$  for Hong Kong, and  $(0, 0, 1)$  for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation:  $\text{logit}(p) = -3.905 + 0.083 \cdot \text{age} + (-0.929) \cdot \text{sex} + (-0.684) \cdot \text{DPB1*02:01} + 1.814 \cdot c1 + (-0.478) \cdot c2 + 0.782 \cdot c3$ . Significance levels in the analysis of disease progression in CHB patients were set as  $\alpha=0.05/10$  in Japanese,  $\alpha=0.05/11$  in Korean,  $\alpha=0.05/15$  in Hong Kong, and  $\alpha=0.05/15$  in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as  $\alpha=0.05/14$  in Japanese,  $\alpha=0.05/17$  in Korean,  $\alpha=0.05/17$  in Hong Kong, and  $\alpha=0.05/18$  in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency >1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests;  $\alpha=0.05/20$  for *DPA1* alleles,  $\alpha=0.05/57$  for *DPB1* alleles, and  $\alpha=0.05/74$  for *DPA1-DPB1* haplotypes.

## Supporting Information

**Figure S1 Comparison of odds ratios in association analyses for HLA-DP with chronic HBV infection among four Asian populations: (A) HLA-DPA1 alleles; (B) HLA-DPB1 alleles; and (C) HLA DPA1-DPB1 haplotypes. Meta-**

**analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.**

(DOCX)

**Table S1 Individuals with successfully genotyped for HLA-DPA1 and HLA-DPB1.**

(DOCX)

**Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.**

(XLSX)

**Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.**

(XLSX)

**Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.**

(XLSX)

**Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.**

(XLSX)

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## Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

## References

- Chen DS (1993) From hepatitis to hepatoma: lessons from type B viral hepatitis. *Science* 262: 369–370.
- Custer B, Sullivan SD, Hazlet TK, Iloeje U, Veenstra DL, et al. (2004) Global epidemiology of hepatitis B virus. *J Clin Gastroenterol* 38: S158–S168.
- Zidan A, Scheuerlein H, Schule S, Settmacher U, Rauchfuss F (2012) Epidemiological pattern of hepatitis B and hepatitis C as etiological agents for hepatocellular carcinoma in Iran and worldwide. *Hepat Mon* 12: e6894.
- Pungpapong S, Kim WR, Poterucha JJ (2007) Natural history of hepatitis B virus infection: an update for clinicians. *Mayo Clin Proc* 82: 967–975.
- Kim DW, Lee SA, Hwang ES, Kook YH, Kim BJ (2012) Naturally occurring precore/core region mutations of hepatitis B virus genotype C related to hepatocellular carcinoma. *PLoS One* 7: e47372.
- Jiang DK, Sun J, Cao G, Liu Y, Lin D, et al. (2013) Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. *Nat Genet* 45: 72–75.
- Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, et al. (2009) A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. *Nat Genet* 41: 591–595.
- Mbarek H, Ochi H, Urabe Y, Kumar V, Kubo M, et al. (2011) A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. *Hum Mol Genet* 20: 3884–3892.
- Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn SH, et al. (2012) Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. *PLoS One* 7: e39175.
- Guo X, Zhang Y, Li J, Ma J, Wei Z, et al. (2011) Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. *Hepatology* 53: 422–428.
- An P, Winkler C, Guan L, O'Brien SJ, Zeng Z (2011) A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. *J Infect Dis* 203: 943–947.
- Li J, Yang D, He Y, Wang M, Wen Z, et al. (2011) Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. *PLoS One* 6: e24221.
- Vermehren J, Lotsch J, Susser S, Wicker S, Berger A, et al. (2012) A common HLA-DPA1 variant is associated with hepatitis B virus infection but fails to distinguish active from inactive Caucasian carriers. *PLoS One* 7: e32605.
- Sawai H, Nishida N, Mbarek H, Matsuda K, Mawatari Y, et al. (2012) No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations. *BMC Med Genet* 13: 47.
- Chandanayingyong D, Stephens HA, Fan L, Sirikong M, Longta P, et al. (1994) HLA-DPB1 polymorphism in the Thais of Southeast Asia. *Hum Immunol* 40: 20–24.