Table 1. Characteristics of participants in HCC, CHB, resolved HBV and HBV uninfected subjects in Thailand.

	HCC (n = 230)	CHB[a] (n = 219)	Resolved[b] (n = 113)	Uninfected[c] (n = 123)
Age (years)	58.2±12	46.6±10	48.2±6	46.7±6
Male	190 (82.6%)	144 (65.7%)	83 (73.5%)	73 (59.3%)
HBsAg positive	230 (100%)	219 (100%)	0	0
ALT>40 (IU/L)	43 (18.7%)	61 (27.8%)	-	-
Alb (g/dl)	3.7 (2.5–5.6)	4.5 (3–5.2)	-	-
TB (mg/dl)	1.2 (0.17–14.8)	0.56 (0.2–2.67)	-	-

Abbreviation: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen;
ALT, Alanine transaminase; Alb, Albumin; TB, Total bilirubin.
[a]Defined as chronic hepatitis B includes chronic HBV infection but not cirrhosis and HCC.
[b]Defined as HBsAg negative but anti-HBc or/and anti-HBs positive.
[c]Defined as any HBV serological markers negative.
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$p = 0.025$ for rs3077 and OR = 0.55, 95% CI = 0.36–0.82, $p = 0.003$ for rs9277378 and OR = 0.57, 95% CI = 0.36–0.90, $p = 0.015$ for rs1419881, respectively). Comparing HBV carriers and uninfected subjects rather than those with resolved infection regarding rs1419881 was significantly protective association against CHB, but rs3128917 and rs652888 were not associated against CHB (OR = 1.58, 95% CI = 1.02–2.46, $p = 0.042$ for rs3128917 and OR = 1.09, 95% CI = 0.65–1.82, $p = 0.080$ for rs652888). When we consider the Bonferroni corrections (5 SNPs), however, the P value for rs1419881 did not reach the level of significant difference (0.015>0.05/5) between HBV carriers and HBV uninfected subjects. These data suggested that other SNPs, rs1419881, rs3128917 and rs652888 were not associated with HBV carriers in this study.

Results of meta-analysis for 3 SNPs (rs3077, rs9277378 and rs3128917) in the *HLA* gene were shown in Table S2 and S3; HBV carriers were compared to HBV resolved or HBV uninfected subjects, respectively. While the other 2 SNPs were published only from Korean population, thus the meta-analysis appeared only between HBV carriers and HBV uninfected subjects. All SNPs analyzed by the meta-analysis were significantly associated with HBV carriers.

The associations between these 5 SNPs and HBV status are depicted graphically in Figure S1. Each histogram compares HBV carriers with subjects that have resolved HBV infection or were never infected. The results showed that the minor dominant model of rs3077 and rs9277378 was highly protective associated against chronic HBV, while no significant associations were observed with rs3128917 and rs652888. Furthermore, comparing the frequency of rs1419881 between HBV carriers and uninfected subjects also revealed its association against chronic HBV infection but the association with resolved HBV did not achieve statistical significance.

Discussion

Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population. In the human genome, single nucleotide polymorphisms are found in every 300–570 nucleotides. Many SNPs have no effect on the function of the encoded proteins, but some variants do appear in regulatory or coding part of the gene and affect gene expression level or protein function which can give rise to disease [21] such as the 3 SNPs including rs3077, rs9277378 and rs3128917 in *HLA-*

Table 2. Minor allele frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

SNPs	Gene	Minor alleles[a]	HBV carriers[b] (2n = 898)	Resolved (2n = 226)	Uninfected (2n = 246)	HBV carriers vs. Resolved OR (95% CI)	P values	HBV carriers vs. Uninfected OR (95% CI)	P values
rs3077	HLA-DPA1	T	227 (25.3%)	84 (37.2%)	86 (35.0%)	0.57 (0.42–0.78)	<0.001	0.63 (0.47–0.85)	0.008
rs9277378	HLA-DPB1	A	237 (26.4%)	85 (37.6%)	96 (39.0%)	0.59 (0.44–0.81)	0.001	0.56 (0.42–0.75)	<0.001
rs3128917	HLA-DPB1	G	459 (51.1%)	108 (47.8%)	122 (49.6%)	1.14 (0.85–1.53)	0.372	1.06 (0.80–1.41)	0.673
rs1419881	TCF19	C	361 (40.2%)	103 (45.6%)	126 (51.2%)	0.80 (0.60–1.08)	0.142	0.64 (0.48–0.85)	0.002
rs652888	EHMT2	C	329 (36.6%)	76 (33.6%)	84 (34.1%)	1.14 (0.84–1.55)	0.400	1.11 (0.83–1.50)	0.478

Abbreviation: CI, confidence interval; OR, odds ratio.
[a]Defined by using data from public database (NCBI).
[b]Defined as the combination between HCC and CHB.
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Table 3. Genotype frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

SNP	Genotype	HBV carriers[a] (n = 449)	Resolved (n = 113)	Uninfected (n = 123)	HBV carriers vs. Resolved OR (95% CI)	P values	HBV carriers vs. Uninfected OR (95% CI)	P values
rs3077	CC	259 (57.7%)	43 (38.1%)	57 (46.3%)	1.00	-	1.00	-
HLA-DPA1	CT	153 (34.1%)	56 (49.6%)	46 (37.4%)	0.45 (0.29–0.71)	<0.001	0.73 (0.47–1.13)	0.161
	TT	37 (8.2%)	14 (12.4%)	20 (16.3%)	0.44 (0.22–0.88)	0.018	0.41 (0.22–0.75)	0.003
	Dominant[b]				**0.45 (0.30–0.69)**	**<0.001**	**0.63 (0.42–0.95)**	**0.025**
	HWEp	0.038	0.516	0.049				
rs9277378	GG	242 (53.9%)	40 (35.4%)	48 (39.0%)	1.00	-	1.00	-
HLA-DPB1	AG	177 (39.4%)	61 (54.0%)	54 (43.9%)	0.48 (0.31–0.75)	0.001	0.65 (0.42–1.00)	0.051
	AA	30 (6.7%)	12 (10.6%)	21 (17.1%)	0.41 (0.20–0.87)	0.018	0.28 (0.15–0.54)	<0.001
	Dominant				**0.47 (0.31–0.72)**	**<0.001**	**0.55 (0.36–0.82)**	**0.003**
	HWEp	0.757	0.110	0.390				
rs3128917	TT	99 (22.0%)	29 (25.7%)	38 (30.9%)	1.00	-	1.00	-
HLA-DPB1	TG	241 (53.7%)	60 (53.1%)	48 (39.0%)	1.18 (0.71–1.94)	0.525	1.93 (1.19–3.13)	0.008
	GG	109 (24.3%)	24 (21.2%)	37 (30.1%)	1.33 (0.73–2.44)	0.355	1.13 (0.67–1.92)	0.648
	Dominant				**1.22 (0.76–1.97)**	**0.413**	**1.58 (1.02–2.46)**	**0.042**
	HWEp	0.117	0.496	0.015				
rs1419881	TT	162 (36.1%)	31 (27.4%)	30 (24.4%)	1.00	-	1.00	-
TCF19	TC	213 (47.4%)	61 (54.0%)	60 (48.8%)	0.67 (0.41–1.08)	0.097	0.66 (0.41–1.07)	0.088
	CC	74 (16.5%)	21 (18.6%)	33 (26.8%)	0.67 (0.36–1.25)	0.210	0.42 (0.24–0.73)	0.002
	Dominant				**0.67 (0.42–1.06)**	**0.084**	**0.57 (0.36–0.90)**	**0.015**
	HWEp	0.778	0.349	0.792				
rs652888	TT	169 (37.6%)	50 (44.2%)	57 (46.3%)	1.00	-	1.00	-
EHMT2	TC	231 (51.4%)	50 (44.2%)	48 (39.0%)	1.37 (0.88–2.12)	0.162	1.62 (1.05–2.50)	0.027
	CC	49 (10.9%)	13 (11.5%)	18 (14.6%)	1.12 (0.56–2.22)	0.756	0.92 (0.49–1.70)	<0.001
	Dominant				**1.31 (0.87–2.00)**	**0.198**	**1.09 (0.65–1.82)**	**0.080**
	HWEp	0.022	0.926	0.142				

Abbreviation: CI, confidence interval; OR, odds ratio ; HWEp, Hardy-Weinberg equilibrium analysis.
[a]Defined as the combination between HCC and CHB.
[b]Defined as a minor dominant according to the comparison between heterozygous+minor homozygous genotype and major homozygous genotype (eg. rs3077; CT+TT vs. CC).
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DP region of MHC class II. The function of HLA-DP is to present bound peptide antigens, e.g. from HBV, at the surface of antigen-presenting cells. CD4+ T cells recognize these antigens and initiate the adaptive immune response. They assist the MHC class I-restricted CD8+ T cells which are the primary cellular effectors mediating HBV clearance from the liver during acute viral infection [22]. HBV infection will either be cleared by these means, or establish itself as a chronic infection. The reason for the latter is unclear but may be related to variation of HLA-DP alleles. Thus, the position of HLA-DP SNPs might be associated with possibility of clearance or chronicity. The rs3077 and rs9277535 SNPs are located within the 3′ untranslated region (UTR) of HLA-DPA1 and HLA-DPB1, respectively while rs3128917 is located downstream of HLA-DPB1.

Recent investigations have identified 11 risk alleles for CHB related to mRNA expression of HLA-DPA1 and HLA-DPB1 [23]. The results showed that only these two alleles, rs3077 and rs9277535 were strongly associated with the risk of CHB and decreased expression of HLA-DPA1 and HLA-DPB1, respectively. In contrast, while rs3128917 was associated with CHB, it was not associated with the level of HLA-DPB1 expression [23]. Variation

at 5′ and 3′ UTRs can alter the binding sites of regulatory proteins which protect and stabilize newly synthesized RNA, either increasing or decreasing binding [24,25]. Nevertheless, the present study showed that rs3128917 was not associated with HBV carrier status in Thailand. Because rs3128917 is located downstream of the direction of transcription of the gene, this suggests that it does not affect regulation or coding of the gene and would have no effect on HLA protein expression.

The results from the present study not only establish the importance of variation at the HLA-DP gene but also explore two new SNPs, rs1419881 located in TCF19 and rs652888 in the EHMT2 gene [16]. TCF19 (or transcription factor SC1) is a late growth regulatory gene like histone, thymidine kinase etc, maximally expressed at the onset of DNA synthesis at the G1-S boundary and S phase of cell cycle. This protein is also involved in regulations of growth and transcription factors controlling the number and development of peripheral-blood monocytes and erythrocytes [26]. The EHMT2 gene is a histone methyltransferase [18] mainly responsible for mono- and di-methylation of H3K9 in euchromatin. This changes the conformation of chromatin from euchromatin to heterochromatin and then affects gene repression

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[19]. Histone methylation has a critical role in gene transcription and epigenetic events [27–30].

According to recently published GWAS data [11], two SNPs associated with the risk for CHB in the Korea population were identified. These were the top signals in the genome-wide significance level analysis and were independently associated with *HLA-DP* and *HLA-DQ*, respectively. The authors then confirmed the results in a replication sample, showing that the frequency of their two SNPs strongly associated with CHB; OR = 0.76, 95% CI = 0.68–0.86, p = 4.51E-11 for rs1419881 and OR = 1.26, 95% CI = 1.07–1.47, p = 2.78E-06 for rs652888 [16]. Furthermore, another GWAS study focused on HLA, of hepatitis B vaccinated people in Indonesia, showed that rs652888 was also associated with risk of CHB ($p \leq$ 0.0001) in that population [31].

In the present study, however, we found that rs1419881 tended to be associated with chronic HBV infection, based on the results of a comparison between HBV carriers and uninfected subjects. Nonetheless, it did not reach the significance by the Bonferroni corrections, as well as when HBV carriers were compared with patients who had their HBV infection resolved, no association with rs1419881 was observed. The second SNP, rs652888, was not associated with chronic HBV infection in the Thai population. Although our study had sampling error due to small samples, it might be another effect that the result between rs652888 in *EHMT2* gene and chronic hepatitis B in Thai population was not associated. The reason for these negative findings for the two SNPs might be due to the affected gene functions that were not involved with the immune system or processes of persistent infection. Data supporting this notion are to be found in the GWAS data for the Korean population, where pathway analysis of genes involved in the regulation of immune function showed that *TCF19* and *EHMT2* genes are not significantly involved in human immunity [16].

Mapping the position of the two new SNPs showed that rs1419881 located at the 3′ UTR of exon 4, with a tendency towards association with CHB and rs652888 which is not associated with CHB located on an intron. The position of each SNP might affect the phenotype of gene expression and susceptibility to disease, explaining why some are associated with chronic HBV infection, and others not. According to previous publications, the 3′ UTR of the *HLA-DP* region is strongly involved with regulating HLA-DP expression and influences the outcome of HBV infection [32]. In addition, another study showed that variation of the 3′ UTR of HLA-C was strongly associated with HLA-C expression levels and with control of human immunodeficiency virus [33]. This illustrated the general principle that the position of SNPs affects association with diseases.

The prevalence of HBV in Eastern countries, i.e. Asia, sub-Saharan Africa and the Pacific is much higher than in Western Europe and America. Most people in Eastern countries are infected with HBV during childhood and 8–10% of these develop CHB. In contract, the frequency of chronic carriers in Western Europe and North America is ≤1%. Furthermore, previous GWAS and meta-analysis reported that A alleles at rs3077 and rs9277353 have protective effects against CHB. Asian and African populations, especially Chinese, have lower frequencies of A alleles than European and American populations [10,34,35]. Moreover, the previous study showed no associations of rs3077 and rs9277535 with progressive CHB infection; however rs3077 was highly significant associated with HBV infection but not associated with rs9277353 in Caucasian populations [36].

While the frequency of alleles at rs3128917 and rs1419881 in Asian and African populations are quite similar, Northern and Western European populations have high frequencies of the protective T allele at rs3128917 but have low T allele frequencies

(a risk allele for CHB) at rs1419881. The allele frequencies of populations in the worldwide for conspicuous details came from dbSNP Short Genetic Variations available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi. Lastly, both ethnic Eastern and Western populations have similar allele frequencies at rs652888, carrying a risk for CHB, with T allele frequencies very much higher than C allele frequencies, which has a protective effect. In addition, evolution of genomic characteristics, the migratory history of different populations, as well as HBV genotypes [37], HBV carrier rate [38] and pathological procession of liver disease [39] in each country may affect the distribution of *HLA* alleles. This was illustrated by a recent report in two Han Chinese populations (southern and northern) having different distributions of *HLA-DP* genes [39]. Thus, the genetics of the host is one of the factors influencing and predicting disease outcome [40].

According to less number of samples, it might influence statistical power in this study. Thus, we made another statistic meta-analysis of data obtained from previous reports and this study in Table S3. We compared HBV carriers with HBV uninfected subjects, because most previous studies also compared CHB with HBV clearance and/or healthy (negative for any HBV serological markers). Interestingly, all SNPs analyzed by the meta-analysis were significantly associated with HBV carriers. These results could support our data in Thailand. Additionally, no heterogeneity was observed between HBV carriers and HBV-resolved subjects (P_{het} = 0.10 for rs3077, 0.79 for rs9277378, and 0.07 for rs3128917), as well as between HBV carriers and HBV uninfected subjects (P_{het} = 0.10 for rs3077, 0.02 for rs9277378, 0.91 for rs1419881, and 0.04 for rs652888) except for rs9277378 (P_{het} = 0.000), for the minor allele frequency (MAF) of only rs9277378 was different between HapMap-CHB (MAF = 46.3% of G allele) and HapMap-JPT (MAF = 44.8% of T allele).

In the present study, we determined associations of variations at the *HLA-DP* gene with outcome in HBV infected Thai patients and the major homozygous genotypes of rs3077 and rs9277378, but not rs3128917, were significantly associated with HBV carrier status. Although genetic variation of two new SNPs, rs1419881 in the *TCF19* gene and rs652888 in the *EHMT2* gene, were not associated with the outcome of HBV infection in the Thai population, a large-scale study should be required.

Supporting Information

Figure S1 Association of 5 SNPs with HBV carriers, resolved HBV and uninfected subjects in Thailand. The results were compared between percentages of combination of heterozygous genotypes and minor homozygous genotypes (White square) with percentages of major homozygous genotypes (Grey square). Five SNPs applied in this study were rs3077, rs9277378 and rs3128917 in *HLA-DP* gene, rs1419881 in *TCF19* gene and rs652888 in *EHMT2* gene. OR, odds ratio; (lower-upper), 95% confidence interval.
(PPTX)

Table S1 Minor allele frequencies in HCC, CHB, resolved HBV and uninfected subjects in Thailand.
(DOC)

Table S2 The meta-analysis of minor allele frequencies in HBV carriers and resolved HBV.
(DOC)

Table S3 The meta-analysis of minor allele frequencies in HBV carriers and uninfected subject.
(DOC)

Author Contributions

Conceived and designed the experiments: SP TW YP YT. Performed the experiments: NP. Analyzed the data: NP SP SI KM NS. Contributed reagents/materials/analysis tools: PT SO SM. Wrote the paper: NP.

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Role of *HLA-DP* Polymorphisms on Chronicity and Disease Activity of Hepatitis B Infection in Southern Chinese

Danny Ka-Ho Wong[1,2*], Tsunamasa Watanabe[3*], Yasuhito Tanaka[3], Wai-Kay Seto[1], Cheuk-Kwong Lee[4], James Fung[1,2], Che-Kit Lin[4], Fung-Yu Huang[1], Ching-Lung Lai[1,2], Man-Fung Yuen[1,2]*

1 Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong, 2 State Key Laboratory for Liver Research, The University of Hong Kong, Queen Mary Hospital, Hong Kong, 3 Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, 4 Hong Kong Red Cross Blood Transfusion Service, Hospital Authority, Hong Kong

Abstract

Background and Aims: The association between *HLA-DP* single nucleotide polymorphisms (SNPs) and chronic hepatitis B virus (HBV) infection varies between different populations. We aimed to study the association between *HLA-DP* SNPs and HBV infection and disease activity in the Chinese population of Hong Kong.

Methods: We genotyped SNPs rs3077 (near *HLA-DPA1*) and rs9277378 and rs3128917 (both near *HLA-DPB1*) in 500 HBV carriers (hepatitis B surface antigen [HBsAg]-positive), 245 non-HBV infected controls (HBsAg- and antibody to hepatitis B core protein [anti-HBc]-negative), and 259 subjects with natural HBV clearance (HBsAg-negative, anti-HBc-positive). Inactive HBV carriers state was defined by HBV DNA levels <2,000 IU/ml and persistently normal alanine aminotransferase level for least 12 months.

Results: Compared to the non-HBV infected subjects, the HBV carriers had a significantly lower frequency of the rs3077 T allele (p = 0.0040), rs9277378 A allele (p = 0.0068) and a trend for lower frequency of rs3128917 T allele (p = 0.054). These alleles were associated with an increased chance of HBV clearance (rs3077: OR = 1.41, p = 0.0083; rs9277378: OR = 1.61, p = 0.00011; rs3128917: OR = 1.54, p = 0.00017). Significant associations between HLA-DP genotypes and HBV clearance were also found under different genetic models. Haplotype TAT was associated with an increased chance of HBV clearance (OR = 1.64, p = 0.0013). No association was found between these SNPs and HBV disease activity.

Conclusion: *HLA-DP* SNPs rs3077, rs9277378 and rs3128917 were associated with chronicity of HBV disease in the Chinese. Further studies are required to determine whether these SNPs influence the disease endemicity in different ethnic populations.

Introduction

Approximately 400 million people worldwide are chronic carriers of hepatitis B virus (HBV) [1]. The disease spectrum of chronic hepatitis B varies among patients, ranging from inactive non-replicative to active replicative state, which may lead to fulminant hepatic failure, liver cirrhosis, or hepatocellular carcinoma (HCC). While persistence or resolution of HBV infection may be affected by a variety of factors, including viral, environmental and host factors, family or twin studies have suggested that host genetic constitution is also an important factor which influences chronicity of HBV infection [2,3]. Many host genetic variations, including genes coding for cytokines such as interferon-gamma and tumor necrosis factors [4], estrogen

receptor alpha [5], vitamin D receptor [6], mannose-binding protein [7], cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) [8] and human leukocyte antigen (HLA) [9,10,11,12], have been suggested to influence chronicity or clearance of HBV infection. In particular, single nucleotide polymorphisms (SNPs) near the *CTLA-4*, genes coding for an inhibitory receptor expressed by T-lymphocytes, and near the *HLA-DR13* locus, coding for component of the major histocompatibility complex class II cell surface receptors, have been studied in several case-control studies for their association with HBV infection in different populations [8,9,10,11,12]. However, these candidate gene studies were not conducted on a large scale genome-wide approach.

Several genome-wide association studies (GWAS) have been performed with large cohorts to study the association of genetic

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variations with HBV infection. These GWAS studies did not find a strong association between HBV infection and those previously identified candidate HBV-associated SNPs. These GWAS studies demonstrated that certain SNPs near the *HLA-DP* loci, are associated with persistent HBV infection [13,14,15]. In a pioneering GWAS study with 786 Japanese chronic HBV carriers and 2,201 controls, Kamatani and colleagues have identified an association between chronic hepatitis B and 11 SNPs in the *HLA-DP* region, two of which, namely rs3077 and rs9277535, were further validated in three additional Japanese and Thai cohorts [13]. The association between these *HLA-DP* SNPs with chronicity and/or clearance of HBV infection was further confirmed by two other GWAS studies, one with 2,667 Japanese chronic HBV carriers and 6,496 controls by the same group [14] and one with 181 Japanese chronic HBV carries, 184 healthy controls, and 185 individuals with natural clearance of HBV [15]. The association of some of these *HLA-DP* SNPs with HBV infection has been verified in many studies, but the association differs between studies in different population cohorts, and more SNPs are yet to be identified [16,17,18,19,20,21].

HLA-DP molecules, belonging to HLA class II, are involved in antigen presentation to CD4+ T helper cells. As HLA-DP plays an important role in host-immune response and particularly antigen presentation, it would be interesting to investigate the possible association of the *HLA-DP* loci variations with hepatitis B disease activity, which is immune-mediated. Since the findings are not consistent in different study cohorts [18,20], the association of these *HLA-DP* SNPs with HBV disease activity remains unclear.

In the present study, we primarily aimed to investigate the association of 3 *HLA-DP* SNPs, namely rs3077, rs9277378 and rs3128917, with chronicity of HBV infection in the Chinese population in Hong Kong. In addition, we studied the association of these SNPs with hepatitis B disease activity. This will extend our understanding of the association between *HLA-DP* variations and HBV infection and may provide some evidences to explain the widely different prevalence of chronic HBV in different ethnic groups in the world.

Patients and Methods

Patients

The present study recruited 500 chronic HBV carriers (hepatitis B surface antigen [HBsAg]-positive for more than 6 months) who had been followed up in our liver clinics in the Queen Mary Hospital, Hong Kong. Upon their first and/or follow up visits, these HBV carriers had given verbal informed consent for the storage of blood samples for further studies. We have also recruited 706 consecutive HBsAg-negative control subjects who have donated blood at the Hong Kong Red Cross Blood Transfusion Service, and they all had given verbal informed consent during blood donation for the storage of blood samples. Data were analyzed anonymously for the 706 HBsAg-negative blood donors. Approval has been obtained from the Institution Review Board, Queen Mary Hospital, The University of Hong Kong, for retrieving archived samples for this study. Among the 706 HBsAg-negative subjects, 202 had previous history of hepatitis B vaccination and were excluded from the subsequent analysis. All study patients/subjects are Chinese, and all blood samples were collected during the period January 2010 to March 2011. All subjects were tested negative for hepatitis C virus and human immunodeficiency virus by the Procleix Ultrio Assay (Novartis Diagnostics, Emeryville, CA).

Of the 504 non-vaccinated control subjects, 259 had HBV natural clearance (HBV clearance group), denoted by the presence

of detectable anti-HBc by the Elecsys assay (Roche Diagnostics, Basel, Switzerland). The remaining 245 subjects (non-HBV infected group) were negative for both HBsAg and anti-HBc. Longitudinal clinical data, including alanine aminotransferase (ALT) and HBV DNA measurements, were obtained from the 500 HBV carriers. Inactive asymptomatic HBV carriers were defined by HBV DNA levels <2,000 IU/ml and persistently normal ALT (<58 U/L for male and <36 U/L for female) for least 12 months.

Genotyping Assays

Three SNPs within chromosome 6, namely rs3077 (in the 3' untranslated region of the *HLA-DPA1* gene), rs9277378 and rs3128917 (inside and near the *HLA-DPB1* gene, respectively), were studied (Figure 1). rs3077 was selected for this study because rs3077 and rs9277535 (at the 3' untranslated region of *HLA-DPB1*) have been identified to be strongly associated with persistent HBV infection [13]. We chose to study rs9277378 instead of rs9277535 because, our previous large scale genotypic analysis revealed that, rs9277378 was more readily detected in DNA extracted from sera than rs9277535 (data not shown). Moreover, linkage disequilibrium (LD) analysis by the Haploview software (version 4.2) revealed that rs9277378 has a strong LD with rs9277535 (D' = 1.00, R^2 = 0.954) in the HapMap Han Chinese in Beijing (CHB) and Japanese in Tokyo (JPT) Populations. [22] We also confirmed, in a small subset of 50 randomly selected samples from the current study, that rs9277535 and rs9277378 genotypes were concordant in 48 (96%) samples with a strong LD (D' = 1.00, R^2 = 0.903). The SNP rs3128917 was also chosen for the present study, as this SNP has the highest odds ratio (OR) among 11 SNPs which influence chronicity of HBV infection [16].

The 3 selected SNPs, rs3077, rs9277378 and rs3128917, were genotyped using the TaqMan SNP genotyping assay (Life Technologies, Carlsbad, CA). Briefly, free circulating DNA was extracted from 200 μl of serum samples, using the Purelink Genomic DNA Mini Kit (Life Technologies). The SNP genotyping reaction was performed in a TaqMan real-time PCR format, using SNP-specific primers and FAM- and VIC-labeled allele-specific probes provided in the TaqMan SNP genotyping kit (Life Technologies) and the real-time PCR enzymes and reagents provided in QuantiFast Probe PCR Kit (QIAGEN, Hilden, Germany). The real-time PCR reaction was performed in a RotorGene-Q real-time PCR System (QIAGEN).

Statistical Analyses

Statistical analyses were performed using PLINK v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [23] and SPSS 18.0 (SPSS Inc., Chicago, IL). Logistic regression was performed to compare between case and control groups, and all ORs and p values were adjusted for age and sex. The Student *t* test was used to test normally distributed variables. Categorical variables were tested by the Chi-square test. Statistical significance was defined by p<0.05.

Results

Demographic Characteristics and Hardy-Weinberg Equilibrium

The mean age of the HBV carriers was 46.8±12.1 years, which was significantly higher than that of the non-HBV infected subjects (36.4±9.9 years; p<0.0001) and that of the HBV clearance subjects (40.3±10.9 years; p<0.0001). The proportion of male (304/500; 61%) in the HBV carriers was significantly higher than that in the non-HBV infected group (127/245; 52%;
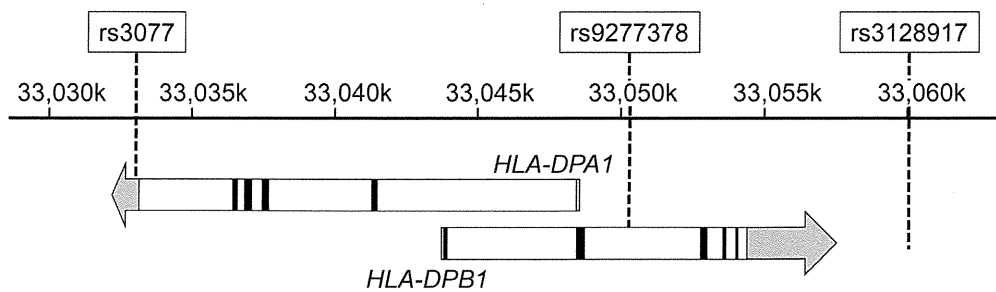
Figure 1. Relative locations of the three studied SNPs and *HLA-DPA1* and *HLA-DPB1* genes on chromosome 6. The names of the three SNPs are shown on top, and the chromosomal positions on chromosome 6 are marked in the ruler in the middle. The *HLA-DPA1* and *HLA-DPB1* genes are shown as arrows in the bottom, with the exons shown as black boxes, introns as white boxes, and un-translated regions as gray boxes/arrows.
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p = 0.020), but did not differ from that in the HBV clearance subjects (152/259; 59%; p = 0.573). Three SNPs (rs3077, rs9277378, and rs3128917) were genotyped in these 500 HBV carriers, 245 non-HBV infected controls and 259 HBV clearance subjects. All 3 polymorphisms in the HBV carriers, non-HBV infected controls and HBV clearance subjects were in Hardy-Weinberg equilibrium, and there was no significant difference between the observed and expected genotypic frequencies in all 3 SNPs in all 3 groups (all p>0.05; Table S1).

Association between HLA-DP Polymorphisms and Chronicity of HBV Infection

The allelic frequencies of the three studied SNPs are listed in Table 1. The minor alleles for rs3077, rs9277378 and rs3128917 determined from the present study cohort were T, A and T, respectively. There was a significantly higher proportion of the rs3077 and rs9277378 minor alleles (T and A, respectively) in the non-HBV infected controls than in the HBV carriers (p = 0.0040 and 0.0068, respectively). There was a trend of a higher proportion of the rs3128917 T allele in the non-HBV infected controls than in the HBV carriers (p = 0.054). The HBV clearance subjects had a significantly higher proportion of rs3077 T allele, rs9277378 A allele, and rs3128917 T allele than in the HBV carriers (p = 0.0083, 0.00011, and 0.00017 for rs3077, rs9277378 and rs3128917, respectively).

Genotype frequencies for the 3 SNPs were compared between the HBV carriers and non-HBV infected controls, as well as between the HBV carriers and HBV clearance group. The genotype distributions of the 3 study groups are listed in Table 2. Compared with the non-HBV infected controls, HBV carriers had a lower prevalence of the minor alleles of rs3077 and rs9277378, as shown by both the dominant-effect (homozygote minor+heter-

ozygote vs. homozygote major) model (p = 0.0089 and 0.0162 for rs3077 and rs9277378, respectively) and the additive-effect (additive dosage of minor allele) model (*P* = 0.0036 and 0.0058 for rs3077 and rs9277378, respectively). There was also a lower frequency of the rs3128917 T allele in the HBV carriers when analyzed using the dominant-effect model (p = 0.0395), but the difference was only marginal when the additive-effect model was applied (p = 0.0561).

Comparison was also made between the HBV carriers and HBV clearance subjects to test the association of these 3 SNPs with natural clearance of HBV infection. As shown in Table 2, rs3077 T allele, rs9277378 A allele, and rs3128917 T allele were associated with an increased chance of clearance of infection in both the dominant-effect model (rs3077: OR = 1.42, 95% confidence interval [CI] = 1.04–1.95, p = 0.0284; rs9277378: OR = 1.61, 95% CI = 1.18–2.2, p = 0.0029; and rs3218917: OR = 1.79, 95% CI = 1.29–2.48, p = 0.00054) and the additive-effect model (rs3077: OR: 1.42, 95% CI = 1.1–1.83, p = 0.0079; rs9277378: OR = 1.62 95% CI = 1.27–2.07, p = 0.00011; and rs3218917: OR = 1.52, 95% CI = 1.22–1.9, p = 0.00024).

Genotypic analysis showed that rs9277378 AA genotype might be most relevant to the clearance of HBV infection (OR = 3.20, p = 8.71×10^{-5}; Table 2). Therefore we performed subgroup analysis to investigate the role of rs3077 and rs3128917 in the patients/subjects with rs9277378 GG genotype, which represent the genotype least likely to clear HBV infection. As shown in Table 2, 398 patients/subjects had rs9277378 GG genotype: 283 (56.6%) HBV carriers and 115 (44.4%) subjects with HBV clearance. Among these 398 patients/subjects, there was no significant difference between the HBV carriers and HBV clearance subjects in the proportion of the protective allele of rs3077 (A allele proportion = 9.2% vs. 11.3%, respectively;

Table 1. Allelic difference and its association with chronicity and clearance of HBV infection.

SNP ID	Minor Allele	HBV carriers (2n = 1000)	Non-HBV infected subjects (2n = 490)	HBV Clearance subjects (2n = 518)	OR (95% CI)*	p*	OR (95% CI)†	p†
rs3077	T	207 (20.7%)	141 (28.8%)	143 (27.6%)	0.67 (0.51–0.88)	0.0040	1.41 (1.09–1.82)	0.0083
rs9277378	A	242 (24.2%)	159 (32.5%)	176 (34.0%)	0.70 (0.54–0.91)	0.0068	1.61 (1.26–2.05)	0.00011
rs3128917	T	335 (33.5%)	202 (41.2%)	231 (44.6%)	0.78 (0.62–1.00)	0.054	1.54 (1.23–1.93)	0.00017

All logistic regression analyses were adjusted for age and sex.
*HBV carriers vs. non-HBV infected subjects.
†Clearance subjects vs. HBV carriers.
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Table 2. Association of HLA-DP genotypes with chronicity and clearance of HBV infection.

SNP ID	Genotype/genetic model	HBV carriers (%) n = 500	Non-HBV infected subjects (%) n = 245	HBV Clearance subjects (%) n = 259	OR (95% CI)*	p*	OR (95% CI)†	p†
rs3077	CC	314 (62.8)	123 (50.2)	136 (52.5)	1.00	–	1.00	–
	TC	164 (33.0)	103 (42.0)	103 (39.8)	0.68 (0.48–0.97)	0.0312	1.31 (0.94–1.82)	0.109
	TT	21 (4.2)	19 (7.8)	20 (7.7)	0.41 (0.20–0.87)	0.0193	2.35 (1.20–4.58)	0.0125
	Dominant				0.64 (0.45–0.89)	0.0089	1.42 (1.04–1.95)	0.0284
	Additive				0.66 (0.50–0.87)	0.0036	1.42 (1.10–1.83)	0.0079
rs9277378	GG	283 (56.6)	109 (44.5)	115 (44.4)	1.00	–	1.00	–
	AG	192 (38.4)	113 (46.1)	112 (43.2)	0.69 (0.49–0.98)	0.0402	1.40 (1.00–1.94)	0.0475
	AA	25 (5.0)	23 (9.4)	32 (12.4)	0.43 (0.22–0.83)	0.0119	3.20 (1.79–5.71)	8.71×10^{-5}
	Dominant				0.66 (0.47–0.93)	0.0162	1.61 (1.18–2.2)	0.0029
	Additive				0.68 (0.52–0.90)	0.0058	1.62 (1.27–2.07)	0.00011
rs3128917	GG	227 (45.4)	83 (33.9)	80 (30.9)	1.00	–	1.00	–
	TG	211 (42.2)	122 (49.8)	127 (49.0)	0.68 (0.47–0.98)	0.0395	1.64 (1.16–2.32)	0.0056
	TT	62 (12.4)	40 (16.3)	52 (20.1)	0.68 (0.41–1.14)	0.141	2.22 (1.40–3.52)	6.84×10^{-4}
	Dominant				0.69 (0.49–0.98)	0.0395	1.79 (1.29–2.48)	0.00054
	Additive				0.79 (0.62–1.00)	0.0561	1.52 (1.22–1.90)	0.00024

All logistic regression analyses were adjusted for age and sex.
*HBV carriers vs. non-HBV infected subjects.
†HBV clearance subjects vs. HBV carriers.
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p = 0.727) and rs3128917 (T allele proportion = 13.8% vs. 17.4%, respectively; p = 0.254).

Haplotype Analysis

LD information of these 3 SNPs for our 3 study groups is shown in Table S2. Haplotype analysis was also performed to assess the effect of the combination of these SNPs on HBV chronicity and clearance of HBV. Of the 8 possible haplotypes out of these 3 SNPs, 5 common haplotypes (with overall haplotype frequencies >0.05) were identified. As shown in Table 3, comparing to the haplotype containing all 3 risk alleles (CGG), the haplotypes TAT and CAT were associated with a higher chance of HBV clearance (for TAT: OR = 1.64, 95% CI = 1.21–2.24, p = 0.0013; and for CAT: OR = 1.98, 95% CI = 1.35–2.9, p = 0.00041). Since both haplotype CAT and TAT were associated with HBV clearance, we also performed haplotype analysis on only the last 2 SNPs (rs9277378 and rs3128917; both at *HLA-DPB1* gene). The haplotype AT was significantly associated with an increased chance of HBV clearance (OR = 1.70, 95% CI = 1.32–2.18, p = 3.66×10^{-5}, with reference to haplotype GG).

Association between HLA-DP Polymorphisms and HBV Disease Activity

Among the 500 HBV carriers, 192 (38.4%) were asymptomatic inactive carriers (HBV DNA levels <2,000 IU/ml and persistently normal ALT for least 12 months). The active carriers were significantly older than the inactive carriers (mean age: 48.1±12.3 vs. 44.7±11.7 years, respectively; p = 0.002), and there was a higher percentage of male in the active carriers (66%) than in the inactive carriers (53%; p = 0.003). Association analysis showed that there were no significant differences in the allele frequency of rs3077, rs9277378, and rs3128917 between the active and inactive HBV carriers (p = 0.175, 0.240, and 0.656, respectively). Similarly, there were no significant genotypic differences between the active and inactive carriers when with the dominant model (p = 0.341, 0.411 and 0.495 for rs3077, rs9277378 and rs3128917, respectively) and additive model (p = 0.172, 0.229 and 0.663 for rs3077, rs9277378 and rs3128917, respectively) were applied. None of the haplotypes was associated with HBV disease activity (all p>0.05).

Discussion

Recent GWAS studies have suggested that certain variations in the *HLA-DP* regions are associated with protection against chronic hepatitis B as well as viral clearance [13,14,15]. In the present study, we have studied 3 SNPs to extend our understanding of the association of these variations with HBV infection in Chinese population in Hong Kong and identified that the rs3077 T allele, rs9277378 A allele and rs3128917 T allele were protective for chronicity of HBV infection. While other studies have demonstrated that *HLA-DP* SNPs rs3077 and rs9277535 are strongly associated with chronic hepatitis B infection [13,14,15,16,17,18,19,20,21], to our knowledge, the present study is the first study to determine the association between rs9277378 and chronicity of HBV infection. Although it is possible that the authentic effect of rs9277378 polymorphism may be due to its high LD with rs9277535, our findings with rs9277378 suggested that more SNPs (or combination of SNPs) in the *HLA-DP* regions may be associated with HBV infection.

Data on the association of HLA-DP variations with chronic HBV infection are relatively scarce. In one study with 201 Caucasian chronic HBV carriers and 235 controls, the rs3077 T allele has also been identified to be protective against chronic HBV infection [18]. However, in that study, the rs3077 T protective allele was the major allele in the Caucasian cohort. This is consistent with the data from the HapMap project, which show that the frequencies of the protective alleles for rs3077 (T), rs9277378 (A) and rs3128917 (T) were higher in people with European ancestry than in the African and Asian populations [22]. Taken together, all these findings of *HLA-DP* genomic variations may shed light on the difference in the geographic distribution of HBV infection: it is possible that the lower prevalence of chronic HBV infection in the European/Caucasian populations is due to the higher prevalence of the protective *HLA-DP* alleles. Similarly, the high prevalence of chronic HBV infection in the Asian/African populations is likely due to the lower prevalence of the protective *HLA-DP* alleles. However, it should be noted that other factors, apart from HLA-DP variations, are also associated with chronicity of HBV infection. If the *HLA-DP* variations were the sole decisive factors for chronicity, the prevalence of chronic hepatitis B would have been much more than 10% in the Chinese. Moreover, a certain proportion of Asian/Chinese who possess the risk *HLA-DP* alleles may not have contacted HBV in their life time. Thus, many other factors, such as viral, environmental and other host genetic factors, are likely to be associated with chronicity of HBV infection. Nevertheless, the findings from the present as well as other genetic association studies, suggest that *HLA-DP* variations are probably one of the genetic factors which plays an important role in the development of chronicity of HBV infection.

Clearance of HBV infection is associated with a high level of CD4+ T cells response [24,25]. HLA-DP molecules, belonging to

Table 3. Haplotype association with chronicity and clearance of HBV infection, with the most common haplotype, CGG, as the reference.

Haplotype	HBV carriers (%)	Non-HBV infected subjects (%)	HBV Clearance subjects (%)	OR (95% CI)*	p*	OR (95% CI)†	p†
CGG	597 (59.7%)	256 (52.2%)	256 (49.6%)	1	–	1	–
TAT	147 (14.7%)	106 (21.6%)	108 (20.9%)	0.62 (0.45–0.86)	0.0044	1.64 (1.21–2.24)	0.0013
CAT	80 (8%)	49 (10%)	66 (12.8%)	0.70 (0.45–1.09)	0.116	1.98 (1.35–2.90)	0.00041
CGT	102 (10.2%)	41 (8.4%)	50 (9.7%)	1.15 (0.76–1.74)	0.495	1.07 (0.74–1.56)	0.458
TGG	53 (5.3%)	28 (5.7%)	30 (5.8%)	0.70 (0.42–1.17)	0.177	1.31 (0.80–2.13)	0.213

All logistic regression analyses were adjusted for age and sex.
SNP order of haplotype: rs3077, rs9277378, rs3128917.
*HBV carriers vs. non-HBV infected subjects.
†Clearance subjects vs. HBV carriers.
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HLA class II, are involved in antigen presentation to CD4+ T helper cells. The antigen-binding sites of HLA-DP molecules are highly polymorphic, and they play an important role in the physical binding of peptides and subsequent recognition by T-cell [26,27]. While it can be expected that variations in the *HLA-DP* coding regions will affect antigen presentation and hence viral clearance, the 3 studied SNPs do not lie within the *HLA-DP* coding region. The SNP rs3077 lies in the 3' untranslated region of *HLA-DPA1*, rs9277378 lies in the second intron of *HLA-DPB1*, and rs3128917 is located ~2.5kb downstream of *HLA-DPB1* (Figure 1). As variations in these SNPs will not cause specific changes in the *HLA-DP* coding sequence, the effect of variations in these 3 SNPs on HLA-DP function and viral clearance is likely to be indirect. There are at least two possible mechanisms. Firstly, it is possible that variation in these SNPs may alter the expression of the *HLA-DP* genes, through the alternation of non-coding RNA sequence or microRNA binding site, as demonstrated in a recent study that variations in rs3077/rs3128917 and rs9277535 affect the expression of *HLA-DPA1* and *HLA-DPB1* respectively [28]. Secondly, as these SNPs are in a strong LD with the *HLA-DP* alleles, it is also likely that variations in these 3 SNPs reflect some yet to be identified variations in *HLA-DP* coding sequence [13,16]. Thus variations in these 3 SNPs may be a marker for the variations in the *HLA-DP* coding sequence, which in turn affect antigen presentation of HBV-derived peptides and alter immune response and chronicity of infection. In fact, it has been demonstrated in a chimpanzee HBV infection model that the outcome of HBV infection is determined by the kinetics of viral spread and CD4 T-cell priming [29]. This suggests that the outcome of HBV infection can be influenced by the physical binding of HBV-derived peptides and their subsequent recognition by CD4 T-cell, which is dependent on *HLA-DP* polymorphism. The correlation between variations in *HLA-DPA1* and *HLA-DPB1* SNPs and the change in *HLA-DP* gene expression and molecule structure deserves a more thorough sequence analysis, and the functional roles of these polymorphisms remain to be studied.

Haplotype-based association analysis is more sensitive than individual SNP association analysis and can capture additional phenotype-related variants with a greater statistical power. This study found that both haplotypes TAT and CAT were associated with an increase chance of HBV clearance, with ORs of 1.64 and 1.98, respectively, both of which were greater than that of the individual SNPs (Table 1). However, there are two caveats. First, although the haplotype CAT showed the greatest OR of 1.98, its relatively greater 95% CI range and low overall haplotype frequency (0.097; data not shown) suggested that its effect on HBV clearance requires further investigations. Second, compared to the OR for individual alleles in the SNPs (for example, for rs9277378, OR = 1.61; Table 1), there was only a small increase in OR by the current haplotype analysis. In this current study, we found that the rs9277378 AA genotype might have the strongest association with HBV clearance (Table 2), and subgroup analysis indicated that the role of other protective SNPs in the rs9277378 GG subgroup was not significant. Therefore individual SNP analysis may be sufficient to provide information on the single most relevant and best-associated SNP with HBV clearance. Nevertheless, haplotype analysis may still have its value by increasing the statistical power in the association analysis and taking into account the effect of variants in other SNPs.

Given the greater genetic distance and weak LD between rs3077 (near *HLA-DPA1*) and the two other SNPs (rs9277378 and

rs3128917; both near *HLA-DPB1*) and the relatively high LD between rs9277378 and rs3128917 (Table S2), it is possible that the two *HLA-DPB1* SNPs form one haplotype block while rs3077 belongs to a distinct haplotype block. Our finding that haplotype of the *HLA-DPB1* SNPs (rs9277378 and rs3128917) alone was associated with HBV clearance (OR = 1.70), independent on the effect of *HLA-DPA1* SNP rs3077, also pointed to this possibility. Although, in our present analysis, the effect of rs3077 alone on HBV clearance appeared to be less than that of the rs9277378-rs3128917 haplotype, it is likely that a more complex network or combination of more SNPs in the HLA region is associated with chronicity of HBV infection. Other recent studies have identified some SNPs in the *HLA-DQ* region which are also associated with susceptibility to HBV infection [14,17]. The interaction between SNPs in the *HLA-DP* and *HLA-DQ* regions, their association with HBV infection in different populations, and their correlation with *HLA-DP* and *HLA-DQ* gene expression remain to be a challenging task to decode the genetic factors involved in HBV infection.

Another important finding from the present study is that we were not able to identify any association between *HLA-DP* genomic variations and HBV disease activity. This is consistent to other studies which also fail to identify any association between other SNPs in the *HLA-DP* region and HBV disease progression [18,20]. Because only a limited number of SNPs was studied in our and other studies, more in-depth studies may be required to elucidate the association between *HLA-DP* variations and HBV disease activity. Similarly, the association between SNPs in the HLA regions and HCC development remains to be confirmed in different study cohorts. Two recent studies had identified 3 SNPs, rs2856718 (*HLA-DQA2/DQB1*), rs3077 (*HLA-DPA1*), and rs9272105 (*HLA-DQA1/DRB1*) to be associated with HBV-related HCC development [17,30], while other studies failed to associate rs3077 and other HLA SNPs with HBV-related HCC development [15,21,31]. Detailed studies in different populations are needed to further elucidate the association between HLA genetic variations and HBV disease activity and HCC development.

In conclusion, we showed that *HLA-DP* SNP rs3077, rs9277378, and rs3128917 were individually associated with chronicity of HBV infection. Haplotype analysis revealed that haplotype TAT was strongly associated with HBV clearance. None of these 3 SNPs was associated with HBV disease activity.

Supporting Information

Table S1 Hardy-Weinberg calculations for all 3 polymorphisms in the HBV carriers, non-HBV infected and HBV clearance subject groups.
(DOCX)

Table S2 Linkage disequilibrium data in the HBV carriers, non-HBV infected and clearance subjects.
(DOCX)

Author Contributions

Conceived and designed the experiments: DKHW TW YT MFY. Performed the experiments: DKHW TW. Analyzed the data: DKHW TW WKS JF. Contributed reagents/materials/analysis tools: DKHW TW FYH. Wrote the paper: DKHW. Recruitment of study subjects: WKS CKL JF CKL

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Case Report

Hepatic failure in pregnancy successfully treated by online hemodiafiltration: Chronic hepatitis B virus infection without viral genome mutation

Shinju Arata,[1] Akito Nozaki,[1] Kenichi Takizawa,[1] Masaaki Kondo,[1] Manabu Morimoto,[1] Kazushi Numata,[1] Sanae Hayashi,[2] Tsunamasa Watanabe,[2] Yasuhito Tanaka[2] and Katsuaki Tanaka[1]

[1]Gastroenterological Center, Yokohama City University Medical Center, Yokohama, and [2]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

A 23-year-old nulliparous woman, a hepatitis B virus (HBV) carrier with stable liver functions, presented with exacerbation of viral replication (HBV DNA level >9.0 log copies/mL) in gestational week 26. During the subsequent follow up without antiviral therapy, she was hospitalized with progression to hepatic failure in gestational week 35. Following initiation of antiviral therapy with lamivudine, emergent cesarean delivery was conducted for fetal safety. Liver atrophy and persistent hepatic encephalopathy (stage 2) necessitated artificial liver support (ALS) involving online hemodiafiltration (HDF) and plasma exchange. She regained full consciousness after the sixth online HDF session. ALS was terminated after the seventh online HDF session. On day 33 of hospitalization, she was discharged home without sequelae. Genetic analysis of the HBV strain isolated from her serum showed that this strain had genotype C. Direct full-length sequencing identified no known mutations associated with fulminant hepatitis B. HBV-related hepatic failure observed in the present case might have been related to perinatal changes in the host immune response.

Key words: artificial liver support, blood purification therapy, flare, fulminant hepatitis, viral mutation

INTRODUCTION

ALTHOUGH POST-PARTUM EXACERBATION of hepatitis B in hepatitis B e-antigen (HBeAg) positive carrier mothers has been recognized,[1] there are few reports documenting hepatic failures triggered by acute exacerbation of hepatitis B virus (HBV) infection in pregnancy,[2-4] and none of the reports present the genome sequence of HBV in these cases. Herein, we report the clinical efficacy of artificial liver support (ALS) in a case with progression of chronic HBV infection to hepatic failure in late pregnancy. We also report that genetic analysis of HBV identified no known mutation associated with fulminant hepatitis B.

CASE REPORT

A 23-YEAR-OLD NULLIPAROUS Chinese woman, who was found to be hepatitis B surface antigen (HBsAg) positive during a routine antenatal virus screening in China in the second pregnancy trimester, was admitted to the Gastroenterological Center, Yokohama City University Medical Center, at gestational week 26, with abdominal pain and elevated liver enzymes. Her previous detailed status of hepatitis viral markers was not known. She had been in good health until that time, she had no history of liver disease and had not had any abnormality identified by medical check-ups in Japan. Her mother, who lived in China, had a chronic liver disease, but the details were unclear. On admission, her only symptom was mild abdominal pain, and physical examination identified no abnormalities. Laboratory findings were: aspartate aminotransferase (AST), 189 IU/L; alanine aminotransferase (ALT), 247 IU/L; and total bilirubin (T-Bil), 0.7 mg/dL. Test results for hepatitis viral markers (HBsAg, 2000 cut-off index;

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HBeAg, 1704.6 signal/cut-off [by chemiluminescence immunoassay [CLIA]]; HBe antibody [CLIA], 0.1%; HBV DNA level, >9.0 log copies/mL [by real-time polymerase chain reaction]) and negativity for anti-hepatitis B core immunoglobulin M suggested exacerbation of HBV infection. She had no medication history suggestive of drug-related liver dysfunction, and the possibility of HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome was etiologically ruled out by the absence of thrombocytopenia, lack of hemolytic findings and normal blood pressure. Hepatic ultrasonography identified no adipose degeneration, a finding that ruled out acute fatty liver of pregnancy. Liver enzyme levels tended to be increased (AST, 350 IU/L; ALT, 432 IU/L) after bedrest without any specific interventions, with concomitant disappearance of abdominal pain. She was discharged home after 10 days of hospitalization for follow-up outpatient care. Two weeks after discharge, liver enzyme levels tended to be slightly decreased (AST, 271 IU/L; ALT, 395 IU/L). Although her physician recommended use of a nucleoside analog for antiviral therapy, she refused and follow up was continued without medication.

Because lower extremity edema with jaundice developed during gestational week 35, on day 3, she was urgently admitted to the Gastroenterological Center, Yokohama City University Medical Center on day 5 of gestational week 35. On admission, she was conscious with systemic jaundice. Laboratory findings at second admission are summarized in Table 1. Elevated liver enzymes, decrease of platelet count and coagulopathy were observed. The results of serological tests strongly suggested flare of hepatitis B.

Administration of lamivudine at a dose of 100 mg/day was immediately initiated. On day 2 of hospitalization, consciousness disorder developed (hepatic encephalopathy stage 2)[5] with a concomitant decrease in prothrombin time international normalized ratio (PT-INR) to 2.33. Gynecological findings were: dilation, 1.0 cm; effacement, 2.0 cm; station, -2; consistency, medium; and Bishop score, 5. The estimated body-weight of the infant was 2547 g. To provide maternal intensive care and ensure fetal safety, an emergent cesarean delivery was conducted on day 2 of hospitalization. A 2414-g healthy female infant was delivered. The operative duration was 39 min. The total maternal fluid loss was estimated to be 2000 mL, including bleeding. To compensate for this fluid loss, 1250 mL of crystalloids, 2 units of red cell concentrates, 10 units of fresh frozen plasma and 10 units of platelet concentrates were

Table 1 Laboratory findings at second admission

Complete blood count		Blood chemistry		Viral markers	
White blood cell	10 690/μL	Total protein	5.5 g/dL	HBsAg	2000 C.O.I.
Red blood cell	329 × 10⁴/μL	Albumin	2.7 g/dL	HBsAb	0.2 mIU/mL
Hemoglobin	10.3 g/dL	AST	613 U/L	HBcAb	11.76S/CO
Hematocrit	30.5%	ALT	364 U/L	Anti-HBc IgM	0.2S/CO
Platelet	5.5 × 10⁴/μL	LDH	412 U/L	HBeAg	1145.7S/CO
		ALP	1228 U/L	HBeAb	0.1%
Coagulation		γ-GTP	100 U/L	HBV DNA	>9.0 log copy/mL
Prothrombin time	39%	Total bilirubin	11.4 mg/dL	HAV Ab	11.3%
PT-INR	1.98	Direct bilirubin	8.4 mg/dL	Anti-HAV IgM	0.1 C.O.I.
APTT	36.9 s	Blood urea nitrogen	5 mg/dL	HCV Ab	0.1 C.O.I.
Fibrinogen	105 mg/dL	Creatinine	0.56 mg/dL	HIV-1/2 Ab	0.1 C.O.I.
		Ammonia	44 μg/dL	HTLV-1 Ab	0.2
				EBV-VCA IgG/IgM	160/<10
				CMV IgG/IgM	16.2/0.55
				VZV IgG	29.1
				HSV IgG	74

Ab, antibody; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; C.O.I., cut-off index; CMV, cytomegalovirus; EBV, Epstein–Barr virus; GTP, glutamyl transpeptidase; HAV, hepatitis A virus; HBcAb, hepatitis B core antibody; HBeAg, hepatitis B e-antigen; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; Ig, immunoglobulin; LDH, lactate dehydrogenase; PT-INR, prothrombin time international normalized ratio; S/CO, signal/cut-off; VCA, viral capsid antigen antibody; VZV, varicella zoster virus.

infused. These infusions stabilized her hemodynamic parameters.

On day 4 of hospitalization, hepatic encephalopathy (stage 2)[5] persisted and the patient's liver volume was estimated to be 722.7 mL by computed tomography (CT). ALS involving online hemodiafiltration (HDF) and plasma exchange was initiated the same day. Online HDF was performed as described previously (Fig. 1).[6] Operational settings at initiation were: blood flow rate (QB), 350 mL/min; dialysate flow rate (QD), 350 mL/h; and substitution fluid flow rate (QS), 350 mL/h. At treatment initiation, the duration of an online HDF session was set to assure that the amount of hemocatharsis (blood flow rate × time) was three times the estimated body fluid volume (actual bodyweight [g] × 0.6 × 3 mL). If the patient recovered from encephalopathy and disorientation disappeared, the duration of online HDF was to be reduced to two-thirds of the initial duration, and if consciousness was maintained under this condition, online HDF was to be given once every other day. If clear consciousness was still maintained under this condition, online HDF was performed with an even longer interval between sessions. Plasma exchange was performed in combination with online HDF if the PT-INR exceeded 2.0, mainly to compensate for blood coagulating activities. ALS was terminated if the patient remained conscious without ALS with PT-INR maintained below 2.0.

An improvement in the patient's consciousness level was observed after the fourth online HDF session, and the patient became fully conscious after the sixth online HDF session. ALS was terminated after the seventh

Figure 1 Circuit of online hemodiafiltration with pre-dilution. Sterile substitution fluid produced online from the dialysate through three ultra-filters. Substitution fluid was infused pre-filter with a substitution fluid pump. ETRF, endotoxin retentive filter; HD, hemodialysis.

online HDF session. On day 20 of hospitalization, liver volume was estimated to be 920.0 mL by CT. The liver biopsy findings on day 23 of hospitalization were consistent with A3/F3–4. On day 33 of hospitalization, she was discharged home without sequelae.

Since hospital discharge, antiviral therapy has been continued with entecavir (switched from lamivudine). Laboratory test findings obtained 6 months after discharge were: AST, 21 IU/L; ALT, 17 IU/L; T-Bil, 0.9 mg/dL; platelet count 115 000/μL; and PT-INR, 1.07. HBV DNA assay revealed a decrease in the viral DNA copy number to less than 2.1 log copies/mL. She is currently in good health with no apparent symptoms of viral hepatitis.

Genetic analysis of the HBV strain isolated from this patient's serum, collected on hospital admission in gestational week 26, showed that this strain had genotype C. Further, direct full-length sequencing was performed at Nagoya City University, the result identified no known mutations associated with other fulminant hepatitis B cases. Comparison of representative core promoter/precore mutations in the HBV strain from this case with the sequences from previous reports are summarized in Table 2. There were no difference between this case and inactive carriers.

DISCUSSION

IN GENERAL, CHRONIC HBV infection is believed to follow a relatively favorable clinical course during pregnancy. A large-scale study comparing perinatal outcomes of pregnant women positive for HBsAg ($n = 824$) with those of non-carrier controls ($n = 6281$) reported no differences between the two groups.[9] On the other hand, post-partum exacerbation of hepatitis B in HBeAg positive carrier mothers is already known,[1] and is a phenomenon that may be related to a sharp post-partum decrease in levels of adrenal corticosteroids that had been elevated during pregnancy. These elevated adrenal corticosteroids during pregnancy, facilitating viral replication, may be associated with an increase in viral load during pregnancy. Rawal *et al.*[10] reported a case of reactivation of chronic hepatitis B infection at gestational week 33 who had developed hepatitis flare. A tendency for the ALT level to increase during late pregnancy in HBsAg positive women has also been reported, although without a significant change in viral load during pregnancy.[11] According to Li *et al.*,[12] fulminant hepatitis in pregnancy occurs most frequently during late pregnancy and tends to have more severe complications than fulminant hepatitis not developing in preg-
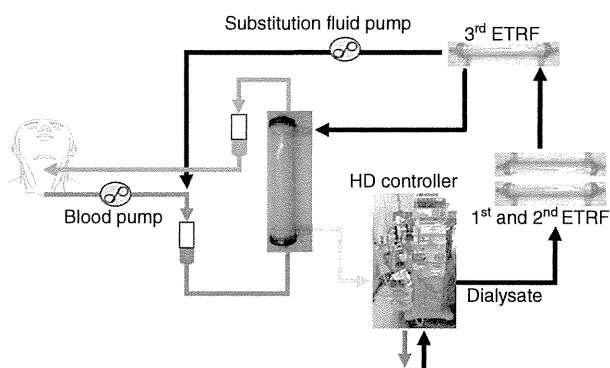
Table 2 Representative core promoter/precore mutations in the HBV strain from this case compared with the sequences from patients with fulminant hepatitis and inactive carriers

Isolated clone	Genotype	Nucleotide number†					
		1753	1754	1762/1764	1862	1896	1899
Fulminant hepatitis‡	B or C	G	G	T/A	T	A	A
Inactive carrier§	C	T	T	A/G	G	G	G
Present case	C	T	T	A/G	G	G	G

†Representative core promoter/precore mutations are indicated in bold type.
‡These mutations are common in hepatitis B virus (HBV) strains from patients with fulminant hepatitis.[7,8]
§The sequences are based on five HBV clones isolated from hepatitis B e-antigen positive inactive carriers whose serum viral titers were over 10^8 copies/mL (X04615, AB033550, AB033553, AB033556, AB033557). The five HBV clones were all genotype C.

nancy. Considering prior case reports of hepatic failure in pregnancy,[2–4] careful monitoring for viral replication in pregnant HBV carriers is important for the prevention of hepatitis exacerbation and for improving perinatal outcomes.

In addition to the reported influences of HBV genotype and mutations on outcomes of acute HBV infection,[7,8] associations of HBV genotype B[13,14] and basal core promoter/precore mutations[13–15] with HBV-related acute-on-chronic liver failure have been suggested. Because genetic analysis of the HBV strain isolated from the present patient identified none of the known aforementioned sequence mutations, the HBV-related hepatic failure observed in the present case may be ascribable to perinatal changes in host immune responses to HBV and HBV-infected liver cells rather than viral genomic factors.

A randomized placebo-controlled study showed that lamivudine given during late pregnancy safely and significantly reduced perinatal vertical transmission of HBV in pregnant HBV carriers.[16] Compared to the untreated control group, a significant reduction in serum HBV was observed in the study group receiving lamivudine from the 24th to 32nd week of pregnancy, although there was no significant difference in the rate of ALT normalization.[16] Furthermore, the perinatal vertical transmission rate in the study group was significantly lower than that of the control group with no significant between-group differences in the incidence of congenital malformations.[17] These findings appear to provide strong evidence for the efficacy and safety of lamivudine in pregnancy. More recently, the efficacy and safety of other anti-HBV agents, namely, tenofovir and telbivudine, have been reported.[18,19] In the present case, initiation of antiviral therapy at gestational week 26 when a high viral load was demonstrated may have prevented progression to hepatic failure.

We previously reported the first clinical application of online HDF (originally intended as a renal replacement therapy) for treating hepatic encephalopathy associated with acute hepatic failure as well as the outcomes of this treatment strategy.[6] Online HDF can supply an unlimited amount of substitution fluid prepared from the dialysate using an online system. This resulted in full recovery of consciousness from hepatic encephalopathy after approximately four online HDF sessions. Furthermore, concomitant use of plasma exchange ensures compensation of liver functions in ahepatic patients. Online HDF has two advantages over conventional HDF techniques, namely, lower cost and improved safety. The latter advantage results from a lower frequency of blood circuit change required for addressing issues caused by blood clotting within the circuit. As a consequence, online HDF is expected to serve as a first-line ALS technique. It is also extremely well suited to serving as a bridge until liver regeneration or liver transplantation.

We reported a case with progression of chronic HBV infection to hepatic failure in pregnancy successfully treated by online HDF. The HBV-related hepatic failure observed in the present case may be related to perinatal changes in host immune responses, because no genetic mutations associated with fulminant hepatitis B were detectable within the genome of the HBV strain isolated from this patient. Further investigation is needed to elucidate the indications for antiviral therapy during pregnancy.

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Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

T. Sakamoto,[1,2] Y. Tanaka,[1] T. Watanabe,[1] S. Iijima,[1] S. Kani,[1] M. Sugiyama,[1] S. Murakami,[1] K. Matsuura,[1,2] A. Kusakabe,[1,2] N. Shinkai,[1,2] S. Fuminaka[1,2] and M. Mizokami[1,3]   [1]*Department of Virology & Liver unit;* [2]*Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; and* [3]*Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan*

SUMMARY. Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by worldwide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/ G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter–driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

Keywords: co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

Correspondence: Masashi Mizokami, MD, PhD, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine 1-7-1, Kohnodai, Ichikawa 272-8516, Japan. E-mail: mmizokami@hospk.ncgm.go.jp

Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

MATERIALS AND METHODS

Plasmid constructs of HBV DNA and sequencing

Hepatitis B virus DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hind*III and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

HBV DNA mutagenesis and construct design

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to

selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CAACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the ε loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP +core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

Cell culture and transfection

After 16 h of culture, Huh7 cells were transfected with 5 μg of DNA construct per 10-cm diameter dish using the Fugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (Toyobo, Osaka, Japan) [17]. Three experiments were conducted for each clone.
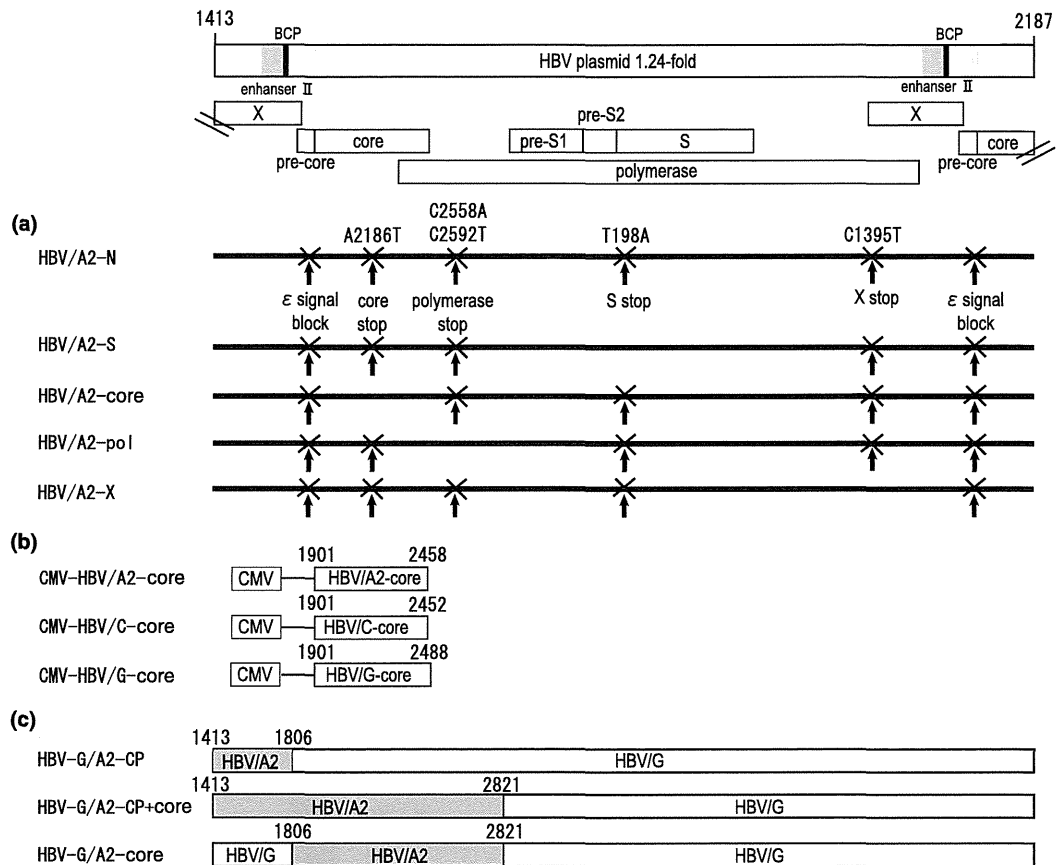
Fig. 1 HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation (ε signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding ε signal.

Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100 μg/mL of RNase A and 200 μg/mL of DNase I for 2 h at 37° C, in the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20 μg of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

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membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12–mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at 200 000 × g for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Chaska, MN, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

Immunoprecipitation

Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'Magnosphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100-μL aliquot of sample was mixed with 100 μL of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

RESULTS

The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower

(0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G
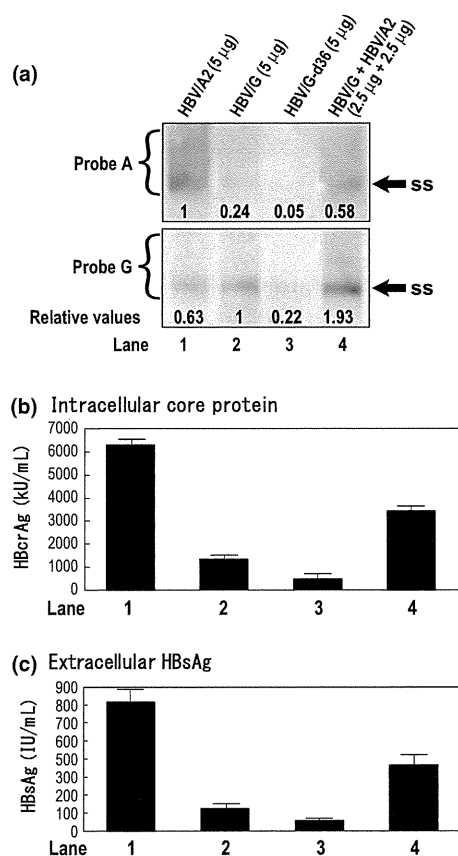

Fig. 2 (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). All experiments were tested at least three times.

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