Discussion

This investigation is the first report on the impact of the DNA polymerase delta catalytic main subunit p125 on the invasive potential of cancer cells in tumor progression. DNA polymerase delta is an essential agent of DNA replication [1] and is therefore crucial for tumor and somatic cell replication. Li and Lee [10] described the transcriptional regulation of *POLD1* in relation to mutant p53. However, no further investigation was reported for some time. In this study, we found that the p125 induced by mutant p53 plays an important role in tumor invasion and contributes to the cellular differentiation of human HCC. Those findings were limited to the early stage of HCC and not necessarily to more advanced stages where those findings may differ, so further investigation was needed.

Wild-type p53 is generally not detected by immunohistochemical staining because of its short half-life. Mutations of the p53 gene may induce conformational changes that stabilize the protein, resulting in the accumulation of nuclear p53 that is detectable by immunohistochemical staining [22, 23]. p53 mutation analysis revealed that immunohistochemical staining of p53-positive cases had a significantly high p53 mutation rate compared with p53-negative cases. Immunohistochemical staining of p53 was therefore thought to be an abnormal protein that is induced by p53 mutation. In our study, immunohistochemical staining revealed a relationship between p125 and p53 expression; especially in heterozygously stained multiple nodular-type HCC, the staining pattern of p53 was the same as that of p125. This is consistent with some previous findings in which a promoter assay of *POLD1* showed that it was suppressed by wild-type p53 or activated by a mutant type of p53 that blocks the binding of wild-type p53 to a specific sequence [10]. These results suggest that p125 expression in HCC is regulated by transcription induced by abnormal p53. A correlation between the expression profile of p53 and the degree of differentiation has been reported [20, 37]. These reports support our finding that p125 might contribute to tumor cell dedifferentiation. The amount of p125 correlated with mRNA levels, suggesting that p125 overexpression in HCC is regulated by transcription.

We performed further investigations using HCC cell lines to investigate whether cellular invasiveness is regulated by p125 expression. There was an inverse correlation between p125 and p53 expression in Huh7, HepG2, and Hep3B cells. We tried to reveal the alteration of the invasive potential of Huh-7 by wild-type p53 stable transfection. Despite repeated transfection experiments, however, we could not establish a sufficient number of wild-type p53 stably transfected Huh-7. Both wild- and mutant-type p53 were detected by mouse monoclonal antibody DO-7 in cell lysates [38]. Huh7 cells have a p53 mutation at codon 220 that induces abnormal p53 [29]. However, not all genes suppressed by wild-type p53 were upregulated in Huh7 cells [39]. Different p53 mutations could have different capacities to suppress target genes. p125 expression might not be affected by p53 mutations in Huh7 cells; hence, p125 may be normally suppressed by p53 in Huh7 cells. The invasive potential of HCC cell lines correlated with p125 expression. Furthermore, the exogenous mutant of p53 induced p125 and increased the invasive potential of HepG2 cells. Inversely, p125 expression and cellular invasive potential were reduced in the RNA interference assay. These findings were supported by immunohistochemical results showing that HCC with high p125 levels had a high rate of venous invasion and p53 overexpression.

The *POLD1* promoter has consensus sequences at the AP-1 and E2F binding sites. E2F is thought to be important for the promoter activity of *POLD1* [9]. Transcriptional activation by E2F is directly inhibited by the tumor suppressor gene pRB resulting from E2F-RB complex formation [40]. Therefore, p125 also might play an important role in tumor progression related to pRB-dependent carcinogenesis.

In conclusion, we demonstrated that p125 expression is upregulated in HCC characterized by venous invasion, poor cellular differentiation, and a poor prognosis. We also showed that the invasive potential of HCC cell lines increased with p125 expression induced by abnormal p53. Our results suggest that p125 induced by p53 mutation plays an important role in tumor invasion and contributes to the cellular differentiation of human HCC. The p125 subunit of the DNA polymerase catalytic domain is therefore a potential therapeutic target for human HCC.

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Disclosure Statement

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References

1 Hubscher U, Maga G, Spadari S: Eukaryotic DNA polymerases. Annu Rev Biochem 2002; 71:133–163.
2 Johnson A, O'Donnell M: Cellular DNA replicases: components and dynamics at the replication fork. Annu Rev Biochem 2005;74: 283–315.
3 Lydeard JR, Jain S, Yamaguchi M, Haber JE: Break-induced replication and telomerase-independent telomere maintenance require pol32. Nature 2007;448:820–823.
4 Maloisel L, Fabre F, Gangloff S: DNA polymerase delta is preferentially recruited during homologous recombination to promote heteroduplex DNA extension. Mol Cell Biol 2008;28:1373–1382.
5 Kunkel TA, Bebenek K: DNA replication fidelity. Annu Rev Biochem 2000;69:497–529.
6 Pavlov YI, Shcherbakova PV, Rogozin IB: Roles of DNA polymerases in replication, repair, and recombination in eukaryotes. Int Rev Cytol 2006;255:41–132.
7 Garg P, Burgers PM: DNA polymerases that propagate the eukaryotic DNA replication fork. Crit Rev Biochem Mol Biol 2005;40: 115–128.
8 Liu L, Mo J, Rodriguez-Belmonte EM, Lee MY: Identification of a fourth subunit of mammalian DNA polymerase delta. J Biol Chem 2000;275:18739–18744.
9 Zhao L, Chang LS: The human POLD1 gene: identification of an upstream activator sequence, activation by sp1 and sp3, and cell cycle regulation. J Biol Chem 1997;272: 4869–4882.
10 Li B, Lee MY: Transcriptional regulation of the human DNA polymerase delta catalytic subunit gene POLD1 by p53 tumor suppressor and sp1. J Biol Chem 2001;276:29729–29739.
11 Harris CC: P53 tumor suppressor gene: from the basic research laboratory to the clinic – an abridged historical perspective. Carcinogenesis 1996;17:1187–1198.
12 el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1992;1:45–49.
13 El-Serag HB, Rudolph KL: Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132: 2557–2576.
14 Kubo S, Tanaka H, Takemura S, Yamamoto S, Hai S, Ichikawa T, Kodai S, Shinkawa H, Shuto T, Hirohashi K: Surgical treatment for hepatocellular carcinoma detected after successful interferon therapy. Surg Today 2007; 37:285–290.
15 Shirabe K, Takenaka K, Taketomi A, Kawahara N, Yamamoto K, Shimada M, Sugimachi K: Postoperative hepatitis status as a significant risk factor for recurrence in cirrhotic patients with small hepatocellular carcinoma. Cancer 1996;77:1050–1055.
16 Sanefuji K, Kayashima H, Iguchi T, Sugimachi K, Yamashita Y, Yoshizumi T, Soejima Y, Nishizaki T, Taketomi A, Maehara Y: Characterization of hepatocellular carcinoma developed after achieving sustained virological response to interferon therapy for hepatitis C. J Surg Oncol 2009;99:32–37.
17 Ueno M, Uchiyama K, Ozawa S, Nakase T, Togo N, Hayami S, Yamaue H: Prognostic impact of treatment modalities on patients with single nodular recurrence of hepatocellular carcinoma. Surg Today 2009;39:675–681.
18 Qin LX, Tang ZY, Ma ZC, Wu ZQ, Zhou XD, Ye QH, Ji Y, Huang LW, Jia HL, Sun HC, Wang L: P53 immunohistochemical scoring: an independent prognostic marker for patients after hepatocellular carcinoma resection. World J Gastroenterol 2002;8:459–463.
19 Caruso ML, Valentini AM: Overexpression of p53 in a large series of patients with hepatocellular carcinoma: a clinicopathological correlation. Anticancer Res 1999;19:3853–3856.
20 Hsu HC, Tseng HJ, Lai PL, Lee PH, Peng SY: Expression of p53 gene in 184 unifocal hepatocellular carcinomas: association with tumor growth and invasiveness. Cancer Res 1993;53:4691–4694.
21 Ng IO, Lai EC, Chan AS, So MK: Overexpression of p53 in hepatocellular carcinomas: a clinicopathological and prognostic correlation. J Gastroenterol Hepatol 1995;10: 250–255.
22 Iggo R, Gatter K, Bartek J, Lane D, Harris AL: Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 1990;335:675–679.
23 Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B, Hamilton SR: An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. J Pathol 1994;172:5–12.
24 Tanaka S, Toh Y, Adachi E, Matsumata T, Mori R, Sugimachi K: Tumor progression in hepatocellular carcinoma may be mediated by p53 mutation. Cancer Res 1993;53:2884–2887.
25 Goldsby RE, Hays LE, Chen X, Olmsted EA, Slayton WB, Spangrude GJ, Preston BD: High incidence of epithelial cancers in mice deficient for DNA polymerase delta proofreading. Proc Natl Acad Sci USA 2002;99: 15560–15565.
26 Sigurdson AJ, Hauptmann M, Chatterjee N, Alexander BH, Doody MM, Rutter JL, Struewing JP: Kin-cohort estimates for familial breast cancer risk in relation to variants in DNA base excision repair, BRCA1 interacting and growth factor genes. BMC Cancer 2004;4:9.
27 Weber F, Shen L, Fukino K, Patocs A, Mutter GL, Caldes T, Eng C: Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. Am J Hum Genet 2006;78:961–972.
28 Venkatesan RN, Treuting PM, Fuller ED, Goldsby RE, Norwood TH, Gooley TA, Ladiges WC, Preston BD, Loeb LA: Mutation at the polymerase active site of mouse DNA polymerase delta increases genomic instability and accelerates tumorigenesis. Mol Cell Biol 2007;27:7669–7682.
29 Hsu IC, Tokiwa T, Bennett W, Metcalf RA, Welsh JA, Sun T, Harris CC: P53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. Carcinogenesis 1993;14:987–992.
30 Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, Ozturk M: Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:1973–1977.
31 May E, Jenkins JR, May P: Endogenous HeLa p53 proteins are easily detected in HeLa cells transfected with mouse deletion mutant p53 gene. Oncogene 1991;6:1363–1365.
32 Liver Cancer Study Group of Japan: General Rules for the Clinical and Pathological Study of Primary Liver Cancer, ed 2. Tokyo, Kanehara, 2003.
33 Itoh S, Maeda T, Shimada M, Aishima S, Shirabe K, Tanaka S, Maehara Y: Role of expression of focal adhesion kinase in progression of hepatocellular carcinoma. Clin Cancer Res 2004;10:2812–2817.
34 Scheffner M, Takahashi T, Huibregtse JM, Minna JD, Howley PM: Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. J Virol 1992;66:5100–5105.
35 Vogelstein B, Kinzler KW: p53 function and dysfunction. Cell 1992;70:523–526.
36 Varnavski AN, Young PR, Khromykh AA: Stable high-level expression of heterologous genes in vitro and in vivo by noncytopathic DNA-based kunjin virus replicon vectors. J Virol 2000;74:4394–4403.
37 Donato MF, Arosio E, Del Ninno E, Ronchi G, Lampertico P, Morabito A, Balestrieri MR, Colombo M: High rates of hepatocellular carcinoma in cirrhotic patients with high liver cell proliferative activity. Hepatology 2001;34:523–528.
38 Vojtesek B, Bartek J, Midgley CA, Lane DP: An immunochemical analysis of the human nuclear phosphoprotein p53: new monoclonal antibodies and epitope mapping using recombinant p53. J Immunol Methods 1992; 151:237–244.
39 Vikhanskaya F, Lee MK, Mazzoletti M, Broggini M, Sabapathy K: Cancer-derived p53 mutants suppress p53-target gene expression – potential mechanism for gain of function of mutant p53. Nucleic Acids Res 2007;35:2093–2104.
40 Harbour JW, Dean DC: Rb function in cell-cycle regulation and apoptosis. Nat Cell Biol 2000;2:E65–E67.

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A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population

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Hepatitis B virus (HBV) infection is a major health issue worldwide which may lead to hepatic dysfunction, liver cirrhosis and hepatocellular carcinoma. To identify host genetic factors that are associated with chronic hepatitis B (CHB) susceptibility, we previously conducted a two-stage genome-wide association study (GWAS) and identified the association of *HLA-DP* variants with CHB in Asians; however, only 179 cases and 934 controls were genotyped using genome-wide single nucleotide polymorphism (SNP) arrays. Here, we performed a second GWAS of 519 747 SNPs in 458 Japanese CHB cases and 2056 controls. After adjustment with the previously identified variants in the *HLA-DP* locus (rs9277535), we detected strong associations at 16 loci with *P*-value of $<5 \times 10^{-5}$. We analyzed these loci in three independent Japanese cohorts (2209 CHB cases and 4440 controls) and found significant association of two SNPs (rs2856718 and rs7453920) within the *HLA-DQ* locus (overall *P*-value of 5.98×10^{-28} and 3.99×10^{-37}). Association of CHB with SNPs rs2856718 and rs7453920 remains significant even after stratification with rs3077 and rs9277535, indicating independent effect of *HLA-DQ* variants on CHB susceptibility (*P*-value of 1.52×10^{-21}– 2.38×10^{-30}). Subsequent analyses revealed *DQA1*0102-DQB1*0604* and *DQA1*0101-DQB1*0501* [odds ratios (OR) =0.16, and 0.39, respectively] as protective haplotypes and *DQA1*0102-DQB1*0303* and *DQA1*0301-DQB1*0601* (OR = 19.03 and 5.02, respectively) as risk haplotypes. These findings indicated that variants in antigen-binding regions of *HLA-DP* and *HLA-DQ* contribute to the risk of persistent HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of infectious liver diseases, and about 400 million people are suffering from chronic viral infection worldwide. Routes of infection include vertical transmission during neonatal period and horizontal transmission in childhood (bites, lesions and sanitary habits) or adulthood (sexual contact, drug use and medical exposure). In Japan, most of the chronic hepatitis B (CHB) patients were infected through vertical transmission and become HBV carrier (1). Nearly 90% of the HBV carrier will clear HBV (negative for HBsAg and positive for HBc ab) during adolescence, and only 10% of the HBV carrier indicate persistent liver dysfunction and develop chronic hepatitis (2). CHB dramatically increases the risk to progress to liver cirrhosis and hepatocellular carcinoma over a period of several decades (3,4). Currently, CHB is a serious public health problem worldwide, however pathogenesis of HBV-related diseases still remains elusive.

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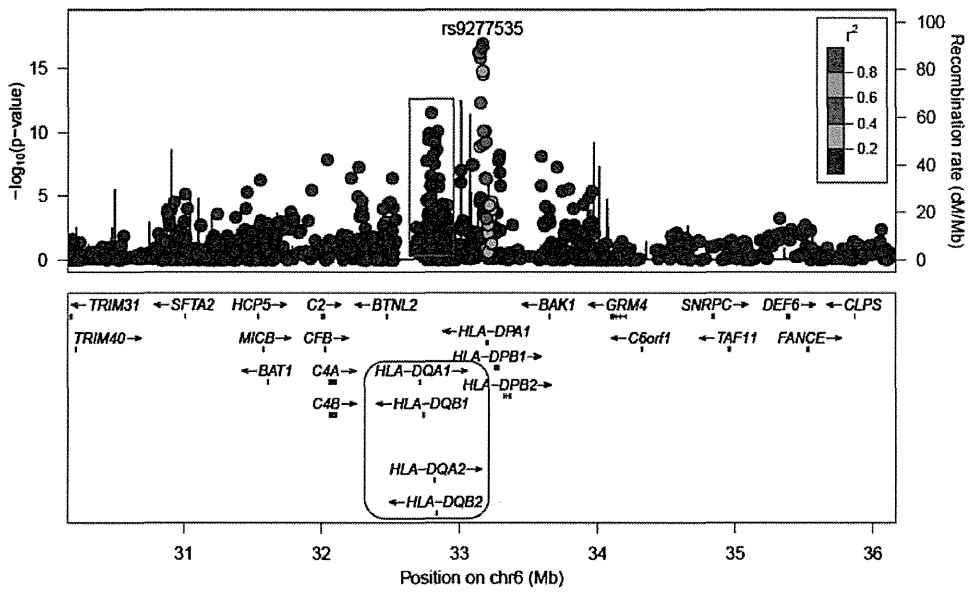

Figure 1. Signal of association with CHB in the *HLA* region of the GWAS stage. This figure shows the regional plots of the negative decadic logarithm trend *P*-values in a ~ 3000 kb window centered on the association peak, located at rs9277535 in *HLA-DPB1*. The top panel shows all SNPs in this region plotted according to the significance of their association with CHB and color coded according to their LD (r^2) with the most significant SNP, rs9277535 (see right corner of the plot). Vertical blue lines indicate local recombination rate. The bottom panel shows the genes in the region. The strongest signal on 6p21.32 localizes to *HLA-DP* genes and the second strongest signal localizes to *HLA-DQ* genes.

In addition to the viral and environmental factors, host genetic factors are considered to govern the pathology of disease development, progression or regression. Genetic epidemiological studies provide robust evidence that genetic variations contribute to progression from acute to chronic hepatitis (5). In 2009, our group conducted a genome-wide association study (GWAS) in the Asian population and identified a strong association of CHB with variants in the *HLA-DP* genes (6). In addition to our report, several association studies have suggested that genetic factors such as *HLA* (7–9), cytokines (10–12) and immune response-related genes (13–15) could influence the outcomes of HBV infection. However, these susceptibility loci were not identified in our previous study probably due to smaller sample size or smaller phenotypic effects of these loci. Here we conducted a second GWAS in the Japanese population to identify new susceptibility loci for CHB by increasing the number of samples in the screening stage from 179 cases and 934 controls to 458 case and 2056 controls.

RESULTS

We performed a two-stage GWAS followed by two independent replications as described in the Supplementary Material, Figure S1. In the GWAS stage, we genotyped 458 Japanese patients with CHB and 2056 control individuals using Illumina gene chip and obtained the genotyping results of 423 627 single nucleotide polymorphisms (SNPs) after quality control (QC). Examination of the quantile–quantile plots of the GWAS stage indicated no evidence for inflation of the test statistics, which could occur in the presence of population substructure ($\lambda = 1.028$) and also revealed an enrichment of significant *P*-values, suggesting the possible existence of candidates (Supplementary Material, Fig. S2A). The results of genome-wide association analysis are represented in Supplementary Material, Table S2, where a total of 34 SNPs in the major histocompatibility complex (MHC) region satisfied the genome-wide significance level ($P < 5.0 \times 10^{-8}$). We also found 54 SNPs (40 in the MHC region and 14 in the non-MHC region) with suggestive associations ($P < 5.0 \times 10^{-5}$) (Supplementary Material, Fig. S2B and Tables S2 and S3). We confirmed the most significant association at the *HLA-DP* locus as described in our previous report (rs9277535 and rs3077, $P = 3.72 \times 10^{-17}$ and 1.28×10^{-16}, respectively) (6) and found another significant peak around the *HLA-DQ* locus which is located ~300 kb telomeric to the *HLA-DP* locus (Fig. 1). To identify SNPs that are associated with CHB independently from *HLA-DP* SNPs, we conducted the association analysis after adjustment for a top SNP in the *HLA-DP* locus (rs9277535) using a logistic regression model (Fig. 2). As a result, five SNPs in the MHC region indicated suggestive associations ($P < 5.0 \times 10^{-5}$) even after stratification with rs9277535. Finally, 5 SNPs in the MHC region and 11 SNPs in the non-MHC region were selected for further analysis (Supplementary Material, Table S4).

Subsequently, we analyzed these 16 SNPs in the first replication set consisting of 606 cases and 2022 controls and found 2 SNPs within the MHC region [rs2856718, $P = 1.6 \times 10^{-5}$, odds ratios (OR) =1.33; rs7453920, $P = 5.72 \times 10^{-4}$, OR = 1.43] to be significantly associated with CHB after stratification for rs9277535 ($P_{corrected} < 3.0 \times 10^{-3}$, Supplementary Material, Table S5). The SNP rs2856718 is located in the intergenic region between *HLA-DQA2* and *HLA-DQB1*, while rs7453920 is located in intron 1 of *HLA-DQB2*

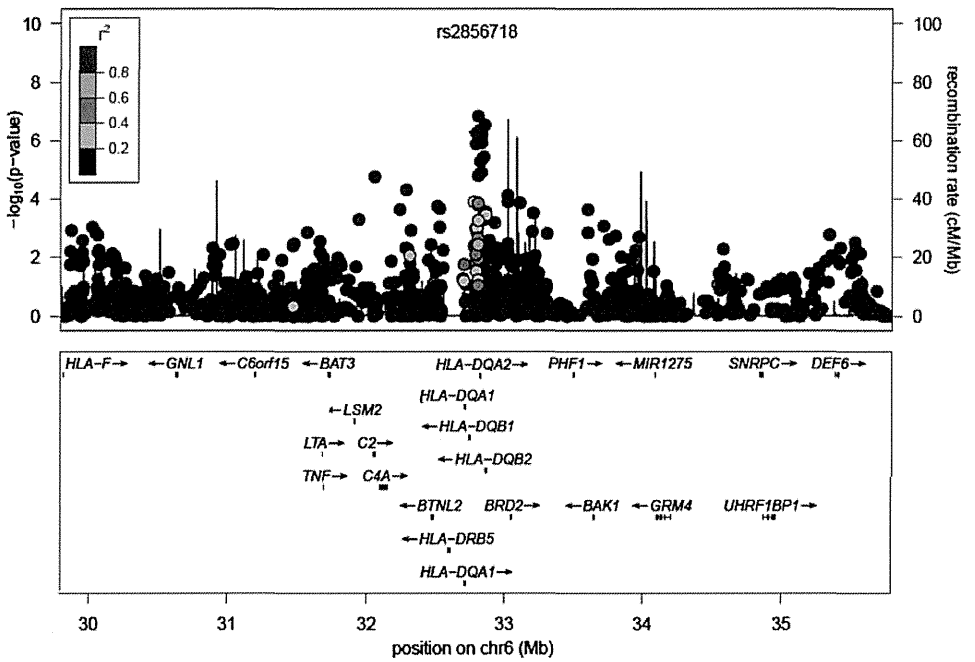

Figure 2. Regional association plot of the 6p21.32 locus after adjustment for the top SNP (rs9277535) in the *HLA-DP* locus in the GWAS stage. This figure shows the evidence of independent association with CHB based on logistic regression analysis. Only one strong peak remained after adjustment for rs9277535. This peak, represented by three top SNPs: rs3892710, rs7453920 and rs2856718, is located in the *HLA-DQ* locus (6p21.32).

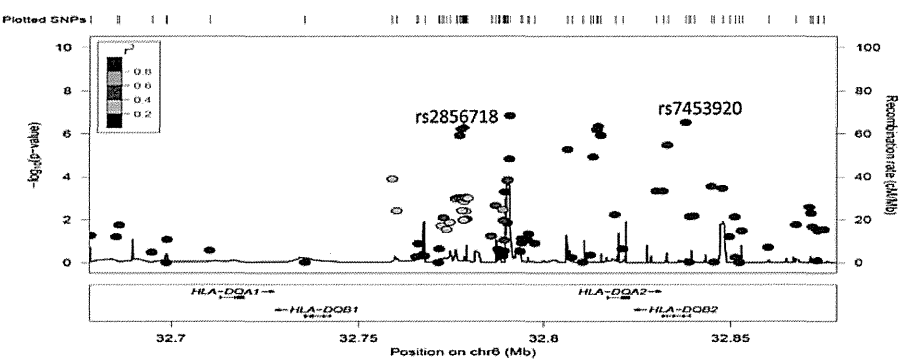

Figure 3. Regional association plot of the *HLA-DQ* locus. This figure indicates a ~200 kb region centered on the association peak, located between rs2856718 and rs7453920. The middle panel shows the genes in this region including the *HLA-DQ* locus.

(Fig. 3). To further validate these results, we analyzed these SNPs in two additional Japanese cohorts consisting of 381 cases and 1539 controls from Biobank Japan as well as 1222 cases and 879 controls from Hiroshima University. Association for these SNPs loci was confirmed in both replication sets (P-value $= 3.14 \times 10^{-5}$–3.59×10^{-12}; Table 1). To combine these studies, we conducted a meta-analysis with a fixed-effects model using the Mantel–Haenszel method. As shown in Table 1 and Supplementary Material, Figure S3, the OR were quite similar among the four studies and no heterogeneity was observed. Mantel–Haenszel P-values for independence were 3.99×10^{-37} for rs2856718 [OR = 1.77, 95% confidence interval (CI) = 1.65–1.91], and 5.98×10^{-28} for rs7453920 (OR = 1.81, 95% CI = 1.62–2.01). Two previously reported SNPs on the *HLA-DP* locus (rs9277535 on *HLA-DPB1* and rs3077 on *HLA-DPA1*) were also associated with CHB ($P_{\text{meta-analysis}} = 2.55 \times 10^{-54}$ and 1.57×10^{-61}) (Table 1).

To test whether the strong association observed in these regions is due to the effect of one of them, we performed logistic regression analysis based on the effect of each top SNP in both *HLA-DP* and *HLA-DQ* loci. Notably, rs2856718 and rs7453920 did show strong association with CHB after adjusting for the effect of rs3077 ($P = 8.12 \times 10^{-27}$ and $P = 1.52 \times 10^{-21}$, respectively) and rs9277535 ($P = 2.38 \times 10^{-30}$ and $P = 2.21 \times 10^{-22}$, respectively), indicating variants at the *HLA-DQ* locus are associated with CHB independent of the effect of *HLA-DP* polymorphisms (Table 2).

Table 1. Summary of results for GWAS and replication study

Chr (position)	SNP	Nearest gene	Allele (1/2)	Stage	Case 11	12	22	MAF[a]	Control 11	12	22	MAF[a]	*P*-value[b]	OR[c] (95% CI)	P^{d}_{het}
6 (33141000)	rs3077	*HLA-DPA1*	A/G	GWAS	38	156	264	0.25	330	991	735	0.40	1.28×10^{-16}	1.98 (1.68–2.32)	
				First replication	42	240	324	0.27	313	947	762	0.39	1.93×10^{-14}	1.74 (1.51–2.01)	
				Second replication	36	139	204	0.28	268	742	529	0.42	9.52×10^{-12}	1.84 (1.55–2.19)	
				Third replication	115	430	681	0.27	155	420	304	0.42	1.53×10^{-21}	1.93 (1.69–2.2)	
				Meta-analysis[e]									1.57×10^{-61}	1.87 (1.73–2.01)	0.62
6 (33162839)	rs9277535	*HLA-DPB1*	A/G	GWAS	40	179	239	0.28	384	1020	652	0.43	3.72×10^{-17}	1.95 (1.67–2.28)	
				First replication	58	254	294	0.31	364	963	696	0.42	3.70×10^{-12}	1.63 (1.42–1.87)	
				Second replication	42	145	192	0.30	301	758	480	0.44	5.43×10^{-12}	1.83 (1.54–2.17)	
				Third replication	133	464	628	0.30	160	429	290	0.43	1.02×10^{-16}	1.75 (1.54–1.99)	
				Meta-analysis[e]									2.55×10^{-54}	1.77 (1.65–1.91)	0.40
6 (32778233)	rs2856718	*HLA-DQB1*	A/G	GWAS	158	226	73	0.41	477	1001	568	0.48	4.41×10^{-10}	1.59 (1.37–1.85)	
				First replication	209	266	127	0.43	484	966	572	0.48	1.07×10^{-7}	1.43 (1.27–1.64)	
				Second replication	128	191	62	0.41	325	746	468	0.45	7.49×10^{-11}	1.72 (1.45–2)	
				Third replication	465	530	227	0.40	216	420	243	0.48	3.59×10^{-12}	1.59 (1.39–1.79)	
				Meta-analysis[e]									3.99×10^{-37}	1.56 (1.45–1.67)	0.24
6 (32837990)	rs7453920	*HLA-DQB2*	A/G	GWAS	4	72	382	0.09	67	582	1407	0.17	1.27×10^{-10}	2.20 (1.73–2.81)	
				First replication	5	127	471	0.11	50	575	1397	0.17	5.47×10^{-6}	1.56 (1.28–1.9)	
				Second replication	4	75	302	0.11	53	422	1064	0.17	3.14×10^{-5}	1.69 (1.32–2.17)	
				Third replication	14	198	1011	0.09	19	245	615	0.16	2.21×10^{-11}	1.88 (1.56–2.27)	
				Meta-analysis[e]									5.98×10^{-28}	1.81 (1.62–2.01)	0.16

[a]MAF, minor allele frequency.
[b]*P*-value of the Cochrane–Armitage trend test for each stage.
[c]OR and CI are calculated using the non-susceptible allele as reference.
[d]*P*-value of the Breslow–Day test.
[e]Results of meta-analysis were calculated by the Mantel–Haenzel method.

Table 2. Logistic regression results for the top SNPs in HLA-DP and HLA-DQ loci associated with CHB in all stages

SNP	*P*-value[a]	$P_{adjusted\ for\ rs3077}$	OR (95% CI)	$P_{adjusted\ for\ rs9277535}$	OR (95% CI)	$P_{adjusted\ for\ rs2856718}$	OR (95% CI)	$P_{adjusted\ for\ rs7453920}$	OR (95% CI)
rs3077	1.57×10^{-61}	NA	–	2.05×10^{-10}	1.43 (1.3–1.67)	7.45×10^{-48}	1.7 (1.58–1.83)	9.42×10^{-51}	1.73 (1.61–1.85)
rs9277535	2.55×10^{-54}	1.67E−05	1.25 (1.15–1.45)	NA	–	6.80×10^{-47}	1.67 (1.55–1.79)	9.03×10^{-48}	1.67 (1.56–1.8)
rs2856718	3.99×10^{-37}	8.12E−27	1.43 (1.33–1.54)	2.38×10^{-30}	1.43 (1.37–1.56)	NA	–	6.34×10^{-26}	1.43 (1.34–1.53)
rs7453920	5.98×10^{-28}	1.52E−21	1.66 (1.49–1.85)	2.21×10^{-22}	1.67 (1.51–1.85)	4.96×10^{-18}	1.60 (1.44–1.77)	NA	–

Trend *P*-values are shown with or without adjusting the analysis for the most associated SNPs in *HLA-DP* and *HLA-DQ* loci.
[a]Meta-analysis *P*-value was calculated by the Mantel–Haenzel method.

Subsequently, we examined the interaction of four SNPs in *HLA-DP* and *HLA-DQ* genes on CHB susceptibility. We only found evidence for interactive effects between HLA-DP SNPs and also between HLA-DQ SNPs (Supplementary Material, Table S6). For all other pairwise combinations, each locus had an independent role in CHB ($P_{interaction} > 0.10$). CHB risk increases with increasing number of risk alleles for four SNPs (Fig. 4 and Supplementary Material, Table S7). Individuals with seven or eight risk alleles have more than 5-fold higher CHB risk than those with two or less risk alleles. Taken together, our findings clearly indicated the additive effects of variants in *HLA-DP* and *HLA-DQ* loci on CHB susceptibility.

HLA-DQ molecules function as a heterodimer of α and β subunits, those are encoded by the *HLA-DQA1* and the *HLA-DQB1* genes, respectively. The SNP rs2856718 is located in a linkage disequilibrium (LD) block including *HLA-DQB1* and *HLA-DQA1* genes, and rs7453920 and rs2856718 are in LD with r^2 of 0.1 and D' of 0.73 (Fig. 3 and Supplementary Material, Fig. S4). Similar to *HLA-DPs*, *HLA-DQs* are highly polymorphic especially in exon 2 which encode antigen-binding sites. We therefore considered that the association of these SNPs with CHB might reflect variations in antigen-binding sites of *HLA-DQA1* and *DQB1* that would affect the immune response to HBV. Hence, we genotyped *HLA-DQA1* and *DQB1* alleles by direct sequencing of exon 2 (cases and controls from the GWAS and first replication sets) and found *HLA-DQB1*0303* and *DQB1*0602* were significantly associated with CHB susceptibility ($P = 1.49 \times 10^{-6}$ and 1.87×10^{-5}, OR = 1.64 and 2.51, respectively), while *DQB1*0501* and *DQB1*0604* were significantly associated with protection from persistent HBV infection ($P = 3.61 \times 10^{-4}$ and 5.38×10^{-16}, OR = 0.50 and 0.22, respectively) (Supplementary Material, Table S8). To further investigate the relationship between *HLA-DQ* alleles and CHB susceptibility, we performed logistic regression analysis using SNPs rs2856718 and rs7453920 as covariates. Interestingly, *HLA-DQB1*0303* and**0604* showed strong association with CHB after adjustment for rs2856718 and rs7453920 ($P = 6.3 \times 10^{-4}$ and $P = 2.59 \times 10^{-8}$, respectively). In addition, we performed logistic regression analysis using the top *HLA-DQ* alleles that show the strongest association (*DQB1*0303*, **0602*, **0501*, **0604*) as covariate. As expected, HLA-DQ SNPs rs2856718 and rs7453920 failed to find the association between CHB and those SNPs ($P = 0.36$, and $P = 0.08$, respectively). Finally, we performed conditional analysis of the *DQB1, DPA1* and *DPB1* alleles together. As a result, HLA-DP SNPs rs3077 and rs9277535 as well as HLA-DQ SNPs rs2856718 and rs7453920 did not show any further association beyond these *HLA-DQ* and *DP* alleles (rs9277535, $P = 0.55$, OR = 0.88; rs3077, P = NA; rs2856718, $P = 0.63$, OR = 0.95 and rs7453920, $P = 0.30$, OR = 0.85). We also performed conditional analysis of the *DPA1* and *DPB1* and we found that *HLA-DQ* alleles **0303*, **0602* and **0604* still showed strong association ($P = 0.0006$, OR = 1.5; $P = 0.00047$, OR = 2.28 and $P = 6.66 \times 10^{-7}$, OR = 0.31) except for *DQB1*0501* ($P = 0.35$, OR = 0.81) which already showed weak association before adjustment as shown in Supplementary Material, Table S8. Collectively, these results together confirmed our findings for the

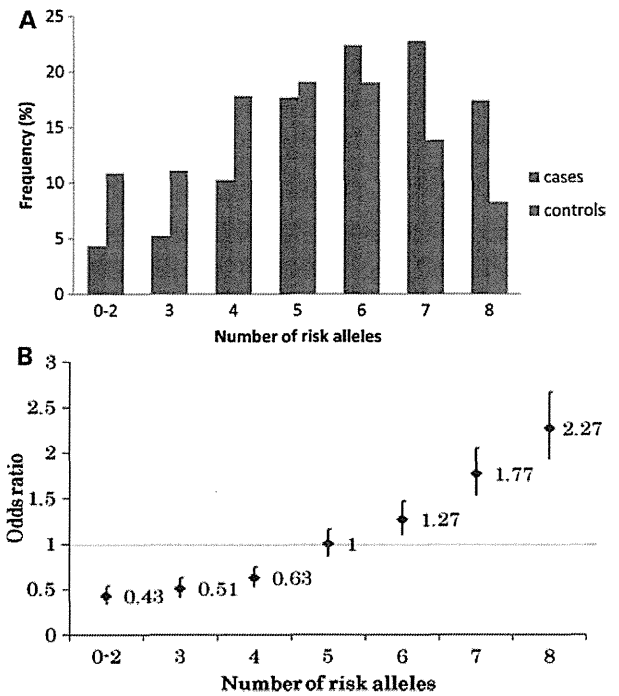

Figure 4. Cumulative effects of CHB risk alleles. (**A**) Distribution of risk alleles in CHB cases (red bars) and controls (blue bars). (**B**) Plot of the increasing OR for CHB with increasing number of risk alleles. The OR are relative to the median number of four risk alleles (rs3077, rs9277535, rs2856718 and rs7453920). Vertical bars correspond to 95% CIs. Horizontal line marks the null value (OR = 1).

causality of *HLA-DQ* and *HLA-DP* alleles and their independent effects on the CHB susceptibility. We further performed haplotype analysis and found four haplotypes showing the highest association ($8.39 \times 10^{-5} - 3.42 \times 10^{-13}$); *DQA1*0102-DQB1*0604* and *DQA1*0101-DQB1*0501* were considered to have protective effects ($P = 3.42 \times 10^{-13}$, OR = 0.16 and $P = 1.06 \times 10^{-5}$, OR = 0.39, respectively), whereas *DQA1*0102-DQB1*0303* and *DQA1*0301-DQB1*0601* increased a risk of CHB ($P = 8.39 \times 10^{-5}$, OR = 19.03, and $P = 7.34 \times 10^{-5}$, OR = 5.02, respectively, Table 3). Furthermore, we performed integrated analysis to test the haplotypic relationship between HLA-DP and DQ. We found seven associated haplotypes: *DQA1*0501-DQB1*0301-DPA1*0202-DPB1*0501*, *DQA1*0301-DQB1*0401 DP A1*0103DPB1*0201*, *DQA1*0301-DQB1*0302-DPA1*0202-DPB1*0501* and *DQA1*0102-DQB1*0604-DPA1*0103-DPB1*0401* showed protective effects ($P = 1.90 \times 10^{-4}$, OR = 0.18; $P = 5.30 \times 10^{-3}$, OR = 0.27; $P = 5.90 \times 10^{-3}$, OR = 0.43 and $P = 9.70 \times 10^{-3}$, OR = 0.41, respectively), whereas *DQA1*0301-DQB1*0301-DPA1*0103-DPB1*0201*, *DQA1*0102-DQB1*0602-DPA1*0202-DPB1*0501* and *DQ A1*0301-DQB1*0601-DPA1*0202-DPB1*0501* were associated with susceptibility to CHB ($P = 2.30 \times 10^{-3}$, OR = 4.9; $P = 9.30 \times 10^{-4}$, OR = 4.8 and $P = 3.30 \times 10^{-5}$, OR = 11, respectively, Supplementary Material, Table S9). Taken together, our findings strongly implicated the significant association of *HLA-DQ-DP* haplotypes with CHB.

Recent GWASs have identified several SNPs that are associated with viral and non-viral liver diseases as well as response to HBV vaccination and liver function test (16–18). More recently, Zhang *et al.* (19) performed a GWAS of hepatocellular carcinoma in chronic HBV carriers of Chinese ancestry. They successfully identified one intronic SNP rs17401966 in *KIF1B* on chromosome 1p36.22 that was highly associated with HBV-related hepatocellular carcinoma. We analyzed those loci in our GWAS data, but failed to find the association between CHB and those SNPs (Supplementary Material, Table S10).

DISCUSSION

Here, we present the results of the two-stage GWAS followed by two independent replications on a total of 2667 cases with CHB and 6496 controls in Japanese population. In this study, we genotyped additional 279 cases and 1122 controls by using Illumina Human610-Quad BeadChip. As a result, we increased the number of samples in the first screening from 179 cases and 934 controls in the previous study to 458 cases and 2056 controls in current study. As a result, the statistic power to detect SNPs with moderate effects (i.e. OR of 1.4 and risk allele frequency of 0.2) increased from 23 to 85% at a significance threshold of 5×10^{-5}. Indeed, two SNPs in *HLA-DQ* locus did not indicate significant association in the GWAS stage of our previous GWAS ($P = 5.62 \times 10^{-2}$ for rs2856718 and $P = 4.88 \times 10^{-2}$ for rs7453920), confirming the importance of sample size in GWAS (20).

Most of significant SNPs with *P*-value of smaller than 5×10^{-5} (74 among 88 SNPs) are located in the MHC region which encompasses a large number of genes involved in our immunological response.

Three groups of *HLA* class II genes produce cell-surface Ag, designated *HLA-DR*, *HLA-DQ* and *HLA-DP*. It is suggested that the host immune response to HBV is under T lymphocyte control, and this response has been shown to be HLA-restricted (21). The *HLA-DQ* locus is located ~300 kb telomeric of the *HLA-DP* locus in a different LD block. Indeed, the analysis of the HLA complex revealed several recombination hot spots distributing across the HLA complex, including two hot spots near *DP* and *DQ* genes (22,23). The result of conditional analyses also demonstrated that the association of the *HLA-DQ* locus with CHB is independent from that of the *HLA-DP* locus.

Previous reports showed an association of *HLA* class II alleles with susceptibility of persistent HBV infection (24–27), but the results were inconsistent even within the same population except for *HLA-DR13*. *HLA-DR13* (corresponding to *HLA-DRB1*1301* and **1302* alleles) was consistently associated with HBV clearance across the population, and we found that rs11752643 which is strongly linked with *HLA-DR13* (28) showed a strong association in the GWAS stage ($P = 1.26 \times 10^{-10}$). The SNP rs3892710 which is in strong LD with rs11752643 ($r^2 = 0.8$, $D' = 1$) and showed higher association in the GWAS stage ($P = 4.49 \times 10^{-12}$) was selected for replication in the first independent replication set. However, rs3892710 failed to clear Bonferroni correction

Table 3. Haplotype analysis

No.	Haplotype *HLA.DQA1*	*HLA.DQB1*	Haplotype frequencies Case (%)	Control (%)	*P*-value[a]	OR[a] (95% CI)
1	*0102	*0604	1.22	6.59	3.42×10^{-13}	0.16 (0.09–0.29)
2	*0101	*0501	1.68	4.77	1.06×10^{-5}	0.39 (0.24–0.65)
3	*0501	*0301	3.06	5.79	1.52×10^{-3}	0.53 (0.35–0.79)
4	*0301	*0401	9.73	13.40	2.98×10^{-3}	0.76 (0.57–1.02)
5	*0301	*0302	5.08	7.56	1.67×10^{-2}	0.72 (0.50–1.02)
6	*0301	*0402	2.55	3.49	1.73×10^{-1}	0.74 (0.45–1.22)
7	*0401	*0402	1.31	1.62	4.91×10^{-1}	0.72 (0.36–1.44)
8	*0101	*0503	4.23	4.34	8.69×10^{-1}	0.94 (0.62–1.42)
9	*0103	*0601	18.70	18.90	9.11×10^{-1}	Reference
10	*0601	*0301	1.38	0.89	2.53×10^{-1}	1.46 (0.68–3.11)
11	*0301	*0503	1.48	0.95	2.06×10^{-1}	1.65 (0.74–3.68)
12	*0301	*0301	2.46	1.79	1.97×10^{-1}	1.33 (0.76–2.33)
13	*0101	*0502	2.09	1.39	1.89×10^{-1}	1.67 (0.90–3.11)
14	*0301	*0303	16.90	13.10	7.50×10^{-3}	1.32 (1–1.74)
15	*0102	*0602	3.39	1.55	3.47×10^{-3}	2.24 (1.28–3.92)
16	*0102	*0303	1.91	0.25	8.39×10^{-5}	19.03 (2.53–143.39)
17	*0301	*0601	2.45	0.42	7.34×10^{-5}	5.02 (1.87–13.45)

[a]*P*-values, OR and its 95% CIs of each haplotype were calculated as described in Materials and Methods.

for multiple testing after adjustment for rs9277535 ($P = 4.73 \times 10^{-2}$). In addition, the association of hepatitis B with *HLA-DQ* SNPs rs2856718 and rs7453920 remarkably attenuated after adjustment for rs11752643 using the logistic regression model ($P = 2.53 \times 10^{-6}$ and $P = 5.84 \times 10^{-4}$, respectively). Unlike HLA-DP SNPs, rs3077 and rs9277535 remained highly significant ($P = 7.74 \times 10^{-13}$ and 2.52×10^{-12}, respectively). Therefore, our findings clearly indicated that hepatitis B is associated with the variants on *HLA-DP* loci independent of the association with SNP rs11752643 that is closely linked with *HLA-DR13* and also reinforce the previous report of *HLA-DQ-DR* linkage. Thus, our study demonstrated that the association of CHB with the variants in the *HLA-DQ* locus was more prominent and consistent than those with *HLA-DR13* in the Japanese population. However, the 19 major haplotypes shown in Supplementary Material, Table S9 accounted for only 51.80% of cases and 57.92% of controls, and other 314 haplotypes were missed due to low haplotype frequency (<1% in both cases and controls). Therefore, the result of *DP-DQ* haplotype analyses should be carefully interpreted. Subsequently, further functional analysis including *HLA-DR*, *DQ* and *DP* is essential to fully elucidate the molecular mechanism whereby these variations confer CHB susceptibility.

In summary, we have demonstrated that genetic variations in the *HLA-DQ* genes were strongly associated with CHB in the Japanese population, and this association was independent from the *HLA-DP* genes which we reported previously. Considering the importance of the MHC region in the clearance after the infection of HBV, our findings should provide a novel insight that the antigen presentation on the HLA-DP and HLA-DQ molecules might be critical for virus elimination and play an important role in the development of CHB. We are confident that our findings would serve to allow better understanding of the pathogenesis of hepatitis B and contribute to better clinical outcome of the disease.

MATERIALS AND METHODS

Study population

A total of 2667 cases and 6496 control subjects were analyzed in this study. Characteristics of each cohort are shown in Supplementary Material, Table S1. DNA samples from both CHB patients and non-HBV controls used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo (29) except for samples for the third replication. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guideline for diagnosis and treatment of chronic hepatitis from The Japan Society of Hepatology (http://www.jsh.or.jp/medical/gudelines/index.html). The control groups for the GWAS and first replication as well as for the second replication consisted of subjects with diseases other than CHB (uterine cancer, esophageal cancer, hematological cancer, pulmonary tuberculosis, ovarian cancer, keloid, peripheral artery disease and ischemic stroke) that were also negative for HBsAg. Case and control samples for the third replication cohort were collected from hospitals participating to the Hiroshima Liver Study Group (listing of participating doctors in this study group can be obtained at http: //home. hiroshima-u.ac.jp/naika1/research_profile/pdf/liver_ study_ group_e.pdf) and Toranomon Hospital. All the participants provided written informed consent. This project was approved by the ethical committees at each institute.

SNP genotyping and QC

In the GWAS stage, 458 patients with CHB and 2056 non-HBV controls were genotyped using Illumina Infinium HumanHap550v3 or Illumina Infinium Human610-Quad DNA Analysis Genotyping BeadChip. SNP QC for all sets of samples was applied as follows: SNP call rate of

≥ 0.99 in both cases and controls and *P*-value of the Hardy–Weinberg equilibrium test of $\geq 1.0 \times 10^{-6}$ in controls. SNPs with minor allele frequency of ≤ 0.01 in both case and control samples were excluded from the further analysis. In the first replication, we genotyped an additional panel of 616 cases by multiplex polymerase chain reaction (PCR)-based Invader assay (Third Wave Technologies, Madison, WI, USA) (30). After excluding 10 cases with the call rate of <0.95, all cluster plots were visually analyzed by trained staffs and SNPs with ambiguous calls were excluded. Randomly selected 94 case samples in the GWAS stage were re-genotyped in the first replication and SNPs with concordance rates of $<98\%$ between two assays (Illumina and Invader) were excluded. In the subsequent replication analyses, we used the TaqMan genotyping system (Applied Biosystems, Foster City, CA, USA) or the multiplex PCR-based Invader assay.

HLA-DQA1 and HLA-DQB1 genotyping

We analyzed *HLA-DQ* genotypes using 748 cases and 614 controls (from GWAS and first replication sets). The second exons of the *HLA-DQA1* and *HLA-DQB1* genes were amplified and directly sequenced according to the protocol reported previously (31–33). *HLA-DQA1* and *DQB1* alleles were determined based on the alignment database of dbMHC.

Statistical analysis

In the GWAS stage and replication analyses, statistical significance of the association with each SNP was assessed using 1-df Cochrane–Armitage trend test and logistic regression analysis adjusted with top SNP (rs9277535) in the *HLA-DP* locus. Significance levels after Bonferroni correction for multiple testing were $P = 3.0 \times 10^{-3}$ (0.05/16) in the first replication and $P = 0.025$ (0.05/2) in second and third replication. OR and CIs were calculated using the non-susceptible allele as a reference. The meta-analysis was conducted using the Mantel–Haenszel method. Heterogeneity among studies was examined by the Breslow–Day test. To assess the association of each *HLA* allele, we used Fisher's exact test on two-by-two contingency tables with or without each *HLA* allele. To analyze the association of haplotypes, we used R package haplo.stats. *P*-values for each haplotype were given by the results of a score test, and OR and 95% CIs were calculated from coefficients of the generalized linear model. OR of each haplotype were calculated relative to the major haplotype. All of these statistical values were calculated by function haplo.cc.

Software

For general statistical analysis, we used R statistical environment version 2.11.1 (http://cran.r-project.org) or plink-1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/). Estimation of haplotype frequencies and analysis of haplotype association were performed by R package haplo.stats (34). Sequence variants in the second exons of *HLA-DQA1* and *HLA-DQB1* were analyzed by Sequencher 4.8. Haploview software was employed to analyze LD values and draw LD map.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Pungpapong, S., Kim, W.R. and Poterucha, J.J. (2007) Natural history of hepatitis B virus infection: an update for clinicians. Mayo Clin. Proc., **82**, 967–975.
2. Okada, K., Kamiyama, I., Inomata, M., Imai, M. and Miyakawa, Y. (1976) e Antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N. Engl. J. Med., **294**, 746–749.
3. Chemin, I. and Zoulim, F. (2009) Hepatitis B virus induced hepatocellular carcinoma. Cancer Lett., **286**, 52–59.
4. McMahon, B. (2009) The natural history of chronic hepatitis B virus infection. *Hepatology*, **49**, S45–S55.
5. Lin, T., Chen, C., Wu, M., Yang, C., Chen, J., Lin, C., Kwang, T., Hsu, S., Lin, S. and Hsu, L. (1989) Hepatitis B virus markers in Chinese twins. Anticancer Res., **9**, 737–741.
6. Kamatani, Y., Wattanapokayakit, S., Ochi, H., Kawaguchi, T., Takahashi, A., Hosono, N., Kubo, M., Tsunoda, T., Kamatani, N., Kumada, H. *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.
7. Thio, C., Carrington, M., Marti, D., O'Brien, S., Vlahov, D., Nelson, K., Astemborski, J. and Thomas, D. (1999) Class II HLA alleles and hepatitis B virus persistence in African Americans. J. Infect. Dis., **179**, 1004–1006.
8. Kummee, P., Tangkijvanich, P., Poovorawan, Y. and Hirankarn, N. (2007) Association of HLA-DRB1*13 and TNF-alpha gene polymorphisms with clearance of chronic hepatitis B infection and risk of hepatocellular carcinoma in Thai population. J. Viral. Hepat., **14**, 841–848.
9. Hwang, S., Sohn, Y., Oh, H., Hwang, C., Lee, S., Shin, E. and Lee, K. (2007) Human leukocyte antigen alleles and haplotypes associated with chronicity of hepatitis B virus infection in Koreans. Arch. Pathol. Lab. Med., **131**, 117–121.
10. Ben-Ari, Z., Mor, E., Papo, O., Kfir, B., Sulkes, J., Tambur, A., Tur-Kaspa, R. and Klein, T. (2003) Cytokine gene polymorphisms in patients infected with hepatitis B virus. Am. J. Gastroenterol., **98**, 144–150.
11. Höhler, T., Kruger, A., Gerken, G., Schneider, P., Meyer zum Büschenefelde, K. and Rittner, C. (1998) A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. Clin. Exp. Immunol., **111**, 579–582.
12. Migita, K., Maeda, Y., Abiru, S., Nakamura, M., Komori, A., Miyazoe, S., Nakao, K., Yatsuhashi, H., Eguchi, K. and Ishibashi, H. (2007) Polymorphisms of interleukin-1beta in Japanese patients with hepatitis B virus infection. J. Hepatol., **46**, 381–386.
13. Chong, W., To, Y., Ip, W., Yuen, M., Poon, T., Wong, W., Lai, C. and Lau, Y. (2005) Mannose-binding lectin in chronic hepatitis B virus infection. *Hepatology*, **42**, 1037–1045.

14. Thio, C., Mosbruger, T., Kaslow, R., Karp, C., Strathdee, S., Vlahov, D., O'Brien, S., Astemborski, J. and Thomas, D. (2004) Cytotoxic T-lymphocyte antigen 4 gene and recovery from hepatitis B virus infection. J. Virol., **78**, 11258–11262.
15. Zhou, J., Lu, L., Yuen, M., Lam, T., Chung, C., Lam, C., Zhang, B., Wang, S., Chen, Y., Wu, S. *et al.* (2007) Polymorphisms of type I interferon receptor 1 promoter and their effects on chronic hepatitis B virus infection. J. Hepatol., **46**, 198–205.
16. Davila, S., Froeling, F., Tan, A., Bonnard, C., Boland, G., Snippe, H., Hibberd, M. and Seielstad, M. (2010) New genetic associations detected in a host response study to hepatitis B vaccine. Genes Immun., **11**, 232–238.
17. Kamatani, Y., Matsuda, K., Okada, Y., Kubo, M., Hosono, N., Daigo, Y., Nakamura, Y. and Kamatani, N. (2010) Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat. Genet., **42**, 210–215.
18. Romeo, S., Kozlitina, J., Xing, C., Pertsemlidis, A., Cox, D., Pennacchio, L., Boerwinkle, E., Cohen, J. and Hobbs, H. (2008) Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat. Genet., **40**, 1461–1465.
19. Zhang, H., Zhai, Y., Hu, Z., Wu, C., Qian, J., Jia, W., Ma, F., Huang, W., Yu, L., Yue, W. *et al.* (2010) Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. Nat. Genet., **42**, 755–758.
20. Wellcome Trust Case Control Consortium. (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, **447**, 661–678.
21. Milich, D. (1988) T- and B-cell recognition of hepatitis B viral antigens. *Immunol. Today*, **9**, 380–386.
22. Jeffreys, A., Kauppi, L. and Neumann, R. (2001) Intensely punctate meiotic recombination in the class II region of the major histocompatibility complex. Nat. Genet., **29**, 217–222.
23. Cullen, M., Perfetto, S., Klitz, W., Nelson, G. and Carrington, M. (2002) High-resolution patterns of meiotic recombination across the human major histocompatibility complex. Am. J. Hum. Genet., **71**, 759–776.
24. Liu, C. and Cheng, B. (2007) Association of polymorphisms of human leucocyte antigen-DQA1 and DQB1 alleles with chronic hepatitis B virus infection, liver cirrhosis and hepatocellular carcinoma in Chinese. Int. J. Immunogenet., **34**, 373–378.
25. Xun, Y., Guo, J., Shi, W., Shi, J. and Liu, C. (2009) Association between HLA-DQA1 gene polymorphism and the outcomes of hepatitis B virus infection. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, **23**, 430–433.
26. Thio, C., Thomas, D., Karacki, P., Gao, X., Marti, D., Kaslow, R., Goedert, J., Hilgartner, M., Strathdee, S., Duggal, P. *et al.* (2003) Comprehensive analysis of class I and class II HLA antigens and chronic hepatitis B virus infection. J. Virol., **77**, 12083–12087.
27. Singh, R., Kaul, R., Kaul, A. and Khan, K. (2007) A comparative review of HLA associations with hepatitis B and C viral infections across global populations. World J. Gastroenterol., **13**, 1770–1787.
28. Thursz, M., Kwiatkowski, D., Allsopp, C., Greenwood, B., Thomas, H. and Hill, A. (1995) Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N. Engl. J. Med., **332**, 1065–1069.
29. Nakamura, Y. (2007) The BioBank Japan Project. *Clin. Adv. Hematol. Oncol.*, **5**, 696–697.
30. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. J. Hum. Genet., **46**, 471–477.
31. van der Zwan, A., Griffith, B., Rozemuller, E., Williams, T. and Tilanus, M.G.J. (2002) Sequence-based typing for HLA-DQB1 strategy for ABI sequencing equipment. In Tilanus, M.G.J. (ed.), International Histocompatibility Working Group, Seattle, Washington, 2002.
32. van Dijk, A., Melchers, R., Tilanus, M. and Rozemuller, E. (2007) HLA-DQB1 sequencing-based typing updated. *Tissue Antigens*, **69**(Suppl. 1), 64–65.
33. Witter, K., Zahn, R., Volgger, A. and Reininger, A.J. (2010) HLA-DQB1*0404, a novel DQB1-allele detected in a volunteer blood platelet donor typed for HLA. *Tissue Antigens*, **76**, 256–258.
34. Schaid, D.J., Rowland, C.M., Tines, D.E., Jacobson, R.M. and Poland, G.A. (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am. J. Hum. Genet., **70**, 425–434.

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Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

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Immunofluorescence method

ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

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into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV precore/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of precore/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the *S* gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

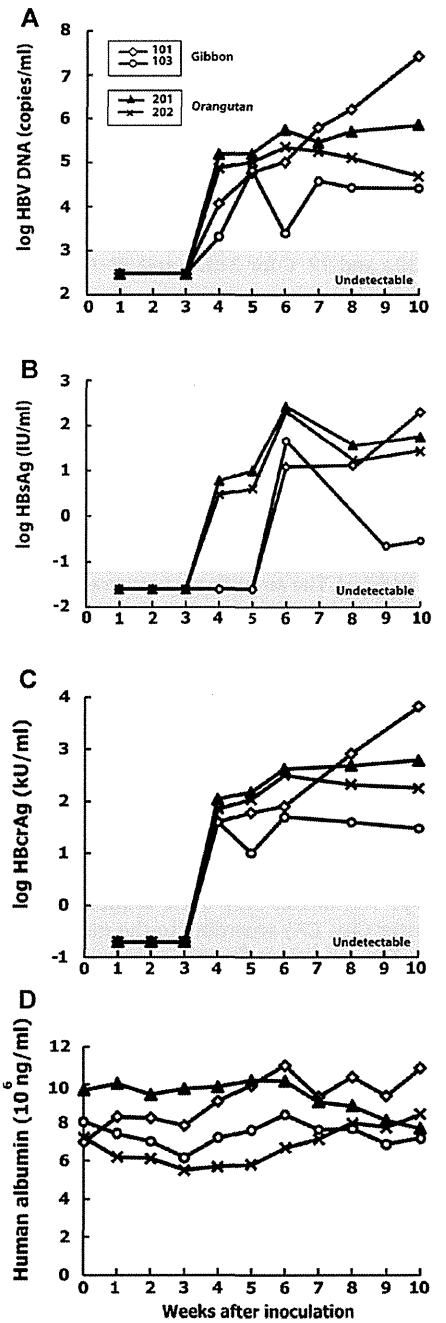

Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR (<10^3 copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

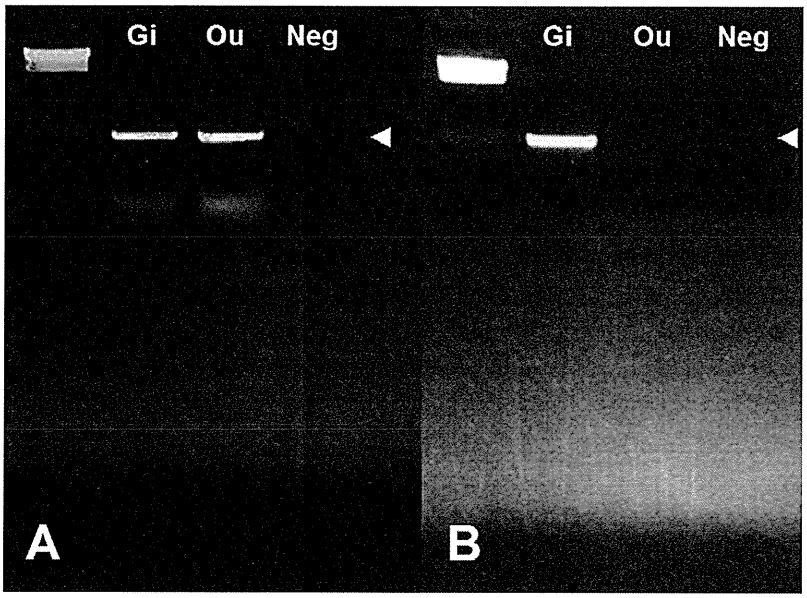

Fig. 2. CccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

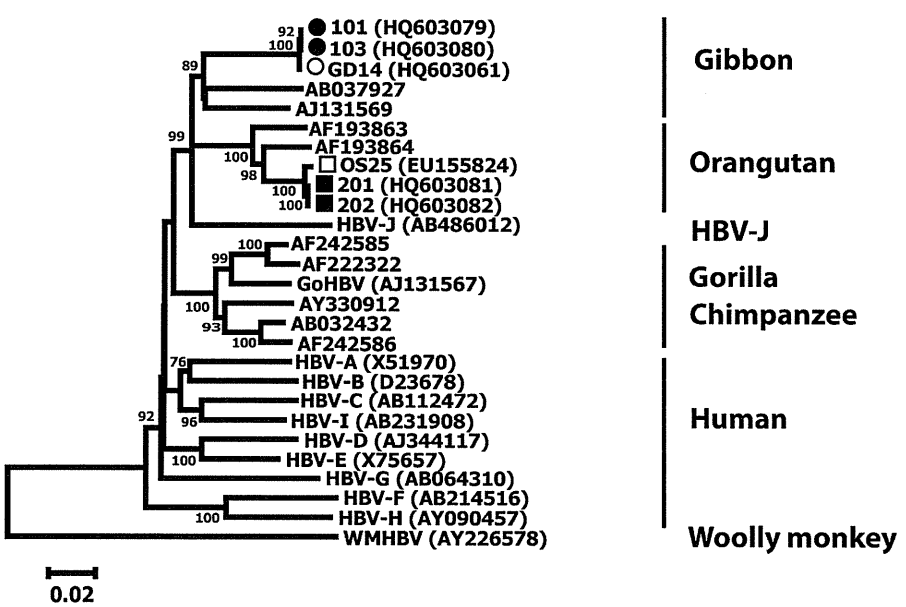

Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

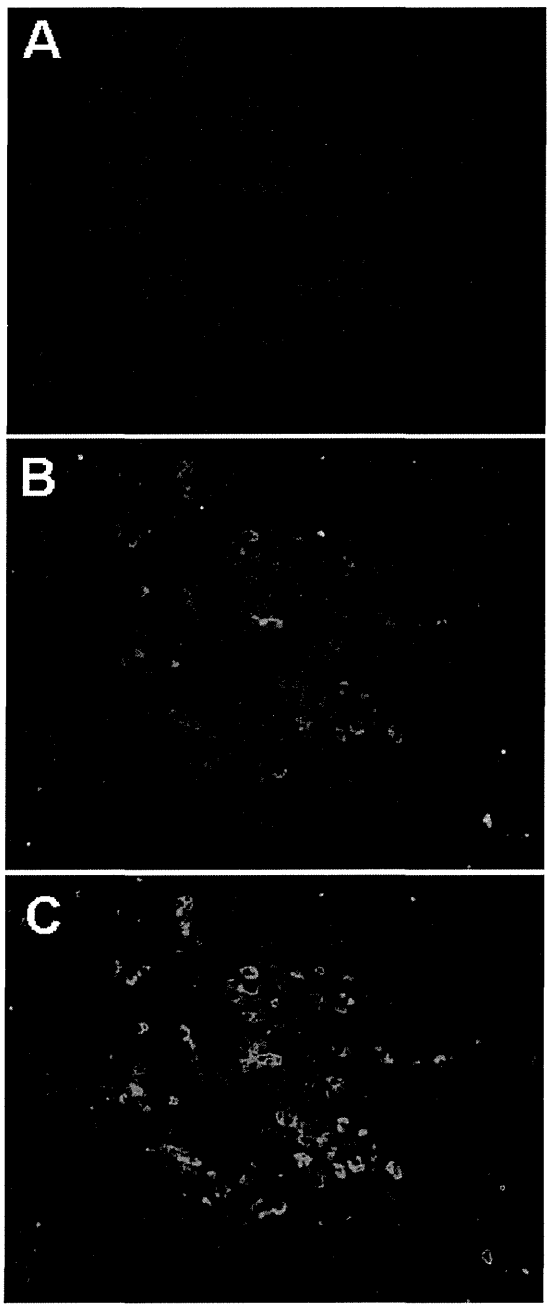

Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

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References

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba, M., Kohara, M., 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J. Clin. Microbiol. 37, 2899–2903.

Alter, H.J., Purcell, R.H., Gerin, J.L., London, W.T., Kaplan, P.M., McAuliffe, V.J., Wagner, J., Holland, P.V., 1977. Transmission of hepatitis B to chimpanzees by hepatitis B surface antigen-positive saliva and semen. Infect. Immun. 16, 928–933.

Ansell, J.D., Bancroft, G.J., 1989. The biology of the SCID mutation. Immunol. Today 10, 322–325.

Bancroft, W.H., Snitbhan, R., Scott, R.M., Tingpalapong, M., Watson, W.T., Tanticharoenyos, P., Karwacki, J.J., Srimarut, S., 1977. Transmission of hepatitis B virus to gibbons by exposure to human saliva containing hepatitis B surface antigen. J. Infect. Dis. 135, 79–85.

Dandri, M., Burda, M.R., Török, E., Pollok, J.M., Iwanska, A., Sommer, G., Rogiers, X., Rogler, C.E., Gupta, S., Will, H., Greten, H., Petersen, J., 2001. Repopulation of

mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33, 981–988.
Ganem, D., Prince, A.M., 2004. Hepatitis B virus infection—natural history and clinical consequences. N. Engl. J. Med. 350, 1118–1129.
Grethe, S., Heckel, J.O., Rietschel, W., Hufert, F.T., 2000. Molecular epidemiology of hepatitis B virus variants in nonhuman primates. J. Virol. 74, 5377–5381.
Hu, X., Margolis, H.S., Purcell, R.H., Ebert, J., Robertson, B.H., 2000. Identification of hepatitis B virus indigenous to chimpanzees. Proc. Natl. Acad. Sci. U.S.A. 97, 1661–1664.
Kim, S.H., Kim, S.H., Oh, H.K., Ryu, C.J., Park, S.Y., Hong, H.J., 2008. In vivo hepatitis B virus-neutralizing activity of an anti-HBsAg humanized antibody in chimpanzees. Exp. Mol. Med. 40, 145–149.
Kimura, T., Ohno, N., Terada, N., Rokuhara, A., Matsumoto, A., Yagi, S., Tanaka, E., Kiyosawa, K., Ohno, S., Maki, N., 2005. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore without C-terminal arginine-rich domain. J. Biol. Chem. 280, 21713–21719.
Kimura, T., Rokuhara, A., Sakamoto, Y., Yagi, S., Tanaka, E., Kiyosawa, K., Maki, N., 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J. Clin. Microbiol. 40, 439–445.
Kneteman, N.M., Mercer, D.F., 2005. Mice with chimeric human livers: who says supermodels have to be tall? Hepatology 41, 703–706.
Kramvis, A., Kew, M., François, G., 2005. Hepatitis B virus genotypes. Vaccine 23, 2409–2423.
Lavanchy, D., 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J. Viral. Hepat. 11, 97–107.
Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337, 1733–1745.
Lin, Y.L., Liao, C.L., Chen, L.K., Yeh, C.T., Liu, C.I., Ma, S.H., Huang, Y.Y., Huang, Y.L., Kao, C.L., King, C.C., 1998. Study of Dengue virus infection in SCID mice engrafted with human K562 cells. J. Virol. 72, 9729–9737.
MacDonald, D.M., Holmes, E.C., Lewis, J.C., Simmonds, P., 2000. Detection of hepatitis B virus infection in wild-born chimpanzees (*Pan troglodytes verus*): phylogenetic relationships with human and other primate genotypes. J. Virol. 74, 4253–4257.
Makuwa, M., Souquière, S., Telfer, P., Leroy, E., Bourry, O., Rouquet, P., Clifford, S., Wickings, E.J., Roques, P., Simon, F., 2003. Occurrence of hepatitis viruses in wild-born non-human primates: a 3 year (1998–2001) epidemiological survey in Gabon. J. Med. Primatol. 32, 307–314.
Mason, A.L., Xu, L., Guo, L., Kuhns, M., Perrillo, R.P., 1998. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27, 1736–1742.
Mason, W.S., Burrell, C.J., Casey, J., Gerlich, W.H., Howard, C.R., Kann, M., Lanford, R., Newbold, J., Schaefer, S., Taylor, J.M., Will, H., 2005. The DNA and RNA transcribing viruses. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 371–382.
McQuillan, G.M., Townsend, T.R., Fields, H.A., Carroll, M., Leahy, M., Polk, B.F., 1989. Seroepidemiology of hepatitis B virus infection in the United States. 1976 to 1980. Am. J. Med. 87, 5S–10S.
Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.
Meuleman, P., Libbrecht, L., De Vos, R., de Hemptinne, B., Gevaert, K., Vandekerckhove, J., Roskams, T., Leroux-Roels, G., 2005. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology 41, 847–856.
Mimms, L.T., Solomon, L.R., Ebert, J.W., Fields, H., 1993. Unique preS sequence in a gibbon-derived hepatitis B virus variant. Biochem. Biophys. Res. Commun. 195, 186–191.
Noppornpanth, S., Haagmans, B.L., Bhattarakosol, P., Ratanakorn, P., Niesters, H.G., Osterhaus, A.D., Poovorawan, Y., 2003. Molecular epidemiology of gibbon hepatitis B virus transmission. J. Gen. Virol. 84, 147–155.
Petersen, J., Dandri, M., Gupta, S., Rogler, C.E., 1998. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. 95, 310–315.
Robertson, B.H., 2001. Viral hepatitis and primates: historical and molecular analysis of human and nonhuman primate hepatitis A, B, and the GB-related viruses. J. Viral. Hepat. 8, 233–242.
Rokuhara, A., Sun, X., Tanaka, E., Kimura, T., Matsumoto, A., Yao, D., Yin, L., Wang, N., Maki, N., Kiyosawa, K., 2005. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. J. Gastroenterol. Hepatol. 20, 1726–1730.
Rokuhara, A., Tanaka, E., Matsumoto, A., Kimura, T., Yamaura, T., Orii, K., Sun, X., Yagi, S., Maki, N., Kiyosawa, K., 2003. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J. Viral. Hepat. 10, 324–330.
Sall, A.A., Starkman, S., Reynes, J.M., Lay, S., Nhim, T., Hunt, M., Marx, N., Simmonds, P., 2005. Frequent infection of *Hylobates pileatus* (pileated gibbon) with species-associated variants of hepatitis B virus in Cambodia. J. Gen. Virol. 86, 333–337.
Sa-nguanmoo, P., Thongmee, C., Ratanakorn, P., Pattanarangsan, R., Boonyaritichaikij, R., Chodapisitkul, S., Theamboonlers, A., Tangkijvanich, P., Poovorawan, Y., 2008. Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon. J. Med. Primatol. 37, 277–289.
Scott, R.M., Snitbhan, R., Bancroft, W.H., Alter, H.J., Tingpalapong, M., 1980. Experimental transmission of hepatitis B virus by semen and saliva. J. Infect. Dis. 142, 67–71.
Sharma, S.K., Saini, N., Chwla, Y., 2005. Hepatitis B virus: inactive carriers. Virol. J. 2, 82.
Shinkai, N., Tanaka, Y., Orito, E., Ito, K., Ohno, T., Hirashima, N., Hasegawa, I., Sugauchi, F., Ueda, R., Mizokami, M., 2006. 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. 36, 272–276.
Starkman, S.E., MacDonald, D.M., Lewis, J.C., Holmes, E.C., Simmonds, P., 2003. Geographic and species association of hepatitis B virus genotypes in non-human primates. Virology 314, 381–393.
Sugauchi, F., Mizokami, M., Orito, E., Ohno, T., Kato, H., Suzuki, S., Kimura, Y., Ueda, R., Butterworth, L.A., Cooksley, W.G., 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J. Gen. Virol. 82, 883–892.
Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S.K., Gish, R.G., Kramvis, A., Shimada, T., Izumi, M., Kaito, M., Miyakawa, Y., Mizokami, M., 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44, 915–924.
Sugiyama, M., Tanaka, Y., Kurbanov, F., Maruyama, I., Shimada, T., Takahashi, S., Shirai, T., Hino, K., Sakaida, I., Mizokami, M., 2009. Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. Gastroenterology 136, 652–662.
Suzuki, F., Miyakoshi, H., Kobayashi, M., Kumada, H., 2009. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J. Med. Virol. 81, 27–33.
Tabor, E., FrÖsner, G., Deinhardt, F., Gerety, R.J., 1980. Hepatitis B e antigen and antibody: detection by radioimmunoassay in chimpanzees during experimental hepatitis B. J. Med. Virol. 6, 91–99.
Tabuchi, A., Tanaka, J., Katayama, K., Mizui, M., Matsukura, H., Yugi, H., Shimada, T., Miyakawa, Y., Yoshizawa, H., 2008. Titration of hepatitis B virus infectivity in the sera of pre-acute and late acute phases of HBV infection: transmission experiments to chimeric mice with human liver repopulated hepatocytes. J. Med. Virol. 80, 2064–2068.
Takahashi, K., Brotman, B., Usuda, S., Mishiro, S., Prince, A.M., 2000. Full-genome sequence analyses of hepatitis B virus (HBV) strains recovered from chimpanzees infected in the wild: implications for an origin of HBV. Virology 267, 58–64.
Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., Miyakawa, Y., Mizokami, M., 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J. Virol. 83, 10538–10547.
Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., Yoshizato, K., 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.
Warren, K.S., Niphuis, H., Heriyanto, Verschoor, E.J., Swan, R.A., Heeney, J.L., 1998. Seroprevalence of specific viral infections in confiscated orangutans (*Pongo pygmaeus*). J. Med. Primatol. 27, 33–37.
Wong, D.K., Tanaka, Y., Lai, C.L., Mizokami, M., Fung, J., Yuen, M.F., 2007. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J. Clin. Microbiol. 45, 3942–3947.

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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).