JVI Journals.ASM.org

HLA-DP Polymorphisms Affect the Outcomes of Chronic Hepatitis B Virus Infections, Possibly through Interacting with Viral Mutations

Qi Zhang,[a] Jianhua Yin,[a] Yuwei Zhang,[a] Yang Deng,[a] Xiaowei Ji,[a] Yan Du,[a] Rui Pu,[a] Yifang Han,[a] Jun Zhao,[b] Xue Han,[c] Hongwei Zhang,[a] Guangwen Cao[a]

Department of Epidemiology, Second Military Medical University, Shanghai, China[a]; Department of Hepatobiliary Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China[b]; Division of Chronic Diseases, Center for Disease Control and Prevention of Yangpu District, Shanghai, China[c]

Genetic polymorphisms of *HLA-DP* have been associated with hepatitis B virus (HBV) persistence. We aimed to determine the effect of *HLA-DP* polymorphisms on the generation of HBV mutations and their interactions on the outcomes of HBV infection. rs3077, rs3135021, rs9277535, and rs2281388 were genotyped in 1,342 healthy controls, 327 HBV clearance subjects, and 2,736 HBV-positive subjects, including 1,108 hepatocellular carcinoma (HCC) patients, using quantitative PCR. HBV mutations were determined by sequencing. Multiplicative interactions of *HLA-DP* polymorphisms and viral mutations were assessed by multivariate logistic regression. rs3077 (from subjects with genotype CT combined with those from subjects with genotype TT [CT+TT] versus CC), rs3135021 (GA+AA versus GG), rs9277535 (GA+AA versus GG), and rs2281388 (CC versus CT+TT) significantly decreased HBV persistence. This effect was found only in genotype B HBV-infected subjects compared to HBV clearance subjects. *HLA-DP* polymorphisms promoting HBV clearance were associated with a lower prevalence of mutations increasing HCC risk (C1653T, T1674C/G, A1846T, G1896A and pre-S2 mutations and pre-S deletion in genotype C) and a higher prevalence of mutations decreasing HCC risk (G1652A, T1673C, T1674C, G1719T, G1730C, and G1799C in genotype B and A1727T in genotype C). Significant effects of viral mutations on cirrhosis and HCC were selectively evident in those with *HLA-DP* polymorphisms promoting HBV persistence. The interactions of C1653T, T1674C/G, and G1896A mutations with *HLA-DP* polymorphisms promoting HBV clearance significantly decreased cirrhosis risk. The interaction of rs9277535 AA with the T1674C/G or G1719T mutation in genotype C significantly decreased HCC risk. In conclusion, *HLA-DP* polymorphisms affect genotype B HBV clearance, regulate immune selection of viral mutations, and influence cirrhosis and HCC risks contributed by HBV mutations.

Chronic infection with hepatitis B virus (HBV) currently affects 350 million to 400 million people worldwide, and over 200,000 and 300,000 HBV-infected subjects die from decompensated hepatic cirrhosis (HC) and hepatocellular carcinoma (HCC), respectively, each year (1, 2). Chronic HBV infection results in approximately one-third of all HC cases and more than one-half of all HCC cases worldwide (3). The World Health Organization includes HBV in "group 1" human carcinogens (4). According to a sequence divergence of >8% in the entire genome, HBV has been classified into at least 8 genotypes so far. HBV genotypes have distinct geographic distributions and have been shown to differ with regard to clinical liver diseases, outcomes, and responses to interferon treatment (5). In East Asia, where HBV genotypes B and C are endemic, viral factors of HBV, including genotype C infection, hepatitis B virus e antigen (HBeAg) expression, high viral load ($>10^4$ copies/ml), and mutations in the enhancer II/basal core promoter/precore (EnhII/BCP/PC) and the pre-S regions, as well as active hepatic inflammation contribute greatly to the development of advanced liver diseases, especially HCC (6–16). Some of the mutations can happen years before a diagnosis of HCC is made and gradually accumulate during the progression of chronic liver diseases (9, 13–16). Some of the mutations can promote the growth and aggressiveness of HCC cells and predict unfavorable prognoses of HCC patients after surgery (17–19). Thus, HBV mutations can predict the occurrence and prognosis of HCC in HBV-infected subjects.

Chronic infection by HBV frequently occurs in individuals infected perinatally (90%) or during childhood (20% to 30%), when the immune system is thought to be immature (2). About 8.5% of adult patients with acute hepatitis B in mainland China will develop a chronic infection, and those who develop a chronic infection are infected mostly with genotype C HBV (20). In the initial immunotolerant phase of chronic HBV infection, HBeAg is positive, viral load is high, and immune pressure is weak. With the progression of chronic infection, especially during HBeAg seroconversion, HBV mutations gradually occur (21). HBV reverse transcriptase lacks proofreading activity, resulting in an estimated mutation rate of 4.57×10^{-5} nucleotide (nt) substitutions per site per year (22). Inflammatory factors promote HBV mutations, at least partially, via activating cytidine deaminases (23, 24). Insufficient immune responses elicited by HBV antigens select disease-related HBV mutations during the long-term evolutionary process. Only the HBV strains/variants best adapted to the host immune system will survive and thrive in liver. HBV accumulates mutations via minimizing the total number of epitopes recognized by $CD8^+$ T cells, particularly in the HBx and the pre-S1/pre-S2/S regions, to avoid immune clearance (25). These HBV mutations are probably selected via virus-immune interactions in the inflammatory microenvironment.

Received 27 July 2013 **Accepted** 27 August 2013
Published ahead of print 4 September 2013
Address correspondence to Guangwen Cao, gcao@smmu.edu.cn.
Q.Z. and J.Y. contributed equally to this work.

doi:10.1128/JVI.02073-13

Human leukocyte antigen (HLA) plays a pivotal role in the immune response against HBV infection. The complexes of HLA class I molecules and HBV-specific antigen peptides are recognized by $CD8^+$ cytotoxic lymphocytes and trigger hepatocytolysis to eliminate HBV-infected hepatocytes. HLA class II molecules are classified into three isotypes: HLA-DR, -DQ, and -DP. The importance of polymorphic residues in HBV peptide binding and T cell recognition, mainly in the HLA-DR and HLA-DQ molecules, has been intensely studied. However, less is known about HLA-DP molecules. A recent genome-wide association study (GWAS) revealed that 11 single-nucleotide polymorphisms (SNPs) in the *HLA-DPA1* and *HLA-DPB1* regions were significantly associated with HBV persistence in Asians (26). Subsequent studies further reported that some of the 11 SNPs were significantly associated with HBV persistence/clearance in Eastern Asians (27–32). The associations of *HLA-DP* SNPs and HBV-caused advanced liver diseases have not been fully elucidated, except for one study reporting a borderline-significant effect of rs3077 on genetic susceptibility to HCC (28). The effects of *HLA* SNPs on the generation of HC- or HCC-related HBV mutations and their interactions on the outcomes of HBV infection have not been reported.

In this study, we investigated the associations of *HLA-DP* SNPs with the persistence/clearance of HBV and the generation of HC- and HCC-related HBV mutations in subjects chronically infected with genotype B or genotype C. The effects of interactions of HBV mutations with *HLA* SNPs on the risks of HC and HCC were also evaluated. This study highlights the effect of *HLA-DP* polymorphisms on the evolution of the HBV genome during chronic infection and also suggests that HBV mutants affect the occurrence of HC and HCC via interacting with *HLA-DP* polymorphisms.

MATERIALS AND METHODS

Study participants. Healthy controls were recruited between September 2009 and November 2012 at the health examination center of Changhai Hospital (Shanghai, China). They were free of previous or current HBV infection and had no history of liver diseases. Hepatitis B virus surface antigen (HBsAg) seroclearance subjects were defined as those who were seronegative for HBsAg and HBV DNA but seropositive for antibodies to both HBsAg and hepatitis B virus core antigen without HBV vaccination history. Asymptomatic HBsAg carrier (ASC) subjects were seropositive for HBsAg without any clinical liver disease and with a normal alanine aminotransferase (ALT) level (<40 U/liter). Chronic hepatitis B (CHB) patients, HC patients, and HCC patients were diagnosed according to criteria described previously (1, 11). HBsAg seroclearance subjects and ASCs were initially recruited from our community-based cohort established in Yangpu District, Shanghai, in 2010. We enrolled only those subjects who yielded a 100% concordant result between the baseline and the follow-up examinations carried out in June to December 2011. The CHB, HC, and HCC patients were recruited from Changhai Hospital, Changzheng Hospital, and Eastern Hepatobiliary Surgery Hospital, affiliated with this university (Shanghai, China); the 88th Hospital (Shandong, China); Southwest Hospital (Chongqing, China); and Zhangjiagang First People's Hospital (Jiangsu, China) between October 2009 and February 2013. Subjects who were positive for antibodies against hepatitis C virus (HCV), hepatitis delta virus (HDV), and/or human immunodeficiency virus (HIV) were excluded. All participants were self-reported Han Chinese and provided written informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Second Military Medical University.

Serological viral marker testing and HBV genotyping. HBV serological markers and anti-HDV antibody were detected by using enzyme-linked immunosorbent assay kits (Kehua, Shanghai, China, and Wantai Bio-Pharm, Beijing, China, respectively) according to the manufacturers' instructions. Serum anti-HCV, anti-HIV, and ALT levels were examined in the hospitals where the study participants were recruited. Extraction and quantification of HBV DNA and HBV genotyping were carried out as previously described (20, 33).

HBV mutation analysis. Amplification and sequencing of the HBV EnhII/BCP/PC region and pre-S region were carried out as previously described (11, 12, 33). We defined wild-type nucleotides and mutations of HBV genotype B and genotype C, respectively. A nucleotide with the highest frequency in the sequences of HBV from the ASCs seropositive for HBeAg was termed a wild-type nucleotide because HBeAg-positive HBV has been traditionally treated as a wild-type strain (21, 34). Nucleotide substitutions with the other 3 nucleotides and a deletion at each site were termed HBV mutations. A site with a mutation frequency of >10% in all genotype B or genotype C HBV-infected subjects, including HCC patients, was termed a "hot spot" of the mutation.

Selection of *HLA* SNPs and genotyping. Of the 11 *HLA-DP* SNPs indentified by the GWAS (26), we selected the SNPs with a minor allele frequency of >5% in the Chinese Han population according to the International HapMap Project (http://www.hapmap.org/). rs3077 (located in the 3′ untranslated region [UTR] of *HLA-DPA1*) was selected because it was the representative of the DPA1 haplotype block, and rs9277535 (in the 3′ UTR of *HLA-DPB1*) and rs2281388 (in the downstream region of *HLA-DPB1*) were selected because they were functionally different SNPs in the DPB1 haplotype block, as determined by using Haploview 4.2 software (available at http://hapmap.ncbi.nlm.nih.gov/). rs3135021 (in intron 1 of *HLA-DPB1*) was also selected because it did not belong to any haplotype block (data not shown). Genomic DNA was extracted from 200-μl peripheral blood samples by using QIAamp blood kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was carried out with a LightCycler480 instrument (Roche, Basel, Switzerland), using fluorescent-probe real-time quantitative PCR with the following steps: an initial denaturation step for 10 s at 95°C, 10 s at 95°C and 30 s at 60°C for 45 cycles, and 1 s at 40°C. Primers and probes for rs3077 were 5′-TCAGCTTTTCTTCTCACTTCATGTG-3′ (forward primer), 5′-TTGAGGTAATGGATAAGGACAGAGC-3′ (reverse primer), 6-carboxyfluorescein (FAM)-AAACTACCCCAGTGGC-MGB, and 6-hexachloro-fluorescein (HEX)-AAACTACTCCAGTG GCT-MGB; those for rs3135021 were 5′-TAGACCTCTCCACACCCCTCC-3′ (forward primer), 5′-TGAGGGGCTGTATTCAGGAGAT-3′ (reverse primer), FAM-CACACCTAGAAGGTAC-MGB, and HEX-CACACCTAAAAGGTAC-MGB; those for rs9277535 were 5′-CAATGGTGAGCAGACTGCAAATC-3′ (forward primer), 5′-AATGATAAAACATGCTCTCAGTAAGGTATATG-3′ (reverse primer), FAM–CCTGATAGGACCCGTATTCCCACAGC–6-carboxytetramethylrhodamine (TAMRA), and HEX-CCTGATAGGACCCATATTCCCACAGCA-TAMRA; and those for rs2281388 were 5′-AGGTAAGCGTCTTTCCCAAGG-3′ (forward primer), 5′-TCTCTGCAATACCCTCAATGACTG-3′ (reverse primer), FAM-AGCCCAACACCCTCGTCTGCC-TAMRA, and HEX-AGCCCAACACCTTCGTCTGCCAT-TAMRA. For quality control, >10% of the samples were randomly selected for repeat testing, yielding 100% concordance. The rates of successful genotyping for rs3077, rs3135021, rs9277535, and rs2281388 were 98.7%, 98.9%, 98.2%, and 98.2%, respectively, for all study participants.

Statistical analysis. Hardy-Weinberg equilibrium (HWE) was examined for each SNP (http://ihg.gsf.de/ihg/snps.html). HBV DNA with a skewed distribution was adjusted to a normal distribution by logarithmic transformation. Student's *t* test and the chi-square test were employed to evaluate the differences for continuous variables and categorical variables, respectively. Since HBV wild types differ considerably between HBV genotypes B and C (11, 12), HBV mutation analysis was carried out in each stratum after stratification by HBV genotype. Correlation between HBV mutations was evaluated by using the phi coefficient. For the effect of

TABLE 1 Characteristics of study participants[f]

Characteristic	Value for group						
	Healthy controls (n = 1,342)	HBsAg seroclearance subjects (n = 327)	HBV-infected subjects without HCC			HBV-infected subjects with HCC (n = 1,108)	P value(s)
			ASCs (n = 316)	CHB patients (n = 845)	HC patients (n = 467)		
No. (%) of male patients	953 (71.01)	186 (56.88)	186 (58.86)	510 (60.36)	342 (73.23)	931 (84.03)	<0.001,[a,b,c,d] 0.019[e]
Mean age of patients (yr) ± SD	53.06 ± 17.43	57.55 ± 12.02	45.31 ± 10.62	54.03 ± 12.59	52.20 ± 11.08	52.24 ± 11.18	<0.001[d,e]
No. (%) of patients infected with HBV genotype							
B			97 (34.28)	96 (23.41)	56 (21.88)	108 (15.21)	<0.001[b]
C			186 (65.72)	314 (76.59)	200 (78.12)	602 (84.79)	
Mean HBV DNA level ($\log_{10}$ copies/ml) ± SD			3.88 ± 1.80	3.83 ± 1.57	3.96 ± 1.37	3.84 ± 1.21	0.625[b]
Mean ALT level (U/liter) ± SD	21.83 ± 14.65	25.33 ± 24.98	26.29 ± 17.53	108.20 ± 315.98	89.66 ± 155.72	72.16 ± 160.34	

[a] Between HBV-infected subjects with HCC and healthy controls.
[b] Between HBV-infected subjects with HCC and HBV-infected subjects without HCC.
[c] Between HBV-infected subjects without HCC and healthy controls.
[d] Between HBV-infected subjects with HCC and HBV natural clearance cases.
[e] Between HBV-infected subjects without HCC and HBV natural clearance cases.
[f] ALT, alanine aminotransferase; ASC, asymptomatic hepatitis B virus surface antigen carrier; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HC, hepatic cirrhosis; HCC, hepatocellular carcinoma.

HLA-DP SNPs on HBV persistence/clearance and the generation of HBV mutations, an unconditional logistic regression model was conducted to calculate odds ratios (ORs) and their 95% confidence intervals (CIs), adjusting for age and gender. An unconditional logistic regression model was also conducted to evaluate the associations of HBV mutations with the risks of HC and HCC without the adjustment. Effects of multiplicative interactions of HBV mutations with *HLA-DP* SNPs on the risks of HC and HCC were evaluated by multivariate logistic regression, adjusting for age and gender. All statistical tests were two sided and conducted by using SPSS 16.0 for Windows (SPSS, Chicago, IL). A P value of <0.05 was considered statistically significant. For multiple comparisons, P values were adjusted by Bonferroni correction.

Nucleotide sequence accession numbers. Sequences in the pre-S and BCP/EnhII/PC regions of HBV determined in this study were deposited in GenBank with accession numbers KC934199 to KC934467 (BCP/EnhII/PC) and KC934468 to KC934744 (preS).

RESULTS

Characteristics of the participants. Participant characteristics are listed in Table 1. Briefly, age was matched among the HCC patients, the HBsAg-positive subjects without HCC, and healthy controls (P = 0.515). The HBsAg seroclearance subjects were older than healthy controls and the HBsAg-positive subjects with or without HCC. The proportions of men and genotype C HBV-infected subjects were higher for the HCC patients than for the HBsAg-positive subjects without HCC.

Associations of *HLA-DP* polymorphisms with HBV persistence, HBsAg seroclearance, and HBV-caused liver diseases. The genotype frequencies of the 4 polymorphisms conformed to HWE in both healthy controls and HBsAg seroclearance subjects ($P > 0.05$ for each). The variant genotypes of rs3077, rs3135021, and rs9277535 in dominant genetic models were inversely associated with HBV persistence, whereas the variant genotypes of rs2281388 were significantly associated with HBV persistence, compared with healthy controls. Interestingly, the variant genotypes of rs3077, rs3135021, and rs9277535 and the wild-type genotype of rs2281388 were inversely associated with HBV persistence compared to the HBsAg seroclearance subjects, and these effects were found solely in the genotype B HBV-infected subjects (Table 2). These data indicate that the variant genotypes of rs3077, rs3135021, and rs9277535 and the wild-type genotype of rs2281388 contribute to genotype B HBV clearance preferentially.

We then compared the distributions of the polymorphisms in ASCs, CHB patients, HC patients, and HCC patients. The heterozygotes of rs3077 and rs3135021 were significantly associated with CHB risk compared to ASCs in the HBV genotype C group (P = 0.048, OR = 1.52, and 95% CI = 1.00 to 2.29, and P = 0.018, OR = 1.68, and 95% CI = 1.09 to 2.57, respectively). The heterozygote of rs3077 and the A allele of rs3135021 were inversely associated with HC risk compared to ASCs plus CHB patients (P = 0.028, OR = 0.77, and 95% CI = 0.61 to 0.97, and P = 0.030, OR = 0.78, and 95% CI = 0.62 to 0.98, respectively). The A allele of rs3135021 was inversely associated with HCC risk compared to the HCC-free subjects in the genotype B group (P = 0.036, OR = 0.58, and 95% CI = 0.35 to 0.97). rs9277535 and rs2281388 were not significantly associated with liver disease.

HC- or HCC-related HBV mutations. The HBV EnhII/BCP/PC region and the pre-S region were successfully sequenced from 1,429 (52.23%) and 1,225 (44.77%) of the HBV-infected subjects, respectively (GenBank accession no. JX556943 to JX559050 and KC934199 to KC934744). Table 3 lists the HBV mutations in the EnhII/BCP/PC region that are significantly associated with HC and HCC. Of these, the C1653T, T1753C, A1762T/G1764A, and G1899A mutations in HBV genotype C and the A1762T/G1764A mutations in HBV genotype B increased in prevalence from the ASC state to HCC successively ($P_{trend} < 0.001$ for each). Significant mutations and their corresponding wild types counterchanged at nt 1652, nt 1673, nt 1727, nt 1730, and nt 1799 between genotype B and genotype C. However, the associations of these mutations with HCC risk were quite consistent in genotype B and genotype C. Interestingly, the T1674C and G1719T mutations

TABLE 2 Associations of *HLA* polymorphisms with HBV persistent infection and HBV natural clearance[a]

HBV genotype	*HLA-DP* SNP	No. of patients with SNP			HBV-positive subjects vs healthy controls		HBV-positive subjects vs HBsAg clearance cases	
		Healthy controls	HBsAg clearance subjects	HBV-positive subjects	AOR (95% CI)	*P* value	AOR (95% CI)	*P* value
rs3077								
Subtotal	CC	537	149	1,465	1.00		1.00	
	CT+TT	793	177	1,226	**0.58 (0.51–0.67)**	**1.60×10^{-14}**	**0.72 (0.56–0.92)**	**8.00×10^{-3}**
Genotype B	CC	537	149	213	1.00		1.00	
	CT+TT	793	177	140	**0.46 (0.36–0.59)**	**3.96×10^{-10}**	**0.55 (0.39–0.77)**	**4.43×10^{-4}**
Genotype C	CC	537	149	669	1.00		1.00	
	CT+TT	793	177	608	**0.34 (0.54–0.74)**	**2.31×10^{-8}**	0.78 (0.60–1.02)	6.68×10^{-2}
rs3135021								
Subtotal	GG	712	180	1,627	1.00		1.00	
	GA+AA	608	147	1,082	**0.79 (0.69–0.90)**	**6.98×10^{-4}**	0.86 (0.67–1.10)	2.36×10^{-1}
Genotype B	GG	712	180	231	1.00		1.00	
	GA+AA	608	147	124	**0.64 (0.50–0.82)**	**4.60×10^{-4}**	**0.69 (0.49–0.97)**	**3.17×10^{-2}**
Genotype C	GG	712	180	762	1.00		1.00	
	GA+AA	608	147	527	**0.82 (0.70–0.97)**	**1.75×10^{-2}**	0.89 (0.69–1.17)	4.10×10^{-1}
rs9277535								
Subtotal	GG	402	118	1,151	1.00		1.00	
	GA+AA	909	207	1,540	**0.60 (0.52–0.69)**	**5.23×10^{-12}**	**0.73 (0.57–0.94)**	**1.62×10^{-2}**
Genotype B	GG	402	118	178	1.00		1.00	
	GA+AA	909	207	174	**0.44 (0.35–0.57)**	**6.42×10^{-11}**	**0.52 (0.37–0.73)**	**1.55×10^{-4}**
Genotype C	GG	402	118	521	1.00		1.00	
	GA+AA	909	207	760	**0.66 (0.56–0.78)**	**8.59×10^{-7}**	0.78 (0.59–1.03)	8.18×10^{-2}
rs2281388								
Subtotal	CC	529	115	758	1.00		1.00	
	CT+TT	798	211	1,915	**1.65 (1.43–1.90)**	**1.02×10^{-11}**	**1.35 (1.05–1.75)**	**2.12×10^{-2}**
Genotype B	CC	529	115	88	1.00		1.00	
	CT+TT	798	211	263	**1.93 (1.47–2.52)**	**1.70×10^{-6}**	**1.59 (1.11–2.28)**	**1.15×10^{-2}**
Genotype C	CC	529	115	377	1.00		1.00	
	CT+TT	798	211	896	**1.53 (1.30–1.81)**	**6.03×10^{-7}**	1.28 (0.97–1.70)	7.94×10^{-2}

[a] Boldface type indicates significant values. AOR, adjusted odds ratio (adjusted for age and gender); CI, confidence interval; HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism.

were protective factors for HCC in genotype B and risk factors for HCC in genotype C. Significant HBV mutations in the pre-S region of HBV genotypes B and C are shown in Table 4. The wild-type nucleotides and mutations counterchanged at almost all hot spots in the pre-S1 region between genotype B and genotype C. Almost all substitution mutations at the pre-S2 region were risk factors for HC and HCC in both genotypes. Importantly, the frequencies of significant HBV mutations (A1C, A31T, T49A, A52C, G105C, C109A, A135C, and G147C in genotype B and A1C, C7A, C10A, A31T, T49A, A52C, G105C, C109A, A135C, and G147C in genotype C) in the pre-S2 region were extremely low (0.0% in genotype B and 0.0% to 4.2% in genotype C) in ASCs but equally high in the CHB patients, HC patients, and HCC patients (92.3% to 100.0% in genotype B and 89.7% to 100.0% in genotype C).

Association of *HLA-DP* polymorphisms with HBV mutations. We then assessed the associations of the *HLA* SNPs with all the significant HC- or HCC-related HBV mutations (Tables 3 and 4) in the subjects with genotype B and those with genotype C infections, respectively. Generally, the *HLA-DP* polymorphisms promoting HBV clearance were significantly associated with a lower prevalence of HBV mutations increasing HCC risk and a higher prevalence of the mutations decreasing HCC risk in both HBV genotypes, in spite of several exceptions (Table 5).

We then investigated the distribution of the *HLA-DP* SNP-affected HBV mutations in the 4 clinical stages of HBV evolution. In the genotype C group, T1674C/G, A1846T, and G1896A mutations were more frequent in the patients with HBV-related liver diseases (CHB, HC, and HCC) than in ASCs ($P < 0.001$ for each comparison). The 3 mutations were more frequent in the CHB patients than in the HC patients, while T1674C/G and A1846T mutations were more frequent in the HCC patients than in the HC patients. However, the C1673T, A1727T, C1730G, and C1799G mutations were more frequent in ASCs and the HC patients than in the CHB patients and the HCC patients, respectively. In the genotype B group, the pattern of the HBV mutations in the EnhII/BCP/PC region in the 4 stages was different from that of the genotype C group. The frequencies of HBV mutations in the pre-S2 region were very low in ASCs but equally high in the CHB, HC, and HCC patients infected with either genotype B or genotype C. The frequencies of C1653T, pre-S deletion, and pre-S2 start codon mutations in genotype C increased successively from the ASC state to HCC ($P_{trend} < 0.001$ for each). These data are presented in Fig 1.

Effects of interactions of the *HLA-DP* polymorphisms with the HBV mutations on the risks of HC and HCC. Multiplicative interactions of the *HLA* SNPs with all the significant HC- or HCC-

TABLE 3 Associations of nucleotide substitutions in the EnhII/BCP/PC region of HBV genotypes B and C with cirrhosis and HCC

Hot spot	OR (95% CI)[c]			
	Genotype B		Genotype C	
	Cirrhosis patients ($n = 44$) vs ASCs ($n = 77$) plus CHB patients ($n = 64$)	HCC patients ($n = 93$) vs HCC-free HBsAg-positive subjects ($n = 185$)	Cirrhosis patients ($n = 162$) vs ASCs ($n = 174$) plus CHB patients ($n = 196$)	HCC patients ($n = 404$) vs HCC-free HBsAg-positive subjects ($n = 532$)
G1652A[a]	**3.37 (1.57–7.24)**[b]	**0.23 (0.12–0.44)**[b]	**0.52 (0.29–0.93)**	**0.37 (0.23–0.59)**[b]
C1653T	–	–	**1.78 (1.09–2.91)**	**2.41 (1.75–3.32)**[b]
T1673C[a]	1.98 (0.94–4.18)	**0.32 (0.17–0.60)**[b]	1.22 (0.76–1.95)	**0.30 (0.19–0.46)**[b]
T1674C	0.36 (0.10–1.29)	**0.41 (0.17–0.99)**	**0.45 (0.26–0.79)**	**1.65 (1.19–2.28)**[b]
T1674G	–	–	0.97 (0.51–1.86)	**1.95 (1.29–2.94)**[b]
G1719T	1.90 (0.89–4.07)	**0.46 (0.23–0.92)**	1.20 (0.82–1.77)	**2.43 (1.84–3.22)**[b]
A1726C	1.44 (0.70–2.95)	**0.47 (0.28–0.79)**	–	–
T1727G	**2.45 (1.11–5.42)**	**0.35 (0.17–0.73)**	–	–
T1727A[a]	–	–	**1.82 (1.05–3.14)**	**0.24 (0.14–0.41)**[b]
A1727G	–	–	**1.64 (1.02–2.62)**	1.32 (0.98–1.77)
G1730C[a]	1.82 (0.90–3.68)	**0.25 (0.13–0.47)**[b]	1.51 (0.94–2.42)	**0.24 (0.15–0.40)**[b]
T1753C	–	–	**1.84 (1.07–3.15)**	**3.06 (2.17–4.32)**[b]
A1762T/G1764A	**2.80 (1.36–5.77)**	**2.51 (1.50–4.21)**[b]	**4.99 (3.25–7.64)**[b]	**4.37 (3.24–5.87)**[b]
G1799C[a]	1.48 (0.74–2.95)	**0.25 (0.13–0.47)**[b]	1.49 (0.94–2.36)	**0.36 (0.24–0.55)**[b]
A1846T	0.95 (0.43–2.11)	1.42 (0.83–2.43)	0.89 (0.57–1.38)	**1.61 (1.22–2.14)**[b]
G1896A	**2.72 (1.30–5.72)**	1.56 (0.93–2.63)	1.42 (0.95–2.13)	**1.91 (1.46–2.50)**[b]
G1899A	1.80 (0.59–5.55)	**2.53 (1.21–5.28)**	**3.59 (1.77–7.27)**[b]	**4.44 (2.91–6.76)**[b]

[a] The wild-type nucleotide and the corresponding mutation at these hot spots exchange between genotype B and genotype C.
[b] The difference is still significant after the Bonferroni correction (cutoff *P* value = 0.0031).
[c] OR, odds ratio. – indicates that this site is not a hot spot in the given genotype. Boldface type indicates significant values.

related HBV mutations in the subjects with genotype B and those with genotype C infections were evaluated, respectively. In the genotype C HBV-infected subjects, a significant association of the C1653T mutation with HC risk was found only for those with the rs3135021 GG genotype, whereas a significant association of the T1674C/G mutation with HC risk was found only for those with the rs2281388 CT genotype. The interactions of rs3135021 (GA versus GG) with the C1653T mutation and of rs2281388 (CT versus CC) with the T1674C/G mutation significantly decreased HC risk, compared to ASCs plus CHB patients, respectively. In genotype B HBV-infected subjects, a significant association of the G1896A mutation with HC risk was found only for those with the rs3077 CC genotype or the rs3135021 GG genotype. The interactions of rs3077 (from subjects with genotype CT combined with those from subjects with genotype TT [CT+TT] versus CC) and rs3135021 (GA versus GG) with the G1896A mutation significantly decreased HC risk (Table 6). Thus, the C1653T mutation in genotype C and the G1896A mutation in genotype B increase HC risk while the T1674C/G mutation in genotype C decreases HC risk solely in those with the *HLA-DP* genotypes promoting HBV persistence. The associations of the T1674C/G and G1719T mutations with HCC risk were stronger in the genotype C HBV-infected subjects with the rs9277535 GG genotype than in those with the AA genotype, indicating that the T1674C/G and G1719T mutations increase HCC risk more significantly in those with the rs9277535 genotype promoting HBV persistence. The interaction of rs9277535 (AA versus GG) with the T1674C/G or with G1719T mutation significantly reduced HCC risk in the genotype C HBV-infected subjects (Table 7). We did not observe any significant interaction of HCC risk in the genotype B HBV-infected subjects. Thus, the effects of HBV mutations on HC and HCC risks depend on the *HLA-DP* genetic background.

DISCUSSION

This study reported that the A/T allele of rs3077, rs3135021, and rs9277535 and the C allele of rs2281388 were inversely associated with HBV persistence, which is quite consistent with previous findings (26–32). Further stratification analysis revealed, for the first time, that this effect was found solely in genotype B HBV-infected subjects compared to HBsAg clearance subjects (Table 2). HBV clearance happens mostly after an acute infection in adults. HBV genotype B is more apt to cause acute infection in young people and to be more easily cleared than genotype C (20). HBV genotype C leads to higher persistence following an acute course and is more apt to cause HC and HCC than genotype B (6, 20, 35). The association of rs3077 with HBsAg seroclearance is more pronounced in younger patients (36). The association of rs9277535 with HBsAg seroclearance is more evident in southern Chinese than in northern Chinese patients (29). A study failed to link rs9277535 with HBsAg seroclearance in northern Chinese patients (30). This may be explained by the fact that the proportion of patients with HBV genotype B infection consecutively increases from north to south China (33). In Taiwan, where HBV genotype B is endemic, CHB patients with the rs9277535 non-GG genotype have a higher chance of clearing HBsAg (37). Thus, the *HLA-DP* polymorphisms promoting HBV clearance may predispose the host to clear genotype B HBV preferentially.

However, the rs9277535 G allele is not or is weakly associated with HBV persistence in Caucasians (38, 39). This disparity might be due to the diversity in HBV genotype endemicity or in the SNP frequencies among different races. According to the NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/), allelic frequencies of the *HLA-DP* polymorphisms differ greatly among races. C/G is the major allele of rs3077 and rs9277535 in the Asian

TABLE 4 Associations of nucleotide substitutions in the pre-S region of HBV genotypes B and C with cirrhosis and HCC

Hot spot	OR (95% CI)[d]			
	Genotype B		Genotype C	
	Cirrhosis patients ($n = 41$) vs ASCs ($n = 54$) plus CHB patients ($n = 78$)	HCC patients ($n = 69$) vs HCC-free HBsAg-positive subjects ($n = 173$)	Cirrhosis patients ($n = 152$) vs ASCs ($n = 76$) plus CHB patients ($n = 279$)	HCC patients ($n = 412$) vs HCC-free HBsAg-positive subjects ($n = 507$)
In pre-S1 region				
A2875C[a,c]	**4.44 (1.96–10.06)[b]**	0.72 (0.33–1.56)	0.81 (0.41–1.61)	**2.26 (1.53–3.33)[b]**
A2946G[a]	**7.35 (2.94–18.39)[b]**	1.32 (0.64–2.74)	**0.26 (0.12–0.59)[b]**	1.25 (0.86–1.80)
T3026C[a]	**11.89 (4.52–31.27)[b]**	0.86 (0.39–1.90)	0.63 (0.29–1.34)	**1.77 (1.17–2.66)**
Pre-S1 start codon mutation	–	–	1.49 (0.77–2.86)	**2.07 (1.37–3.13)[b]**
In pre-S2 region				
A1C	**28.42 (3.75–215.22)[b]**	**Risk factor[b]**	**21.27 (5.13–88.25)[b]**	**15.40 (6.15–38.56)[b]**
C7A	**20.63 (6.60–64.48)[b]**	1.51 (0.69–3.32)	**12.84 (3.97–41.52)[b]**	**1.97 (1.31–2.97)[b]**
C10A	**Risk factor[b]**	**Risk factor[b]**	**17.42 (4.20–72.19)[b]**	**8.19 (3.89–17.24)[b]**
A31T	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**	**10.75 (4.58–25.25)[b]**
T49A	**Risk factor[b]**	**51.00 (6.31–412.39)[b]**	**35.50 (4.87–258.96)[b]**	**7.00 (3.44–14.25)[b]**
A52C/T	**Risk factor[b]**	**Risk factor[b]**	**16.96 (4.08–70.44)[b]**	**11.65 (5.00–27.17)[b]**
C76A	0.82 (0.37–1.78)	**2.95 (1.53–5.70)[b]**	–	–
G105C	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**
C109A	**Risk factor[b]**	**23.54 (2.90–191.06)[b]**	**Risk factor[b]**	**8.11 (3.84–17.10)[b]**
A135C	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**	**14.88 (5.95–37.24)[b]**
G147C	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**	**Risk factor[b]**
Pre-S2 start codon mutation	0.63 (0.17–2.31)	1.60 (0.74–3.45)	1.01 (0.59–1.72)	**1.93 (1.40–2.67)[b]**
Pre-S deletion	3.11 (1.08–8.96)	1.63 (0.72–3.71)	**1.73 (1.08–2.78)**	**2.45 (1.81–3.33)[b]**

[a] The wild-type nucleotide and the corresponding mutation at these hot spots exchange between genotype B and genotype C.
[b] The difference is still significant after the Bonferroni correction (cutoff *P* value = 0.0016).
[c] Representing highly correlated (phi coefficient, >0.7) mutations, including T2931C, A2950G, A2951G, G2962A, A2964C, T3054A, T3060C, G3063C, A3066T, G3069T, T3120A, G3186A, and C3191G.
[d] – indicates that this site is not a hot spot in the given genotype. Boldface type indicates significance.

population, but it is the variant allele in the European population. This might be one of the reasons why HBV persistence is more frequent in Asians than in Europeans.

In this study, we defined the wild-type nucleotides of HBV genotype B and genotype C from ASCs seropositive for HBeAg. The prevalence of C1653T, T1753C, A1762T/G1764A, and pre-S deletion mutations in HBV genotype C and A1762T/G1764A mutations in HBV genotype B increased successively along with HBV evolution, which was quite consistent with our previous meta-analysis (8), supporting the rationality of this definition. Although the HC- and HCC-related HBV mutations counterchanged at some nucleotides between genotype B and genotype C, the associations of these mutations with HCC risk were quite consistent for both genotypes (Table 3). This reflects the inherent law of HBV evolution since chronic HBV infection is established. T1674C and G1719T mutations were protective factors for HCC in genotype B and risk factors for HCC in genotype C infections (Table 3), and the impacts of the A2875C, A2946G, and T3026C mutations were not consistent between genotypes B and C (Table 4). These findings reflect the diversity of the genetic and antigenic natures of the two HBV genotypes. We found that the frequencies of significant HBV mutations in the pre-S2 region of both genotypes were close to 0% in ASCs but reached approximately 100% in CHB patients, HC patients, and HCC patients. These significant mutations may be selected during acute exacerbation upon the ASC state and kept in subsequent steps of HBV evolution. This novel finding is of great clinical and public health significance.

Associations between HLA class I genotypes/alleles and escape mutations in the HBV core gene have been investigated in chronic HBV infection (40, 41). However, associations of *HLA-DP* polymorphisms with HBV mutations have not been reported. In this study, we found a group of disease-related HBV mutations whose frequencies were significantly affected by *HLA-DP* polymorphisms (Table 5). Of these HBV mutations, the G1896A, A1846T, and T1674C/G mutations reached a high prevalence at the CHB and HCC stages, indicating that their emergence may be quickly triggered by the active immune response (42); the C1673T, A1727T, C1730G, and C1799G mutations were found more frequently in ASCs and HC patients than in CHB and HCC patients, possibly because of immune tolerance in ASCs and HC patients (43, 44). The C1653T, T1674C/G, G1719T, A1727T, C1730G, and C1799G mutations were located in the core promoter region (nt 1591 to nt 1882) (45), resulting in amino acid substitutions at H94Y, S101P, V116L, K118N, D119E, and S142C, respectively, in the C-terminal region of HBx. The C1653T mutation also changes the box-α binding site for the transcription factor C/EBP, which enhances the activity of the core promoter and viral replication (46). The C1673T mutation does not cause an amino acid substitution but binds to transcription factors such as C/EBP (47), resulting in possible alterations in viral activities. The same is true for the A1846T mutation (11). The A31T, G105C, A135C, and G147C mutations lead to amino acid substitutions at E133D, G158A/V, N168T, and G172A, respectively, in the pre-S2 region. The C10A, T49A, and C109A mutations do not cause corresponding amino acid substitutions. They might af-

TABLE 5 Significant associations of *HLA-DP* polymorphisms with frequencies of HBV mutations associates with liver disease risk[d]

	Genotype C HBV-infected subjects		Genotype B HBV-infected subjects	
HLA-DP SNP	HBV mutation	Adjusted OR (95% CI)	HBV mutation	Adjusted OR (95% CI)
rs3077				
CC		1.00		1.00
CT	C1653T	**0.68 (0.48–0.95)**		
	C1673T	**0.65 (0.43–0.99)**		
	Pre-S1 start codon mutation	**1.56 (1.02–2.39)**		
TT	A1846T	**0.54 (0.30–0.97)**	G1719T	**2.97 (1.06–8.37)**
	G1896A	**0.47 (0.27–0.80)**	G1730C	**2.63 (1.01–6.85)**
	C10A[a]	**0.40 (0.17–0.94)**		
	Pre-S deletion	**0.43 (0.20–0.91)**		
rs3135021				
GG		1.00		1.00
GA	C1673T[b]	**0.54 (0.35–0.84)**		
	Pre-S1 start codon mutation	**2.04 (1.34–3.11)**		
AA	A1727T	**2.95 (1.20–7.23)**		
	A1846T	**0.47 (0.23–0.95)**		
rs9277535				
GG		1.00		1.00
GA				
AA	T1674C/G	**0.62 (0.39–0.97)**		
	A1846T	**0.62 (0.39–0.98)**		
rs2281388				
CC		1.00		1.00
CT	T1674C/G	**1.47 (1.04–2.06)**	T1674C	**0.35 (0.14–0.87)**
	Pre-S deletion	**1.49 (1.02–2.17)**	G1719T	**0.41 (0.20–0.84)**
	Pre-S2 start codon mutation	**1.54 (1.02–2.33)**	G1652A[c]	**0.46 (0.24–0.91)**
			A1C	**0.33 (0.13–0.82)**
TT	A1846T	**1.61 (1.06–2.44)**	G1719T	**0.41 (0.18–0.93)**
	G1896A	**1.63 (1.11–2.42)**		
	Pre-S2 start codon mutation	**1.99 (1.23–3.24)**		
	Pre-S deletion	**1.90 (1.21–2.98)**		

[a] Represents highly correlated (phi coefficient, >0.7) mutations, including A31T, T49A, G105C, C109A, A135C, and G147C.
[b] Represents highly correlated (phi coefficient, >0.7) mutations, including A1652G, C1730G, and C1799G.
[c] Represents highly correlated (phi coefficient, > 0.7) mutations, including T1673C, G1730C, and G1799C.
[d] Boldface type indicates significant values.

fect viral activities by altering potential transcription factor binding sites (48). The G1896A mutation introduces a stop codon, W28Stop, in the pre-C region, which impairs HBeAg expression (46). The HBV mutations in the core promoter region fall in HLA-A2-restricted epitopes, while A31T and T49A mutations in the pre-S2 region fall in the restricted epitopes of HLA-A2/A3 and HLA-DR1/DR2 as well as HLA-A2 and HLA-DR1, respectively (49). It is unknown if the *HLA-DP* polymorphism-affected HBV mutations form the restricted epitopes of HLA-DP. The *HLA-DP* genotypes promoting HBV persistence were generally associated with a higher prevalence of HBV mutations increasing HCC risk, indicating that they may play an active role in maintaining an evolutionary microenvironment for the selection of these disease-related HBV mutations. This study enrolled HCC-free HBV-infected subjects around 50 years of age. Some of them will develop HCC because HCC incidence in HBV-infected subjects increases sharply after 60 years of age. HCC-associated HBV mutations are usually generated years before HCC occurs (9, 13–16). Thus, *HLA* polymorphisms might affect the occurrence of HCC via regulating immunoselection of HBV mutations.

Interestingly, the associations of the HBV mutations with the risks of HC and HCC were significantly affected by the *HLA-DP* polymorphisms (Tables 6 and 7). Significant effects of the G1896A, C1653T, and T1674C/G mutations on HC risk were significant only for those with the *HLA-DP* polymorphisms promoting HBV persistence and not for those with the ones promoting HBV clearance. The interactions of these mutations with the *HLA-DP* polymorphisms promoting HBV clearance were protective for HC. One of the reasons for this is that rs2281388 variant genotypes were significantly associated with a lower prevalence of T1674C/G (Table 5). Similarly, the effects of the T1674C/G and G1719T mutations on HCC were stronger in those with the *HLA-DP* polymorphisms promoting HBV persistence than in those with the ones promoting HBV clearance. The interactions of the two mutations with the *HLA-DP* polymorphisms promoting HBV clearance were protective for HCC, possibly because the rs9277535 AA genotype was significantly associated with a lower prevalence of the T1674C/G mutation (Table 5). These interactions should be of importance in identifying HBV-infected subjects who are more likely to develop HC or HCC.

HLA-DPA1 and HLA-DPB1 are normally expressed on a sub-

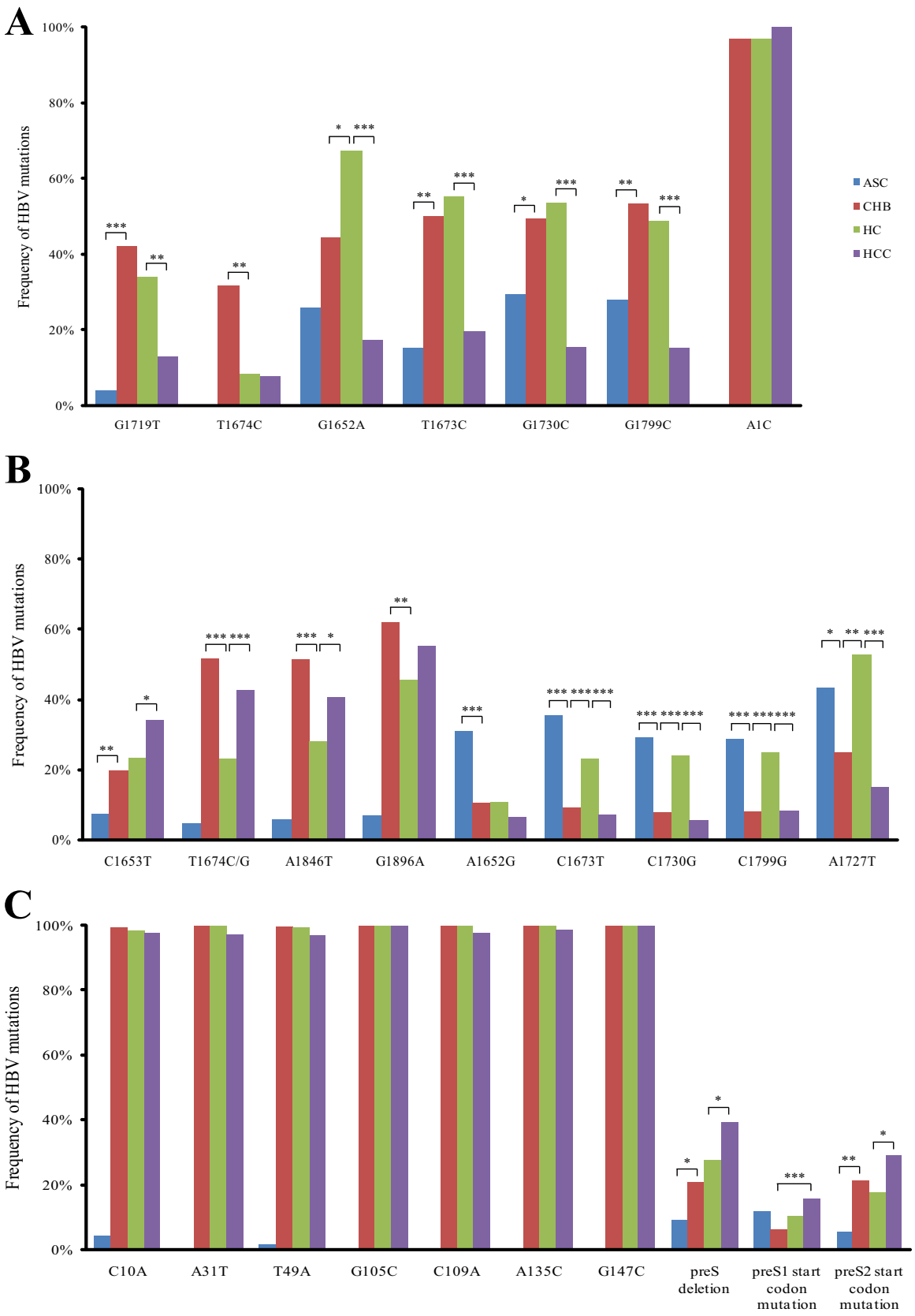

FIG 1 Frequencies of *HLA-DP* genetic polymorphism-affected HBV mutations in asymptomatic hepatitis B surface antigen carriers (ASCs), chronic hepatitis B (CHB) patients, hepatic cirrhosis (HC) patients, and hepatocellular carcinoma (HCC) patients. (A) Mutations in the enhancer II/basal core promoter/precore region and the pre-S region of HBV genotype B. (B) Mutations in the enhancer II/basal core promoter/precore region of HBV genotype C. (C) Mutations in the pre-S region of HBV genotype C. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$.

TABLE 6 Association of the interactions of *HLA* SNPs and HBV mutations with the risk of hepatic cirrhosis in HBV-infected subjects[a]

HLA-DP SNP	HBV mutation	No. of ASCs plus CHB patients	No. of HC patients	Adjusted OR (95% CI)	*P* value
In genotype C HBV-infected subjects					
rs3135021	C1653T				
GG	C	142	72	1.00	
GG	T	22	30	**2.71 (1.45–5.07)**	**0.002**
GA	C	85	42	1.09 (0.67–1.76)	0.734
GA	T	15	5	0.83 (0.49–1.40)	0.481
For interaction				**0.20 (0.06–0.72)**	**0.014**
rs2281388	T1674C/G				
CC	T	74	32	1.00	
CC	C/G	20	15	1.55 (0.66–3.65)	0.316
CT	T	86	56	1.61 (0.92–2.81)	0.094
CT	C/G	67	8	**0.47 (0.30–0.73)**	**0.001**
For interaction				**0.10 (0.03–0.30)**	**<0.001**
In genotype B HBV-infected subjects					
rs3077	G1896A				
CC	G	62	8	1.00	
CC	A	21	17	**4.00 (1.41–11.34)**	**0.009**
CT+TT	G	29	7	1.80 (0.56–5.78)	0.324
CT+TT	A	26	5	0.96 (0.50–1.83)	0.897
For interaction				**0.13 (0.03–0.67)**	**0.015**
rs3135021	G1896A				
GG	G	63	5	1.00	
GG	A	27	18	**6.15 (1.95–19.38)**	**0.002**
GA	G	25	6	3.57 (0.90–14.13)	0.070
GA	A	20	3	0.94 (0.40–2.22)	0.893
For interaction				**0.07 (0.01–0.48)**	**0.007**

[a] Boldface type indicates significant values.

TABLE 7 Associations of the interaction of *HLA* SNPs and HBV mutations with the risk of HCC in genotype C HBV-infected subjects[a]

HLA-DP SNP	HBV mutation	No. of HCC-free HBV-infected subjects	No. of HCC patients	Adjusted OR (95% CI)	*P* value
rs9277535	T1674C/G				
GG	T	118	92	1.00	
GG	C/G	52	75	**1.64 (1.02–2.62)**	**0.039**
AA	T	46	43	1.12 (0.66–1.90)	0.682
AA	C/G	24	14	0.69 (0.46–1.03)	0.067
For interaction				**0.35 (0.14–0.88)**	**0.026**
rs9277535	G1719T				
GG	G	90	37	1.00	
GG	T	94	130	**3.13 (1.94–5.06)**	**<0.001**
AA	G	30	20	1.42 (0.68–3.00)	0.354
AA	T	46	37	**1.40 (1.02–1.91)**	**0.037**
For interaction				**0.40 (0.17–0.97)**	**0.042**

[a] Boldface type indicates significant values.

set of Kupffer cells, resident macrophages in liver, and play important roles in the presentation of viral antigens to $CD4^+$ T helper (Th) lymphocytes. Of Th lymphocytes, Th1 facilitates HBV clearance. The HBV persistence-promoting genotypes of rs3077, rs9277535, and rs2281388 have been associated with decreased transcription of *HLA-DPA1* and *HLA-DPB1* in normal liver (50). Decreased expression of HLA-DPA1 and HLA-DPB1 may contribute to insufficient antiviral immunity and HBV persistence, possibly via decreasing the Th1/Th2 ratio in liver and promoting chronic inflammation. An insufficient and persistent antiviral immune reaction contributes to HBV mutations during HBV evolution. The HBV mutations, to some extent, were consequences of viral adaptation to the host immune responses (51). Further experimental and prospective population-based studies are warranted to clarify the roles of HLA-DPA1 and HLA-DPB1 in HBV evolution.

To the best of our knowledge, this is the first study revealing that *HLA-DP* genetic variants influence immune selection of disease-related HBV mutations and affect HC and HCC risks via interacting with these mutations. Moreover, a large sample size provided sufficient statistical power and gave more convincing data. However, this study had limitations. First, we amplified only the two HBV fragments from half of the HBsAg-positive subjects despite enormous efforts, possibly because of low viral load or viral mutations in the primer binding sites. This might result in a possible preponderance of missing data. Second, the sample sizes in the HBsAg seroclearance group and genotype B HBV-infected subjects were relatively small. Third, this study design was cross-sectional. Further prospective studies are warranted to validate these findings.

In conclusion, the present study indicates that the *HLA-DP* genotypes promoting HBV clearance may predispose the host to clearing genotype B HBV preferentially. *HLA-DP* genetic polymorphisms might affect the outcomes of chronic HBV infection via regulating immune selection of HBV mutations and affect the risks of HC and HCC contributed by the HBV mutations. This study provides epidemiological evidence of the complex host-virus interactions in HBV evolution and should be helpful in identifying HBV-infected subjects who are more likely to develop HC or HCC.

ACKNOWLEDGMENTS

This work was supported by the Outstanding Young Scholar Fund (81025015), the Key Project (91129301), and the Creative Research Group (81221061) from the National Natural Scientific Foundation of China and by grants from the Science and Technology Commission of Shanghai Municipality (12ZR1453600 and 12ZR1429300) and from the Shanghai Health Bureau Fund (20114066).

We thank Chengzhong Li, Qian Zhang, Wu Ni, Xinyan Sun, Xiaoqing Jiang, Bin Li, Huafen Wang, Lei Han, and Peixin Qian for their help in the recruitment of the study subjects.

We declare that we have no conflicts of interest.

REFERENCES

1. **Lok AS, McMahon BJ.** 2007. Chronic hepatitis B. Hepatology **45:**507–539.
2. **Fattovich G, Bortolotti F, Donato F.** 2008. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J. Hepatol. **48:**335–352.
3. **Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP.** 2006. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J. Hepatol. **45:**529–538.
4. **Pollicino T, Saitta C, Raimondo G.** 2011. Hepatocellular carcinoma: the point of view of the hepatitis B virus. Carcinogenesis **32:**1122–1132.
5. **Cao GW.** 2009. Clinical relevance and public health significance of hepatitis B virus genomic variations. World J. Gastroenterol. **15:**5761–5769.
6. **Chan HL, Tse CH, Mo F, Koh J, Wong VW, Wong GL, Lam Chan S, Yeo W, Sung JJ, Mok TS.** 2008. High viral load and hepatitis B virus subgenotype ce are associated with increased risk of hepatocellular carcinoma. J. Clin. Oncol. **26:**177–182.
7. **Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ.** 2002. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N. Engl. J. Med. **347:**168–174.
8. **Liu S, Zhang H, Gu C, Yin J, He Y, Xie J, Cao G.** 2009. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. J. Natl. Cancer Inst. **101:**1066–1082.
9. **Yang HI, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, Wang LY, Lu SN, You SL, Chen DS, Liaw YF, Chen CJ.** 2008. Associations between

hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J. Natl. Cancer Inst. **100:**1134–1143.
10. **Sung JJ, Tsui SK, Tse CH, Ng EY, Leung KS, Lee KH, Mok TS, Bartholomeusz A, Au TC, Tsoi KK, Locarnini S, Chan HL.** 2008. Genotype-specific genomic markers associated with primary hepatomas, based on complete genomic sequencing of hepatitis B virus. J. Virol. **82:**3604–3611.
11. **Yin J, Xie J, Liu S, Zhang H, Han L, Lu W, Shen Q, Xu G, Dong H, Shen J, Zhang J, Han J, Wang L, Liu Y, Wang F, Zhao J, Zhang Q, Ni W, Wang H, Cao G.** 2011. Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. Am. J. Gastroenterol. **106:**81–92.
12. **Yin J, Xie J, Zhang H, Shen Q, Han L, Lu W, Han Y, Li C, Ni W, Wang H, Cao G.** 2010. Significant association of different preS mutations with hepatitis B-related cirrhosis or hepatocellular carcinoma. J. Gastroenterol. **45:**1063–1071.
13. **Munoz A, Chen JG, Egner PA, Marshall ML, Johnson JL, Schneider MF, Lu JH, Zhu YR, Wang JB, Chen TY, Kensler TW, Groopman JD.** 2011. Predictive power of hepatitis B 1762T/1764A mutations in plasma for hepatocellular carcinoma risk in Qidong, China. Carcinogenesis **32:**860–865.
14. **Bai X, Zhu Y, Jin Y, Guo X, Qian G, Chen T, Zhang J, Wang J, Groopman JD, Gu J, Tu H.** 2011. Temporal acquisition of sequential mutations in the enhancer II and basal core promoter of HBV in individuals at high risk for hepatocellular carcinoma. Carcinogenesis **32:**63–68.
15. **Chou YC, Yu MW, Wu CF, Yang SY, Lin CL, Liu CJ, Shih WL, Chen PJ, Liaw YF, Chen CJ.** 2008. Temporal relationship between hepatitis B virus enhancer II/basal core promoter sequence variation and risk of hepatocellular carcinoma. Gut **57:**91–97.
16. **Fang ZL, Sabin CA, Dong BQ, Ge LY, Wei SC, Chen QY, Fang KX, Yang JY, Wang XY, Harrison TJ.** 2008. HBV A1762T, G1764A mutations are a valuable biomarker for identifying a subset of male HBsAg carriers at extremely high risk of hepatocellular carcinoma: a prospective study. Am. J. Gastroenterol. **103:**2254–2262.
17. **Huang Y, Tai AW, Tong S, Lok AS.** 2013. HBV core promoter mutations promote cellular proliferation through E2F1-mediated upregulation of S-phase kinase-associated protein 2 transcription. J. Hepatol. **58:**1068–1073.
18. **Mun HS, Lee SA, Kim H, Hwang ES, Kook YH, Kim BJ.** 2011. Novel F141L pre-S2 mutation in hepatitis B virus increases the risk of hepatocellular carcinoma in patients with chronic genotype C infections. J. Virol. **85:**123–132.
19. **Yeh CT, So M, Ng J, Yang HW, Chang ML, Lai MW, Chen TC, Lin CY, Yeh TS, Lee WC.** 2010. Hepatitis B virus-DNA level and basal core promoter A1762T/G1764A mutation in liver tissue independently predict postoperative survival in hepatocellular carcinoma. Hepatology **52:**1922–1933.
20. **Zhang HW, Yin JH, Li YT, Li CZ, Ren H, Gu CY, Wu HY, Liang XS, Zhang P, Zhao JF, Tan XJ, Lu W, Schaefer S, Cao GW.** 2008. Risk factors for acute hepatitis B and its progression to chronic hepatitis in Shanghai, China. Gut **57:**1713–1720.
21. **Tran A, Kremsdorf D, Capel F, Housset C, Dauguet C, Petit MA, Brechot C.** 1991. Emergence of and takeover by hepatitis B virus (HBV) with rearrangements in the pre-S/S and pre-C/C genes during chronic HBV infection. J. Virol. **65:**3566–3574.
22. **Orito E, Mizokami M, Ina Y, Moriyama EN, Kameshima N, Yamamoto M, Gojobori T.** 1989. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc. Natl. Acad. Sci. U. S. A. **86:**7059–7062.
23. **Vartanian JP, Henry M, Marchio A, Suspene R, Aynaud MM, Guetard D, Cervantes-Gonzalez M, Battiston C, Mazzaferro V, Pineau P, Dejean A, Wain-Hobson S.** 2010. Massive APOBEC3 editing of hepatitis B viral DNA in cirrhosis. PLoS Pathog. **6:**e1000928. doi:10.1371/journal.ppat.1000928.
24. **Xu R, Zhang X, Zhang W, Fang Y, Zheng S, Yu XF.** 2007. Association of human APOBEC3 cytidine deaminases with the generation of hepatitis virus B x antigen mutants and hepatocellular carcinoma. Hepatology **46:**1810–1820.
25. **Maman Y, Blancher A, Benichou J, Yablonka A, Efroni S, Louzoun Y.** 2011. Immune-induced evolutionary selection focused on a single reading frame in overlapping hepatitis B virus proteins. J. Virol. **85:**4558–4566.
26. **Kamatani Y, Wattanapokayakit S, Ochi H, Kawaguchi T, Takahashi A, Hosono N, Kubo M, Tsunoda T, Kamatani N, Kumada H, Puseenam A, Sura T, Daigo Y, Chayama K, Chantratita W, Nakamura Y, Matsuda K.** 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.
27. **Guo X, Zhang Y, Li J, Ma J, Wei Z, Tan W, O'Brien SJ.** 2011. Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. Hepatology **53:**422–428.
28. **Hu L, Zhai X, Liu J, Chu M, Pan S, Jiang J, Zhang Y, Wang H, Chen J, Shen H, Hu Z.** 2012. Genetic variants in human leukocyte antigen/DP-DQ influence both hepatitis B virus clearance and hepatocellular carcinoma development. Hepatology **55:**1426–1431.
29. **Li J, Yang D, He Y, Wang M, Wen Z, Liu L, Yao J, Matsuda K, Nakamura Y, Yu J, Jiang X, Sun S, Liu Q, Song Q, Chen M, Yang H, Tang F, Hu X, Wang J, Chang Y, He X, Chen Y, Lin J.** 2011. Associations of HLA-DP variants with hepatitis B virus infection in southern and northern Han Chinese populations: a multicenter case-control study. PLoS One **6:**e24221. doi:10.1371/journal.pone.0024221.
30. **An P, Winkler C, Guan L, O'Brien SJ, Zeng Z.** 2011. A common HLA-DPA1 variant is a major determinant of hepatitis B virus clearance in Han Chinese. J. Infect. Dis. **203:**943–947.
31. **Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn SH, Park JY, Hige S, Kang JH, Suzuki K, Kurosaki M, Asahina Y, Mochida S, Watanabe M, Tanaka E, Honda M, Kaneko S, Orito E, Itoh Y, Mita E, Tamori A, Murawaki Y, Hiasa Y, Sakaida I, Korenaga M, Hino K, Ide T, Kawashima M, Mawatari Y, Sageshima M, Ogasawara Y, Koike A, Izumi N, Han KH, Tanaka Y, Tokunaga K, Mizokami M.** 2012. Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. PLoS One **7:**e39175. doi:10.1371/journal.pone.0039175.
32. **Wong DK, Watanabe T, Tanaka Y, Seto WK, Lee CK, Fung J, Lin CK, Huang FY, Lai CL, Yuen MF.** 2013. Role of HLA-DP polymorphisms on chronicity and disease activity of hepatitis B infection in Southern Chinese. PLoS One **8:**e66920. doi:10.1371/journal.pone.0066920.
33. **Yin J, Zhang H, He Y, Xie J, Liu S, Chang W, Tan X, Gu C, Lu W, Wang H, Bi S, Cui F, Liang X, Schaefer S, Cao G.** 2010. Distribution and hepatocellular carcinoma-related viral properties of hepatitis B virus genotypes in Mainland China: a community-based study. Cancer Epidemiol. Biomarkers Prev. **19:**777–786.
34. **Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, Serra A, Saracco G, Verme G, Will H, Bonino F.** 1991. Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. Proc. Natl. Acad. Sci. U. S. A. **88:**4186–4190.
35. **Chan HL, Wong GL, Tse CH, Chim AM, Yiu KK, Chan HY, Sung JJ, Wong VW.** 2009. Hepatitis B virus genotype C is associated with more severe liver fibrosis than genotype B. Clin. Gastroenterol. Hepatol. **7:**1361–1366.
36. **Seto WK, Wong DK, Kopaniszen M, Proitsi P, Sham PC, Hung IF, Fung J, Lai CL, Yuen MF.** 2013. HLA-DP and IL28B polymorphisms: influence of host genome on hepatitis B surface antigen seroclearance in chronic hepatitis B. Clin. Infect. Dis. **56:**1695–1703.
37. **Cheng HR, Liu CJ, Tseng TC, Su TH, Yang HI, Chen CJ, Kao JH.** 2013. Host genetic factors affecting spontaneous HBsAg seroclearance in chronic hepatitis B patients. PLoS One **8:**e53008. doi:10.1371/journal.pone.0053008.
38. **Thomas R, Thio CL, Apps R, Qi Y, Gao X, Marti D, Stein JL, Soderberg KA, Moody MA, Goedert JJ, Kirk GD, Hoots WK, Wolinsky S, Carrington M.** 2012. A novel variant marking HLA-DP expression levels predicts recovery from hepatitis B virus infection. J. Virol. **86:**6979–6985.
39. **Vermehren J, Lotsch J, Susser S, Wicker S, Berger A, Zeuzem S, Sarrazin C, Doehring A.** 2012. A common HLA-DPA1 variant is associated with hepatitis B virus infection but fails to distinguish active from inactive Caucasian carriers. PLoS One **7:**e32605. doi:10.1371/journal.pone.0032605.
40. **Abbott WG, Tsai P, Leung E, Trevarton A, Ofanoa M, Hornell J, Gane EJ, Munn SR, Rodrigo AG.** 2010. Associations between HLA class I alleles and escape mutations in the hepatitis B virus core gene in New Zealand-resident Tongans. J. Virol. **84:**621–629.
41. **Khakoo SI, Ling R, Scott I, Dodi AI, Harrison TJ, Dusheiko GM, Madrigal JA.** 2000. Cytotoxic T lymphocyte responses and CTL epitope escape mutation in HBsAg, anti-HBe positive individuals. Gut **47:**137–143.

42. **Yan T, Li K, Li F, Su H, Mu J, Tong S, Patel M, Xia J, Wands JR, Wang H.** 2011. T1846 and A/G1913 are associated with acute on chronic liver failure in patients infected with hepatitis B virus genotypes B and C. J. Med. Virol. **83:**996–1004.
43. **Márquez M, Fernández-Gutiérrez C, Montes-de-Oca M, Blanco MJ, Brun F, Rodríguez-Ramos C, Girón-González JA.** 2009. Chronic antigenic stimuli as a possible explanation for the immunodepression caused by liver cirrhosis. Clin. Exp. Immunol. **158:**219–229.
44. **Schildberg FA, Wojtalla A, Siegmund SV, Endl E, Diehl L, Abdullah Z, Kurts C, Knolle PA.** 2011. Murine hepatic stellate cells veto CD8 T cell activation by a CD54-dependent mechanism. Hepatology **54:**262–272.
45. **Kramvis A, Kew MC.** 1999. The core promoter of hepatitis B virus. J. Viral Hepat. **6:**415–427.
46. **Kitab B, Essaid El Feydi A, Afifi R, Trepo C, Benazzouz M, Essamri W, Zoulim F, Chemin I, Alj HS, Ezzikouri S, Benjelloun S.** 2012. Variability in the precore and core promoter regions of HBV strains in Morocco: characterization and impact on liver disease progression. PLoS One **7:**e42891. doi:10.1371/journal.pone.0042891.
47. **Günther S, Piwon N, Iwanska A, Schilling R, Meisel H, Will H.** 1996. Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. J. Virol. **70:**8318–8331.
48. **Xie JX, Zhao J, Yin JH, Zhang Q, Pu R, Lu WY, Zhang HW, Wang HY, Cao GW.** 2010. Association of novel mutations and haplotypes in the preS region of hepatitis B virus with hepatocellular carcinoma. Front. Med. China **4:**419–429.
49. **Desmond CP, Bartholomeusz A, Gaudieri S, Revill PA, Lewin SR.** 2008. A systematic review of T-cell epitopes in hepatitis B virus: identification, genotypic variation and relevance to antiviral therapeutics. Antivir. Ther. **13:**161–175.
50. **O'Brien TR, Kohaar I, Pfeiffer RM, Maeder D, Yeager M, Schadt EE, Prokunina-Olsson L.** 2011. Risk alleles for chronic hepatitis B are associated with decreased mRNA expression of HLA-DPA1 and HLA-DPB1 in normal human liver. Genes Immun. **12:**428–433.
51. **Desmond CP, Gaudieri S, James IR, Pfafferott K, Chopra A, Lau GK, Audsley J, Day C, Chivers S, Gordon A, Revill PA, Bowden S, Ayres A, Desmond PV, Thompson AJ, Roberts SK, Locarnini SA, Mallal SA, Lewin SR.** 2012. Viral adaptation to host immune responses occurs in chronic hepatitis B virus (HBV) infection, and adaptation is greatest in HBV e antigen-negative disease. J. Virol. **86:**1181–1192.