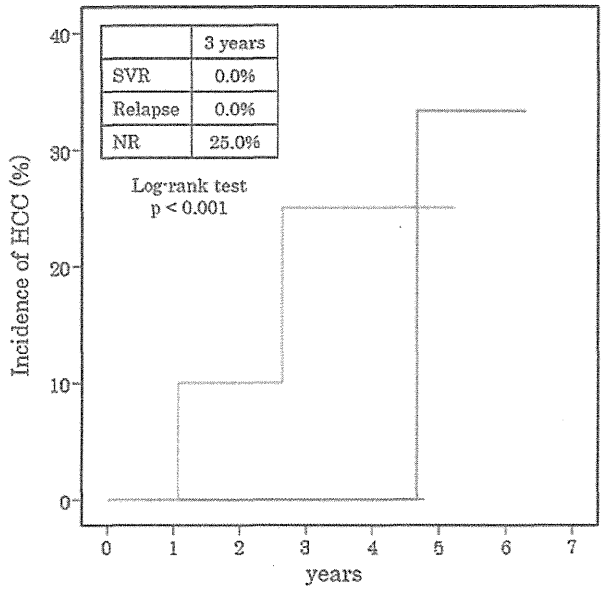

Fig. 4 Cumulative rates of HCC incidence in group B patients (ALT $\leq$ 30 IU/L and PLT $< 15 \times 10^4/mm^3$) with moderate to severe liver fibrosis (F2–4), according to the treatment effect of Peg-IFN plus ribavirin combination therapy. *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

for those with genotype 2. The reason for this was considered to be that groups B and D included many patients with moderate to severe liver fibrosis (F3–4, 17.0 %), which can lead to a lower SVR rate [23, 29, 30].

The present study revealed the cumulative rates of HCC incidence according to the treatment effect in the four groups. In group D, the cumulative rate of HCC incidence in the SVR and relapse patients was significantly lower than that for the NR patients. This result supports the recommendation by the treatment guidelines that patients in group D be managed in the same way as patients with chronic hepatitis C (CH-C) and elevated ALT levels.

In group B patients, the treatment guidelines recommend antiviral therapy for those who have moderate to severe liver fibrosis (F2–4). In our present study, patients with no fibrosis to mild fibrosis (F0–1) did not develop HCC, and in the patients with moderate to severe fibrosis (F2–4), the cumulative rate of HCC incidence tended to be lower in the SVR group than that in the NR group ($p = 0.071$). These results also indicate the appropriateness of the Japanese treatment guidelines. However, further study is needed because of the small number of cases studied here.

It appears that group A patients have time to wait for therapy with the next generation of direct antiviral agents (DAAs), such as Peg-IFN plus ribavirin plus a second-generation protease inhibitor, because none of the patients had developed HCC at 3 years. Even in group C, for which the treatment guidelines recommend antiviral therapy, there was no significant difference in the cumulative rate of HCC incidence among the SVR, relapse, and NR patients, with the incidence being below 5 % at 3 years. Accordingly, patients with PLT counts of more than $15 \times 10^4/mm^3$ (groups A or C) have time to wait until the next generation of DAAs becomes available, because patients with PLT counts of more than $15 \times 10^4/mm^3$ have a low 3-year carcinogenesis rate.

The Japanese treatment guidelines recommend antiviral therapy for patients with $30 <$ ALT ≤ 40 IU/L levels. However, in the present study, in the patients with $30 <$ ALT ≤ 40 IU/L levels, the p value for a significant difference in the cumulative rate of HCC incidence among the patients with SVR, relapse, and NR was 0.059. This result indicates that the patients with $30 <$ ALT ≤ 40 IU/L levels have the potential to be candidates for antiviral therapy, and further study is needed to clarify this. However, these patients may not be candidates for immediate antiviral therapy because the cumulative rates of HCC incidence at 3 years in the patients with SVR, relapse, and NR were low (cumulative rates of HCC at 3 years: 1.6, 0.0, and 3.7 %). On the other hand, as mentioned above, in the patients with PLT counts of $<15 \times 10^4/mm^3$, the cumulative rate of HCC incidence was significantly lower in the SVR and relapse patients than that in the NR patients (cumulative rates of HCC at 3 years: 0.0, 0.0, and 9.3 %; at 5 years: 0.0, 11.1, and 20.8 %; $p < 0.001$). This result suggests that patients with PLT counts of $<15 \times 10^4/mm^3$ may be candidates for antiviral therapy.

A limitation of this study was that the incidence of HCC was not compared between a treatment group and a non-treatment group. This study showed the suppressive effect of antiviral therapy on HCC incidence by comparing patients according to the treatment's antiviral effect. Peg-IFN plus ribavirin combination therapy has become acceptable for patients with chronic HCV infection and N-ALT levels. However, if there were no difference in HCC incidence between patients with SVR and non-SVR in the group receiving Peg-IFN plus ribavirin combination therapy, it would not be necessary for patients with chronic HCV infection and N-ALT to receive this therapy. In this study, we compared the incidence of HCC according to the treatment effect in HCV-infected patients with N-ALT levels categorized by the Japanese treatment guidelines. Indeed, although our results did not demonstrate that N-ALT patients should be treated, they indicated that it could be appropriate to treat N-ALT patients, because the incidence of HCC in these patients with SVR was suppressed compared with that in the NR patients.

In conclusion, in patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ who received Peg-IFN plus ribavirin

combination therapy, the cumulative rate of HCC incidence was significantly lower in those with SVR or relapse than in those with NR. Therefore, HCV-infected patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy for the purpose of reducing the risk of developing HCC.

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References

1. Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. Gastroenterology. 1997;112:463–72.
2. Serfaty L, Aumaitre H, Chazouilleres O, Bonnand AM, Rosmorduc O, Poupon RE, Poupon R. Determinants of outcome of compensated hepatitis C virus-related cirrhosis. Hepatology. 1998;27:1435–40.
3. Hu KQ, Tong MJ. The long-term outcomes of patients with compensated hepatitis C virus-related cirrhosis and history of parenteral exposure in the United States. Hepatology. 1999;29: 1311–6.
4. National Institutes of Health Consensus Development Conference Statement. Management of hepatitis C 2002 (June 10–12, 2002). Gastroenterology. 2002;123:2082–99.
5. Nutt AK, Hassan HA, Lindsey J, Lamps LW, Raufman JP. Liver biopsy in the evaluation of patients with chronic hepatitis C who have repeatedly normal or near-normal serum alanine aminotransferase levels. Am J Med. 2000;109:62–4.
6. Marcellin P. Hepatitis C: the clinical spectrum of the disease. J Hepatol. 1999;31(Suppl 1):9–16.
7. Martinot-Peignoux M, Boyer N, Cazals-Hatem D, Pham BN, Gervais A, Le Breton V, Levy S, et al. Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. Hepatology. 2001;34:1000–5.
8. Sangiovanni A, Morales R, Spinzi G, Rumi M, Casiraghi A, Ceriani R, Colombo E, et al. Interferon alfa treatment of HCV RNA carriers with persistently normal transaminase levels: a pilot randomized controlled study. Hepatology. 1998;27:853–6.
9. Pradat P, Alberti A, Poynard T, Esteban JI, Weiland O, Marcellin P, Badalamenti S, et al. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. Hepatology. 2002;36:973–7.
10. Tassopoulos NC. Treatment of patients with chronic hepatitis C and normal ALT levels. J Hepatol. 1999;31(Suppl 1):193–6.
11. Arora S, O'Brien C, Zeuzem S, Shiffman ML, Diago M, Tran A, Pockros PJ, et al. Treatment of chronic hepatitis C patients with persistently normal alanine aminotransferase levels with the combination of peginterferon alpha-2a (40 kDa) plus ribavirin: impact on health-related quality of life. J Gastroenterol Hepatol. 2006;21:406–12.
12. Bini EJ, Mehandru S. Sustained virological response rates and health-related quality of life after interferon and ribavirin therapy in patients with chronic hepatitis C virus infection and persistently normal alanine aminotransferase levels. Aliment Pharmacol Ther. 2006;23:777–85.
13. Marcellin P, Asselah T, Boyer N. Fibrosis and disease progression in hepatitis C. Hepatology. 2002;36:S47–56.
14. Hiramatsu N, Inoue Y, Oze T, Kurashige N, Yakushijin T, Mochizuki K, Miyagi T, et al. Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case–control study. J Gastroenterol. 2011;46(11):1335–43.
15. Zeuzem S, Diago M, Gane E, Reddy KR, Pockros P, Prati D, Shiffman M, et al. Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. Gastroenterology. 2004;127:1724–32.
16. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996;24:289–93.
17. Tsuji K, Yamasaki K, Yamanishi M, Kawakami M, Shirahama S. Risk of alanine aminotransferase flare-up among asymptomatic hepatitis C virus RNA carriers: a 10-year follow-up study. J Gastroenterol Hepatol. 2001;16:536–40.
18. Puoti C, Castellacci R, Montagnese F. Hepatitis C virus carriers with persistently normal aminotransferase levels: healthy people or true patients? Dig Liver Dis. 2000;32:634–43.
19. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology. 2009;49:1335–74.
20. Puoti C, Guido M, Mangia A, Persico M, Prati D. Clinical management of HCV carriers with normal aminotransferase levels. Dig Liver Dis. 2003;35:362–9.
21. Puoti C, Magrini A, Stati T, Rigato P, Montagnese F, Rossi P, Aldegheri L, et al. Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine transaminase levels. Hepatology. 1997;26:1393–8.
22. Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, et al. Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels. J Hepatol. 2009;50:729–35.
23. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
24. Puoti C, Bellis L, Martellino F, Guarisco R, Dell' Unto O, Durola L, Galossi A. Chronic hepatitis C and 'normal' ALT levels: treat the disease not the test. J Hepatol. 2005;43:534–5.
25. Puoti C, Bellis L, Galossi A, Guarisco R, Nicodemo S, Spilabotti L, Unto OD. Antiviral treatment of HCV carriers with persistently normal ALT levels. Mini Rev Med Chem. 2008;8:150–2.
26. Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, Yasui K, et al. A follow-up study to determine

the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. J Hepatol. 2005;43:599–605.
27. Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, et al. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med. 2002;137:1–10.
28. Ahmed A, Keeffe EB. Chronic hepatitis C with normal aminotransferase levels. Gastroenterology. 2004;126:1409–15.
29. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358:958–65.
30. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.

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Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

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Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $P_{meta} = 1.89 \times 10^{-12}$ for rs3077 and $P_{meta} = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ($P_{meta} = 4.40 \times 10^{-19}$ for rs3077 and $P_{meta} = 1.28 \times 10^{-15}$ for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the HLA-DP molecule.

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Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPB1* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–18]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

Results

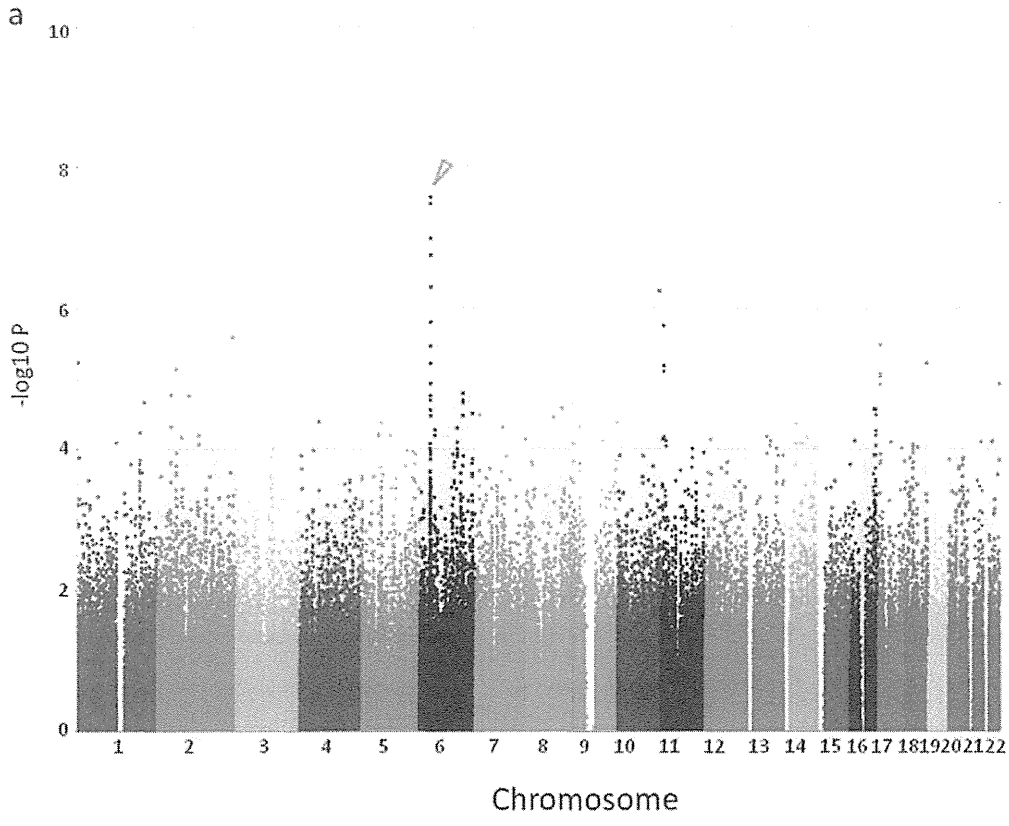
Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate ≥95%; (ii) minor allele frequency (MAF) ≥1% for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3′ UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPB1*, respectively (rs3077, $P = 1.14 \times 10^{-7}$, and rs9277542, $P = 5.32 \times 10^{-8}$, respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion $P < 8.36 \times 10^{-8}$ (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077, $P = 2.70 \times 10^{-8}$, OR = 0.48 and rs9277542, $P = 3.33 \times 10^{-6}$, OR = 0.54; replication-2 (Korean); rs3077, $P = 2.08 \times 10^{-6}$, OR = 0.47 and rs9277542, $P = 8.29 \times 10^{-5}$, OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ($P_{het} = 0.80$ for rs3077 and 0.40 for rs9277542). P_{meta} values were 4.40×10^{-19} for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and 1.28×10^{-15} for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate ≥ 95% and HWE p-value ≥ 0.01. Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel P values of 2.80×10^{-7} (OR = 0.56, 95% CI = 0.45–0.70) and 1.09×10^{-7} (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ($P_{het} = 0.67$ for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed $P_{meta} = 3.94 \times 10^{-45}$, OR = 0.55 for rs3077, $P_{meta} = 1.74 \times 10^{-21}$, OR = 0.61 for rs9277535 and $P_{meta} = 1.69 \times 10^{-15}$, OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of $r^2 = 0.955$ on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with $P_{het} > 0.01$.

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).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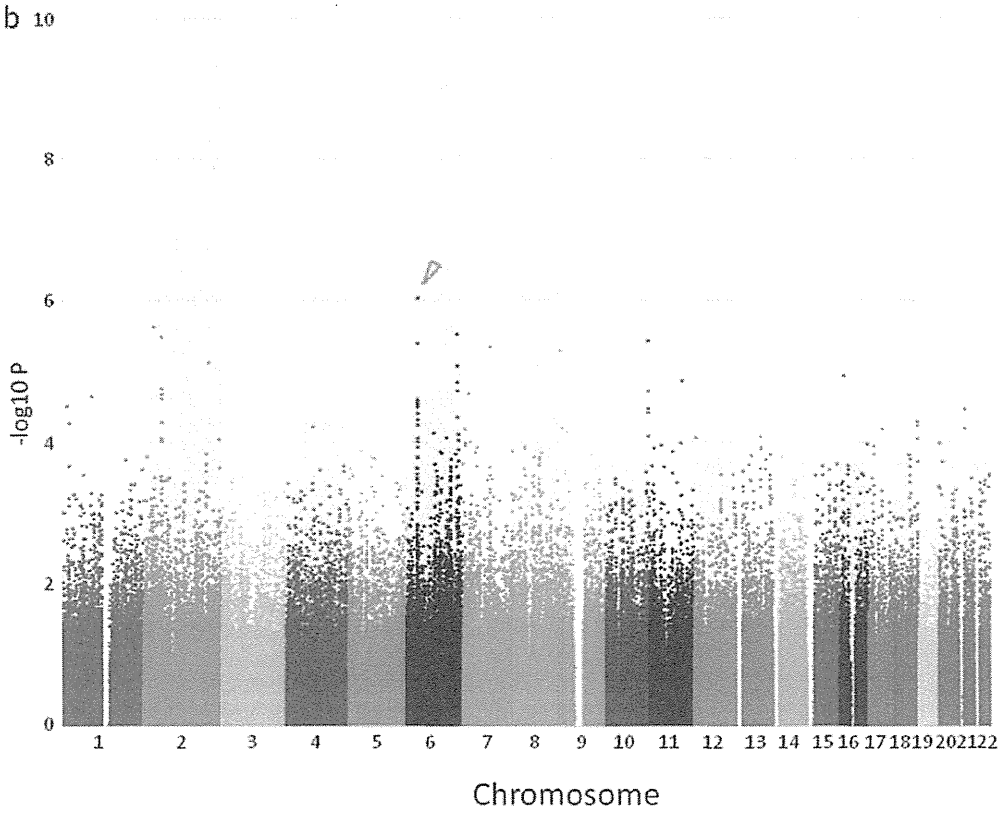
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Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. *P* values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance.
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Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P=9.24\times10^{-7}$ and $P=3.15\times10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1:256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2:344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P=3.32\times10^{-2}$, OR $=0.72$ and rs9277542, $P=1.25\times10^{-2}$, OR $=0.68$; replication-2 (Korean): rs3077, $P=2.35\times10^{-7}$, OR $=0.41$ and rs9277542, $P=4.97\times10^{-6}$, OR $=0.46$; Table 3]. Meta-analysis using random effects model showed $P_{meta}=1.56\times10^{-4}$ for rs3077 (OR $=0.51$, 95% CI = 0.36–0.72), and 5.91×10^{-7} for rs9277542 (OR $=0.55$, 95% CI = 0.43–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het}=0.03$) and no evidence for rs9277542 ($P_{het}=0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta}=3.28\times10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel *P* values of 2.10×10^{-5} (OR $=0.59$) and 1.10×10^{-5} (OR $=0.56$), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het}=0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were observed with $P_{meta}=0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta}=1.48\times10^{-9}$, OR $=0.60$ for rs3077, $P_{meta}=1.08\times10^{-17}$, OR $=0.66$ for rs9277535 and $P_{meta}=5.14\times10^{-5}$, OR $=0.55$ for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het}=0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het}=9.57\times10^{-6}$ across 5 independent studies, including our study.

Table 1. Number of study samples.

population		GWAS Japanese	Replication-1 Japanese	Replication-2 Korean
HBV carriers	Total	**181**	**256**	**344**
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls		**184**	**236**	**151**
Resolved individuals		**185**	**150**	**106**

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
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URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including *HLA-DPA1* and *HLA-DPB1* genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as *HLA-DPA1* and *HLA-DPB1* and protective effects against CHB in Korean and Japanese individuals. Mata-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of *HLA-DP* contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the *HLA-DPA1* and *HLA-DPB1* genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het}=0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta}=5.88\times10^{-24}$, OR $=0.56$ (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het}=0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P=3.32\times10^{-2}$, OR $=0.72$). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta}=1.89\times10^{-12}$, OR $=0.43$ for rs3077 with no evidence of heterogeneity ($P_{het}=0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of *HLA-DPA1/−DPB1* with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR $=0.43$ for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR $=0.46$ for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

Table 2. Results of replication study for protective effects against CHB.

		Position	MAF[a]	Allele	Stage	HBV carriers			Healthy controls				OR[b]		
dbSNP rsID	Chr	Buld 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	HWEp	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10^{-7}	
			(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
					Replication-1	26	95	134	46	125	65	0.309	0.48	2.70×10^{-8}	
					(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
					Replication-2	23	81	111	31	74	40	0.767	0.47	2.08×10^{-6}	
					(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
					Meta-analysis[e]								0.46	4.40×10^{-19}	0.80
													(0.39–0.54)		
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10^{-8}	
			(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
					Replication-1	30	106	118	54	114	67	0.681	0.54	3.33×10^{-6}	
					(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
					Replication-2	30	87	94	35	72	36	0.933	0.54	8.29×10^{-5}	
					(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
					Meta-analysis[e]								0.50	1.28×10^{-15}	0.40
													(0.43–0.60)		

[a]*Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).*
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
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comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2<0.1$) was observed between *HLA-DQB2* locus and *HLA-DPA1/−DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs92775542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with $P_{log}>0.05$ were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DPA1/−DPB1*.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover, there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

Table 3. Results of replication study for clearance of hepatitis B virus.

		Position	MAF[a]	Allele	Stage	HBV carriers			Resolved individuals			OR[b]		
dbSNP rsID	Chr	Buld 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10^{-7}	
			(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
					Replication-1	26	95	134	20	64	60	0.72	3.32×10^{-2}	
					(Japanese)	(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
					Replication-2	23	81	111	29	48	28	0.41	2.35×10^{-7}	
					(Korean)	(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
					Meta-analysis[e]							0.51	1.56×10^{-4}	0.03
												(0.36–0.72)		
					Meta-analysis[e] (GWAS+replication-2)							0.43 (0.34–0.54)	1.89×10^{-12}	0.75
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15×10^{-5}	
			(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
					Replication-1	30	106	118	28	62	52	0.68	1.25×10^{-2}	
					(Japanese)	(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
					Replication-2	30	87	94	30	53	22	0.46	4.97×10^{-6}	
					(Korean)	(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
					Meta-analysis[e]							0.55	5.91×10^{-7}	0.19
												(0.43–0.69)		
					Meta-analysis[e] (GWAS+replication-2)							0.49 (0.39–0.61)	9.69×10^{-10}	0.65

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
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City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

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 portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (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 an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE *P*-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P<0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P<0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P=8.36\times10^{-8}$ (0.05/597,789) and $P=8.47\times10^{-8}$ (0.05/590,278), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P=0.0017$ (0.05/29) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P>0.01$).

The genetic inflation factor λ was estimated by applying the Cochrane-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. *P* values were calculated using chi-squared test for allele frequencies.
(PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm.
(PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals.
(PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT).
(PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S5 Logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) and *HLA-DQ* (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the *HLA-DQ* genotypes individually as a covariate.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

References

1. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO (2002) Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 83: 2059–2073.
2. Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok ASF (2007) Management of hepatitis B: Summary of a clinical research workshop. Hepatology 45: 1056–1075.
3. Yokosuka O, Kurosaki M, Imazeki F, Arase Y, Tanaka Y, et al. (2011) Management of hepatitis B: Consensus of the Japan society of Hepatology 2009. Hepatol Res 41: 1–21.
4. Tada H, Uga N, Fuse Y, Shimizu M, Nemoto Y, et al. (1992) Prevention of perinatal transmission of hepatitis B virus carrier state. Acta Paediatr Jpn 34: 656–659.
5. Stevens CE, Toy PT, Taylor PE, Lee T, Yip HY (1992) Prospects for control of hepatitis B virus infection: implications of childhood vaccination and long-term protection. Pediatrics 90: 170–173.
6. Szmuness W (1979) Large-scale efficacy trials of hepatitis B vaccines in the USA: baseline data and protocols. J Med Virol 4: 327–340.
7. Kwon H, Lok AS (2011) Hepatitis B therapy. Nat Rev Gastroenterol Hepatol 8: 275–284.
8. Kobayashi M, Ikeda K, Arase Y, Suzuki F, Akuta N, et al. (2008) Change of Hepatitis B virus genotypes in acute and chronic infections in Japan. J Med Virol 80: 1880–1884.
9. Yano K, Tamada Y, Yatsuhashi H, Komori A, Abiru S, et al. (2010) Dynamic epidemiology of acute viral hepatitis in Japan. Intervirology 53: 70–75.
10. 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.
11. 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.
12. 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.
13. 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. Hepatol 53: 422–428.
14. Thursz MR, Kwiatkowski D, Allsopp CEM, Greenwood BM, Thomas HC, et al. (1995) Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N Engl J Med 332: 1065–1069.
15. Godkin A, Davenport M, Hill AVS (2005) Molecular analysis of HLA class II associations with hepatitis B virus clearance and vaccine nonresponsiveness. Hepatology 41: 1383–1390.
16. An P, Winkler C, Guan L, O'Brien SJ, Zeng Z, et al. (2011) A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. J Infect Dis 203: 943–947.
17. Wang L, Wu X-P, Zhang W, Zhu D-H, Wang Y, et al. (2011) Evaluation of genetic susceptibility loci for chronic hepatitis B in Chinese: two independent case-control study.
18. Hu L, Zhai X, Liu J, Chu M, Pan S, et al. (2011) Genetic variants in HLA-DP/DQ influence both hepatitis B virus clearance and Hepatocellular carcinoma development. Hepatology (in press).
19. Nishida N, Koike A, Tajima A, Ogasawara Y, Ishibashi Y, et al. (2008) Evaluating the performance of Affymetrix SNP Array 6.0 platform. BMC Genomics 9: 431.
20. Miyagawa T, Nishida N, Ohashi J, Kimura R, Fujimoto A, et al. (2008) Appropriate data cleaning methods for genome-wide association study. J Hum Genet 53: 886–893.
21. Nishida N, Tanabe T, Takasu M, Suyama A, Tokunaga K (2007) Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. Anal Biochem 364: 78–85.
22. Nishida N, Mawatari Y, Sageshima M, Tokunaga K (2012) Highly parallel and short-acting amplification with locus-specific primers to detect single nucleotide polymorphisms by the DigiTag2 assay. PLoS ONE 7: e29967.

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No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations

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Abstract

Background: A recent genome-wide association study (GWAS) using chronic HBV (hepatitis B virus) carriers with and without hepatocellular carcinoma (HCC) in five independent Chinese populations found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HCC. In the present study, a total of 580 HBV-derived HCC cases and 1351 individuals with chronic hepatitis B (CHB) or asymptomatic carrier (ASC) were used for replication studies in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results: We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09, 95 % CI = 0.82-1.43; replication 2: OR = 0.79, 95 % CI = 0.54-1.15), in the Korean cohort (replication 3: OR = 0.95, 95 % CI = 0.66-1.36), or in the Hong Kong Chinese cohort (replication 4: OR = 1.17, 95 % CI = 0.79-1.75). Meta-analysis using these cohorts also did not show any associations with P = 0.97.

Conclusions: None of the replication cohorts showed associations between rs17401966 and HBV-derived HCC. This may be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Other reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. A much wider range of investigations is needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Keywords: Hepatitis B, hepatocellular carcinoma, candidate SNP, replication study, genome-wide association study

Background

Hepatitis B (HB) is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), and approximately 360 million people worldwide are thought to be chronically infected with HBV. The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC). Although some HBV carriers spontaneously eliminate the virus, 2-10 % of individuals with chronic HB (CHB) develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or HCC. Around 600,000 new HCC cases are diagnosed annually worldwide, with HCC being relatively common in Asia-Pacific countries and sub-Saharan Africa; more than 70 % of HCC patients are diagnosed in Asia (with 55 % in China) [1]. However, HCC is relatively uncommon in the USA, Europe and Australia [1,2]. The majority of HCC develops in patients with cirrhosis, which is most often attributable

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to chronic HBV infection followed by chronic HCV in the Asia-Pacific region [3].

A recent genome-wide association study (GWAS) using Japanese CHB cases and controls confirmed that 11 SNPs in a region including *HLA-DPA1* and *-DPB1* were associated with CHB [4]. Moreover, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese populations reported that one SNP (rs17401966) in *KIF1B* was associated with HCC susceptibility [5]. In the present study, we performed replication studies using Japanese, Korean and Hong Kong Chinese cases and controls in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results

We performed SNP genotyping of rs17401966 located in the *KIF1B* gene for the purpose of replication analysis of the previous GWAS report [5]. Four distinct cohorts were used for these replication analyses (Table 1). We first examined two independent Japanese case–control samples including 179 cases and 769 controls from Biobank Japan (replication 1), and 142 cases and 251 controls from various hospitals (replication 2). We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09; 95 % CI = 0.82-1.43, replication 2: OR = 0.79; 95 % CI = 0.54-1.15). We further examined Korean case–control samples comprising 164 cases and 144 controls (replication 3) and Hongkongese 94 HCC cases and 187 CHB controls (replication 4), but again did not detect any association (replication 3: OR = 0.95; 95 % CI = 0.66-1.36, replication 4: OR = 1.17; 95 % CI = 0.79-1.75). Logistic regression analysis adjusted for age and gender also did not show any association (P_{log} = 0.65, 0.27, 0.11, 0.56 for each replication panel). Moreover, we conducted meta-analysis to combine these studies, also not detect any association (P_{meta} = 0.97).

Discussion and conclusions

Zhang et al. [5] reported that SNP rs17401966 was significantly associated with HBV-related HCC (joint OR = 0.61). They conducted a GWAS using 348 cases and 359 controls in a population in Guangxi in southern China, and selected 45 SNPs for the replication study based on the results ($P < 10^{-4}$). In the first replication study, they used 276 cases and 266 controls from Beijing in northern China, and 5 SNPs showed the same direction of association as in the GWAS ($P < 0.05$). They performed a further replication study (of 507 cases and 215 controls) in Jiangsu in eastern China and only one SNP showed the same trend ($P = 3.9 \times 10^{-5}$). Guangdong and Shanghai samples from southern and eastern China were used for further replication studies. The association yielded a p-value of 1.7×10^{-18} on meta-analysis.

We performed four replication analyses using Japanese, Korean and Hong Kong Chinese samples (Table 1). Although sample size of each cohort is smaller than that of the previous GWAS, we conducted mete-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC (P_{meta} = 0.97).

This may be due to differences in genetic diversity among Japanese, Korean and Chinese populations. A maximum-likelihood tree of 126 populations based on 19,934 SNPs showed that Japanese and Korean populations form a monophyletic clade with a 100 % bootstrap value [6]. However, Chinese populations form a paraphyletic clade with two other populations. This indicates that Japanese and Korean populations are genetically closer to one another than the Chinese population.

Table 1 Association between rs17401966 and HBV-derived HCC

cohort	sample size	cases			controls				OR		
	(cases/controls)	GG	AG	AA	GG	AG	AA	HWE p	(95 % CI)	P [a]	P_{het} [b]
replication 1	179/769	13	61	105	45	261	463	0.599	1.09	0.578	
(Japan 1)		(7.2)	(34.1)	(58.7)	(5.9)	(33.9)	(60.2)		(0.82-1.43)		
replication 2	142/251	5	46	91	14	91	146	1	0.79	0.212	
(Japan 2)		(3.5)	(32.4)	(64.1)	(5.6)	(36.2)	(58.2)		(0.54-1.15)		
replication 3	164/144	17	59	88	15	55	74	0.616	0.95	0.790	
(Korea)		(10.4)	(36.0)	(53.6)	(10.4)	(38.2)	(51.4)		(0.66-1.36)		
replication 4	94/187	10	39	44	13	80	94	0.767	1.17	0.432	
(Hong Kong)		(10.6)	(41.5)	(46.8)	(6.9)	(42.8)	(50.3)		(0.79-1.75)		
Meta-analysis [c]									0.996	0.965	0.423
									(0.84-1.18)		

[a]P value of fisher's exact test for allele model.
[b]Result of Breslow-Day test.
[c]Results of meta-analysis were calculated by the Mantel-Haenzel method.

We did not find any association with Hong Kong Chinese cohort (P = 0.43). Moreover, a study using 357 HCC cases and 354 HBV-positive non-HCC controls in Hong Kong Chinese did not show any significant difference (P = 0.91) [7]. Previous population studies have revealed that various Han Chinese populations show varying degrees of admixture between a northern Altaic cluster and a southern cluster of Sino-Tibetan/Tai-Kadai populations in southern China and northern Thailand [6]. Although Hong Kong is located closed to the Guangdong (cohort 3 of Zhang et al study), there is great heterogeneity for rs17401966 between Hong Kong cohorts (our study and Chan's study [7]) and Guangdong cohort (our study versus Zhang's study: $P_{het} = 0.0066$; Chan's study versus Zhang's study: $P_{het} = 0.035$). This result suggests the existence of other confounding factors, which can differentiate the previous study in China and this study.

One of the possible reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. HCC development is a multiple process which links to causative factors such as age, gender, environmental toxins, alcohol and drug abuse, higher HBV DNA levels, and HBV genotype variations [8]. The eight HBV genotypes display distinct geographical and ethnic distributions. Genotypes B and C are prevalent in Asia. Specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C), in basal core promoter (BCP) and in ORF encoding Pre-S1/Pre-S2/S and Pre-C/C. Because there is an overlap between Pre-C or BCP mutations and genotypes, these mutations appear to be more common in genotype C as compared to other genotypes [9].

Aflatoxins are a group of 20 related metabolites and Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. Aflatoxin exposures multiplicatively increase the risk of HCC in people chronically infected with HBV, which illustrates the deleterious impact that even low toxin levels in the diet can have on human health [10–12]. Liu and Wu estimated population risk for aflatoxin-induced HCC around the world [13]. Most cases occur in sub-Saharan Africa, Southeast Asia and China, where populations suffer from both high HBV prevalence and largely uncontrolled exposure to aflatoxin in food. But we could not obtain the information of these confounding factors from both of the previous GWAS study and this study. A much wider range of investigations is thus needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Methods

Samples

Case and control samples used in this study were collected from Japan, Korea and Hong Kong listed in supplementary Additional file 1: Table S1. A total of 179 cases and 769 control subjects were analyzed in the first replication study. DNA samples from both CHB controls and HBV-related HCC cases used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo [14]. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guidelines for diagnosis and treatment of chronic hepatisis from The Japan Society of Hepatology (http://www.jsh.or.jp/medical/gudelines/index.html). The mean (and standard deviation; SD) age was 62.0 (9.4) years for the cases and 54.7 (13.5) years for the controls. The second Japanese replication sample sets for the cases (n = 142) and controls (n = 251) study were obtained from 16 hospitals. The case samples for the second replication included 142 HCC patients and the controls included 135 CHB patients and 116 asymptomatic carriers (ASC). The mean (SD) age was 61.3 (10.2) years for the cases and 56.2 (10.9) years for the controls. The Korean replication samples were collected from Yonsei University College of Medicine. The third replication set was composed of 165 HCC patients and 144 CHB patients. The mean (SD) age was 52.2 (8.9) and 37.3 (11.3) years for the cases and controls, respectively. The samples in Hong Kong were collected from the University of Hong Kong, Queen Mary Hospital. The fourth replication set was composed of 94 HCC patients and 187 CHB patients. The mean (SD) age was 58.0 (10.5) and 56.9 (8.3) years for the cases and controls, respectively. All participants provided written informed consent. This research project was approved by the Research Ethics Committees at the Institute of Medical Science and the Graduate School of Medicine, the University of Tokyo, Yonsei University College of Medicine, the University of Hong Kong, Nationa Center for Global Health and Medicine, Hokkaido University Graduate School of Medicine, Teine Keijinkai Hospital, Iwate Medical University, Saitama Medical University, Kitasato University School of Medicine, Musashino Red Cross Hospital, Kanazawa University Graduate School of Medicine, Shinshu University School of Medicine, Nagoya City University Graduate School of Medical Sciences, Kyoto Prefectural University of Medicine, National Hospital Organization Osaka National Hospital, Kawasaki Medical College, Tottori University, Ehime University Graduate School of Medicine, and Kurume University School of Medicine.

SNP Genotyping

For the first replication samples, we genotyped rs17401966 using PCR-based Invader assay (Third Wave Technologies, Madison, WI) [15], and for the second, third and fourth replication samples, we used TaqMan genotyping assay (Applied Biosystems, Carlsbad, CA). In the TaqMan SNP

genotyping assay, PCR amplification was performed in a 5-μl reaction mixture containing 1 μl of genomic DNA, 2.5 μl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 40 x TaqMan SNP Genotyping Assay probe (ABI) for this SNP. QPCR thermal cycling was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP call rate of each replication panel was 100 %, 100 %, 99.7 % and 99.6 %.

Statistical analysis

We performed Hardy-Weinberg equilibrium test for the case and control samples in each replication study. Fisher's exact test was applied to two-by-two contingency tables for three different genetic models; allele frequency, dominant and recessive model. Odds ratios and confidence intervals were calculated using the major alleles as references. Meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. Genotype-phenotype association for the SNP rs17401966 was assessed using logistic regression analysis adjusted for age and gender in plink 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml).

Additional file

Additional file 1: Table S1. Samples used in this study.

Abbreviations

HB: Hepatitis b; HBV: Hepatitis b virus; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis b; HCV: Hepatitis c virus; GWAS: Genome-wide association study; ASC: Asymptomatic carrier.

Competing interests

The authors declare that they have no competing interests.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P: **Global cancer statistics, 2002.** *CA: a cancer journal for clinicians* 2005, **55**(2):74–108.
2. Parkin DM: **Global cancer statistics in the year 2000.** *The lancet oncology* 2001, **2**(9):533–543.
3. Marrero CR, Marrero JA: **Viral hepatitis and hepatocellular carcinoma.** *Archives of medical research* 2007, **38**(6):612–620.
4. Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, Hosono N, Kubo M, Tsunoda T, Kamatani N, Kumada H, *et al*: **A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians.** *Nature genetics* 2009, **41**(5):591–595.
5. Zhang H, Zhai Y, Hu Z, Wu C, Qian J, Jia W, Ma F, Huang W, Yu L, Yue W, *et al*: **Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers.** *Nature genetics* 2010, **42**(9):755–758.
6. Abdulla MA, Ahmed I, Assawamakin A, Bhak J, Brahmachari SK, Calacal GC, Chaurasia A, Chen CH, Chen J, Chen YT *et al*: **Mapping human genetic diversity in Asia.** *Science (New York, NY* 2009, **326**(5959):1541–1545.
7. Chan KY, Wong CM, Kwan JS, Lee JM, Cheung KW, Yuen MF, Lai CL, Poon RT, Sham PC, Ng IO: **Genome-wide association study of hepatocellular carcinoma in Southern Chinese patients with chronic hepatitis B virus infection.** *PLoS One* 2011, **6**(12):e28798.
8. Sherman M: **Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis.** *Semin Liver Dis* 2010, **30**(1):3–16.
9. Yang HI, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, Wang LY, Lu SN, You SL, Chen DS, *et al*: **Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma.** *J Natl Cancer Inst* 2008, **100**(16):1134–1143.
10. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD: **A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China.** *Cancer Epidemiol Biomarkers Prev* 1994, **3**(1):3–10.
11. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE: **Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma.** *Lancet* 1992, **339**(8799):943–946.
12. Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, Wu MH, Wu WP, Wang LW, Wang Q, *et al*: **Aflatoxin exposure and risk of hepatocellular carcinoma in Taiwan.** *International journal of cancer* 1996, **67**(5):620–625.
13. Liu Y, Wu F: **Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment.** *Environmental health perspectives* 2010, **118**(6):818–824.
14. Nakamura Y: **The BioBank Japan Project.** *Clin Adv Hematol Oncol* 2007, **5**(9):696–697.
15. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y: **A high-throughput SNP typing system for genome-wide association studies.** *Journal of human genetics* 2001, **46**(8):471–477.

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Original Article

Long-term outcomes of add-on adefovir dipivoxil therapy to ongoing lamivudine in patients with lamivudine-resistant chronic hepatitis B

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Aim: Add-on adefovir dipivoxil (ADV) therapy has been a standard rescue treatment for patients with lamivudine (LAM)-resistant chronic hepatitis B, but the overall benefits of long-term add-on ADV therapy are still limited. The aim of this study was to evaluate the long-term efficiency of add-on ADV treatment and to explore predictive factors associated with it.

Methods: A total of 158 patients with LAM-resistant chronic hepatitis B were included in this retrospective, multicenter, nationwide study in Japan. After confirming LAM resistance, ADV was added to LAM treatment. Three types of events were considered as outcomes: virological response, hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization. Virological response was defined as serum hepatitis B virus (HBV) DNA levels of less than 3 log copies/mL. Baseline factors contributing to these outcomes were examined by univariate and multivariate analyses.

Results: The median total duration of ADV treatment was 41 months (range, 6–84). The rate of virological response was 90.8% at 4 years of treatment; HBeAg clearance and ALT normalization were achieved by 34.0% and 82.7%, respectively, at the end of follow up. Each outcome had different predictive factors: baseline HBV DNA and albumin level were predictive factors for virological response, history of interferon therapy and ALT level for HBeAg clearance, and sex and baseline albumin level for ALT normalization.

Conclusion: Long-term add-on ADV treatment was highly effective in LAM-resistant chronic hepatitis B patients in terms of virological and biochemical responses. Lower HBV replication and lower albumin level at baseline led to better outcomes.

Key words: adefovir dipivoxil, alanine aminotransferase normalization, chronic hepatitis B, hepatitis B e antigen clearance, lamivudine resistance, virological response

INTRODUCTION

CHRONIC HEPATITIS B (CHB) is an important cause of morbidity and mortality worldwide.[1–3] The main goals of therapy in CHB patients are to prevent the development of liver failure, due to subsequent liver

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cirrhosis, and the emergence of hepatocellular carcinoma (HCC). All of these are likely be achieved by suppressing hepatitis B virus (HBV) replication, which thereby leads to remission of liver disease.[4]

Lamivudine (LAM) treatment has been used to prevent the progression of CHB and the development of HCC.[5] LAM is an effective and well-tolerated treatment for patients with CHB, but it has the major limitation of drug-resistant mutants arising at a rate of 16–32% during the first year of treatment and increasing by 15% with each additional year of treatment.[6–8] The widespread use of LAM monotherapy in CHB patients before introduction of entecavir, which is more potent, has progressively increased the numbers of patients with LAM-resistant HBV mutant strains.

Adefovir dipivoxil (ADV) has been reported to be effective in suppressing HBV replication and approved as a standard therapy in LAM-resistant patients.[9,10] However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients are still limited. The aims of this study were to evaluate the long-term efficiency of ADV add-on treatment based on virological response (VR), hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization, and to explore the predictive factors associated with ADV add-on treatment.

METHODS

Patients

A TOTAL OF 158 patients (109 males and 49 females) were included in this retrospective study from 21 medical centers of the National Hospital Organization (NHO) in Japan. Both HBeAg positive and negative CHB patients were considered eligible if they had documented LAM resistance confirmed by detection of mutations in the YMDD motif of the reverse transcriptase gene of the virus (genotypic resistance), elevated serum HBV DNA levels (≥4 log copies/mL and/or >1 log copies/mL elevation from the LAM on-treatment nadir) and/or elevated serum ALT levels (>40 IU/L). Patients were excluded if they had decompensated liver cirrhosis, HCC at the initiation of ADV, or if they had co-infections (human immunodeficiency virus, hepatitis C virus) or other concomitant liver diseases such as autoimmune liver disease. Patients with no available clinical, biochemical, serological or virological data at baseline as well as every 6 months during treatment were also excluded.

Patient records were extracted from each institutional database. All data were labeled with their respective institution and pooled. In total, 20 variables were examined to evaluate the long-term responses. The following variables were used as baseline factors: sex, HBeAg status, liver disease, age, body mass index, duration of LAM monotherapy, history of interferon (IFN) therapy, serum HBV DNA level, aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase (γ-GTP), platelet (PLT) counts, and total bilirubin (T-Bil), albumin (Alb), prothrombin time (PT) and α-fetoprotein (AFP) levels. All were measured at the initiation of ADV therapy. For each variable, it was not used in the stepwise analysis if missing data accounted for more than 10% of the cases.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients and approval of this study was obtained from the NHO.

Statistical analysis

Three types of events were considered as outcomes: (i) VR; (ii) HBeAg clearance; and (iii) ALT normalization. VR was defined as serum HBV DNA levels of less than 3 log copies/mL by a quantitative real-time polymerase chain reaction assay, and ALT normalization was defined as a decrease in ALT levels to less than 31 IU/L during the on-treatment follow-up period. Baseline factors that could have an impact in the prediction of VR, HBeAg clearance as well as ALT normalization were investigated. The predictive value of several baseline parameters for VR was evaluated using time-to-event methods, because of the varying length of follow up. Time-to-event analysis was carried out using Kaplan–Meier estimates to draw cumulative incidence curves, compared by log–rank tests, as well as using univariate and multivariate Cox's proportional hazards models in combination with stepwise regression analysis. Factors contributing to HBeAg clearance and ALT normalization during ADV add-on therapy were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A stepwise variable selection procedure was used for variables that were at least marginally associated with the outcomes.

Covariates included in these analyses were binomial or continuous variables. Quartile analysis was initially performed separately for each continuous variable to make the decision regarding cut-off points. At first, we divided each continuous data into quarters to convert numerical values into four categorical values. Then, we estimated whether there was a regular trend among these four ordinal categorical data with outcome and selected a cut-off point among the 25th, 50th and 75th percentiles so that these variables could be appropriately

Table 1 Baseline characteristics at the initiation of add-on ADV therapy in LAM-resistant CHB patients based on HBeAg status

Baseline characteristics	HBeAg positive $n = 99$	HBeAg negative $n = 59$
Age (years)	51.6 (25.5–80.4)	59.3 (33.3–76.9)
Sex (male/female)	73/26	36/23
Liver disease (CH/cirrhosis)	79/20	38/21
Duration of LAM therapy (months)	29.8 (6.0–82.4)	39.3 (8.4–91.2)
History of IFN therapy (months)	39	15
HBV DNA (log copies/mL)	7.5 (2.1–7.6)	5.9 (2.1–7.6)
≤6	15	31
6–7.5	38	21
>7.5	46	7
Total bilirubin (mg/dL)	0.8 (0.3–5.2)	0.9 (0.41–3.7)
AST (IU/L)	60 (18–959)	60 (17–464)
ALT (IU/L)	80 (11–697)	86 (17–724)
γ-GTP (IU/L)	38 (12–325)	53 (10–740)
Albumin (g/dL)	4.3 (2.6–5.4)	4.3 (2.7–5.2)
Platelet count ($\times 10^4/mm^3$)	15.5 (3.7–50.0)	12.3 (1.7–33.2)

Continuous variables are expressed in median (range) and categorized variables in number.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; γ-GTP, γ-glutamyl transpeptidase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LAM, lamivudine.

dichotomized. The hazards ratio (HR) and the odds ratio (OR) are presented with 95% confidence intervals (CI) and *P*-values, with less than 0.05 being considered statistically significant. All data analyses were processed using the R statistical software ver. 2.13.

RESULTS

IN THIS RETROSPECTIVE nationwide analysis of add-on ADV therapy in Japan, a total of 158 patients were enrolled from 2003–2010, consisting of 99 HBeAg positive and 59 HBeAg negative patients. Table 1 summarizes the baseline characteristics of the study population; most were HBV genotype C. At the time of this analysis, the median total duration of ADV treatment was 41 months (range, 6–84), and the median time of LAM monotherapy, prior to initiation of ADV, was 34 months (range, 6–91).

VR

Figure 1 shows a Kaplan–Meier curve displaying the cumulative probability of VR based on HBV DNA levels among HBeAg positive and negative patients. Patients with a lower HBV DNA level displayed earlier VR than those with a higher HBV DNA level among both HBeAg positive and negative patients ($P < 0.001$, $P = 0.002$,

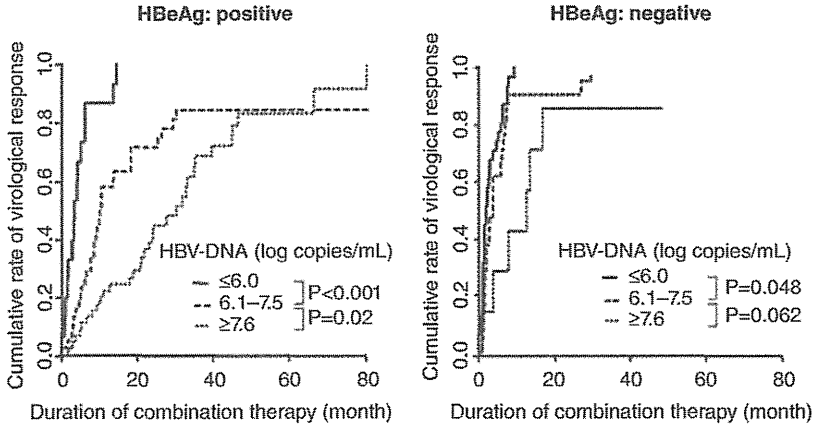

Figure 1 Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response compared with HBeAg positivity or higher HBV replication. —, ≤6.0; ---, 6.1–7.5; ····, ≥7.6.

Table 2 Univariate and multivariate Cox's regression analysis of predictors of virological response

Variable	HBeAg positive $n = 99$				HBeAg negative $n = 59$	
	Univariate		Multivariate		Univariate	
	HR	*P*-value	HR	*P*-value	HR	*P*-value
Age (years) (<45/45≤)	0.91	0.69			0.66	0.34
Sex (male/female)	1.07	0.86			0.71	0.21
Liver disease (CH/cirrhosis)	0.61	0.069			1	0.99
Duration of LAM therapy (months) (<34/34≤)	0.92	0.76			1.72	0.076
History of IFN therapy (−/+)	0.83	0.43			0.89	0.73
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.28	<0.001	<0.001	<0.001	0.44	0.012
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.66	0.067	1.73	0.06	1.54	0.13
AST (IU/L) (<100/100≤)	1.57	0.061			1.11	0.71
ALT (IU/L) (<130/130≤)	1.51	0.085			1.05	0.87
γ-GTP (IU/L) (<70/70≤)	1.53	0.113			1.33	0.3
Albumin (g/dL) (<4.1/4.1≤)	0.51	0.011	0.48	0.0065	1.41	0.32
Platelet count ($\times 10^4/mm^3$) (<15/15≤)	0.93	0.77			1.1	0.74

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

respectively; log–rank test). HBeAg negative patients displayed higher VR rates than HBeAg positive patients at month 12 (89.9% vs 45.5%), month 24 (95.0% vs 61.5%), month 36 (98.4% vs 79.6%) and month 48 (98.4% vs 86.4%) of treatment. Even at a higher HBV DNA level (HBV DNA ≥7.0 log copies/mL), HBeAg negative patients displayed more rapid VR than HBeAg positive patients ($P < 0.001$). Seven patients did not achieve VR during the 4-year treatment, and one HBeAg positive patient developed ADV-resistant mutations without VR at month 44 of treatment. According to the results of the univariate Cox regression model, HBV DNA level and Alb level were associated with VR in HBeAg positive patients, while only the HBV DNA level was in HBeAg negative patients (HR = 0.44, 95% CI = 0.24–0.84, $P = 0.012$). In multivariate analysis, both lower HBV DNA level and lower Alb level were independent predictive factors associated with VR in HBeAg positive patients (HR = 0.26, 0.48, 95% CI = 0.15–0.44, 0.28–0.81, $P < 0.001$, $P = 0.0065$, respectively) (Table 2), while only the HBV DNA level was selected by a stepwise analysis for HBeAg negative patients.

HBeAg clearance or HBeAg seroconversion

Among 99 HBeAg positive patients, HBeAg clearance and seroconversion were achieved by 17.1% and 11.0% at month 24, by 24.3% and 14.3% at month 36 of treatment, and by 34.0% and 16.0% by the end of follow up, respectively. Except for a history of IFN therapy (OR = 2.46, 95% CI = 0.94–6.6, $P = 0.047$), none of the other baseline variables were significantly associated with HBeAg clearance, according to the results of the univariate logistic regression analysis. In multivariate analysis, serum ALT level and history of IFN therapy were independent predictive factors for HBeAg clearance (Table 3). No patient experienced a reappearance of HBeAg or reverse seroconversion to HBeAg positive status during this treatment.

Normalization of ALT levels

The mean ALT level declined from 138.2 to 24.7 IU/L by add-on ADV therapy. Furthermore, addition of ADV to LAM-resistant CHB led to normalization of ALT levels in 75.2%, 79.5% and 82.7% of the patients at months 24 and 36, and at the final follow up, respectively. We next estimated the predictive factors for ALT normalization. Univariate logistic regression analysis revealed that only the baseline Alb level was significantly related to the ALT normalization. In the multivariate model, female patients (OR = 0.19, $P = 0.037$) and lower Alb level (OR = 0.19, $P = 0.0017$) were found to be independent predictors of ALT normalization.

DISCUSSION

ADD-ON ADV therapy has been a standard rescue treatment for patients with LAM-resistant HBV, but the overall benefits of long-term add-on ADV therapy

Table 3 Univariate and multivariate logistic regression analysis of predictors of HBeAg clearance and ALT normalization

Variable	HBeAg loss, $n = 99$				ALT normalization			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value
Age (years) (<45/45≤)	0.42	0.065			0.94	0.85		
Sex (male/female)	3.02	0.075	2.99	0.081	0.4	0.34	0.19	0.037
Liver disease (CH/cirrhosis)	0.76	0.59			0.54	0.73		
Duration of LAM therapy (months) (<34/34≤)	1.1	0.97			0.59	0.39		
History of IFN therapy (−/+)	2.46	0.047	2.67	0.041	1.2	0.78		
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.49	0.15			0.32	0.21		
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.03	0.83			1.83	0.72		
AST (IU/L) (<100/100≤)	1.52	0.47			3.99	0.075		
ALT (IU/L) (<130/130≤)	2.44	0.061	2.74	0.043	3.71	0.13		
γ-GTP (IU/L) (<70/70≤)	2.16	0.17			1.29	0.98		
Albumin (g/dL) (<4.4/4.4≤)	0.9	0.99			0.17	0.0047	0.19	0.0017
Platelet count ($\times 10^4/mm^3$) (<15/15≤)	1.21	0.82			0.52	0.39		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

have not yet been fully assessed. In this multicenter study of 158 patients from 21 hospitals over a mean follow-up period of 43.5 months, we tried to evaluate the long-term efficacy of add-on ADV therapy to LAM-resistant patients, and also to investigate which baseline factors were associated with VR, HBeAg clearance and ALT normalization. We found long-term add-on ADV treatment produced long-term virological and biochemical improvement. In addition, each outcome had different predictive factors; baseline HBV DNA and Alb level were predictive factors for VR in HBeAg positive patients, history of IFN therapy and ALT level for HBeAg clearance, and sex and Alb level for ALT normalization.

The rate of VR was 90.8% at 4 years of treatment. The strongest predictive factor for VR in both HBeAg positive and negative patients were confirmed by previous observations showing that add-on ADV therapy achieves more rapid and higher rates of VR when ADV is initiated in LAM-resistant patients with low viral replication levels.[11–17] We also found that lower Alb level was an independent predictive factor for VR in HBeAg positive patients. In fact, baseline Alb correlated with PLT counts ($r = 0.51$, $P < 0.001$) and T-Bil ($r = -0.38$, $P < 0.001$), indicating that a lower Alb level reflected progression of liver disease. Little attention has been given to the relation of Alb level with VR – further studies will be needed to confirm our findings and understand its underlying mechanisms – but progression of chronic hepatitis might be predictive of VR under the add-on ADV treatment. This is the first report to show the significance of baseline Alb levels as we used a time-to-event method for large populations, which is a more powerful and informative method to assess the association of factors to time-to-event outcomes.

The rate of HBeAg clearance was 34% at the end of follow up, which was compatible with previous observations.[10,18] According to the results of multivariate analysis, IFN history was the strongest predictor of HBeAg clearance. Of the 37 patients, 17 (46%) who had previously received IFN therapy achieved HBeAg loss, suggesting that previous IFN therapy might have some immune modulatory effect on the ongoing combination therapy. IFN-induced HBeAg loss has been reported to be durable after a follow-up period of 4–8 years.[19–21] In addition, baseline ALT levels were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline ALT levels to be the strongest predictor of HBeAg seroconversion in response to IFN therapy[22] as well as nucleos(t)ide analog therapy.[23,24]

Alanine aminotransferase normalization was achieved in 82.7% of the patients. ALT normalization and VR were independent of each other. Actually, among 24 patients who did not achieve ALT normalization, only seven had not achieved VR, suggesting that ALT elevation after sustained suppression of HBV replication might be associated with some conditions other than CHB. In addition, lower baseline Alb was revealed