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Fine mapping analysis of *HLA-DP/DQ* gene clusters on chromosome 6 reveals multiple susceptibility loci for HBV infection

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Abstract Recent genome-wide association studies have revealed the HLA region on chromosome 6p21 as a susceptibility locus for hepatitis B virus (HBV) infection, a finding subsequently replicated in independent samples. However, only limited single nucleotide polymorphisms (SNPs) were analyzed in most of these studies, and it remains to be determined which SNPs contribute to the detected association. After genotyping 140 SNPs within this genomic region in a total of 1657 HBV-positive patients and 1456 HBV-negative controls, we conducted a series of genetic epidemiological and bioinformatics analysis, including individual SNP-based association analysis, haplotype-based association analysis, and conditional analysis. We identified 76 SNPs and 5 LD blocks in HLA-DP/DQ clusters that are significantly associated with HBV infection, with the smallest P value being 3.88×10^{-18} for rs9277535 in HLA-DPB1. With conditional analysis, we further revealed that the genes contributing to the effects of variants in HLA-DP/DQ on infection are independent of each other, and the LD block 5 in the 3′-UTR region of HLA-DPB1 had a predominant effect in the association of HLA-DP with HBV infection. We also found that the SNPs in the 3′-UTR region of HLA-DPB1 were significant between the subgroups of inactive HBV carrier, chronic hepatitis B, or hepatic cirrhosis from the case group and the spontaneous HBV-clearance subgroup from the control group. Finally, we did further association analysis of SNPs in this region with different subgroups from the case group, which revealed no association of these SNPs with the progression of HBV-related diseases. In sum, we showed, for the first time, that the HLA-DP/DQ clusters contribute independently to HBV infection, and the 3′-UTR region of HLA-DPB1 represents an important functional region involved in HBV infection.

Keywords Hepatitis B virus infection · Host susceptibility · *HLA-DP* · *HLA-DQ* · *HLA-DPB* · SNPs · Association analysis · Conditional analysis

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Introduction

Hepatitis B virus (HBV) infection is one of the world's most common and serious infectious diseases. According to the recent World Health Organization report, released in July 2014, there are approximately 240 million people in the world known to have chronic HBV infection. After developing serious diseases such as hepatic cirrhosis (HC) and hepatocellular carcinoma (HCC), about 780,000 infected people die each year. China, currently recognized as a low and high HBV endemicity co-existing region, still has 7.18 % of the general population positive for hepatitis B surface antigen (HBsAg) and more than 50,000 people

die annually from the end stage of the disease (Cui and Jia 2013; Sun and Hou 2010).

Because infection and progression of HBV-related diseases vary, genetic factors are considered to be as important as the viral and environmental factors. As early as the 1970s, a familial aggregation study of hepatitis B infection implied that genetics plays an important role in HBV infection (Szmuness et al. 1975). In 1989, a twin study showed that host genetic background has an impact on infection outcome (Lin et al. 1989). Since then, many candidate gene-based association studies have been conducted to determine the genetic mechanism underlying HBV infection. Genes in the HLA region on chromosome 6 (such as *HLA-DP* and *HLA-DQ*, which are expressed as surface antigens and determine the immune response to virus infection) were associated, not only with HBV infection and progression, but also with the response to HBV vaccination and interferon treatment of hepatitis B (Desombere et al. 1998; Ahn et al. 2000; Jiang et al. 2003; Zang et al. 2004; Xi-Lin et al. 2006).

In 2009, significant associations between chronic hepatitis B (CHB) and single nucleotide polymorphisms (SNPs) rs3077 and rs9277535 in the *HLA-DP* gene cluster were reported in 786 Japanese individuals having CHB and 2201 controls as a result of a two-stage genome-wide association study (GWAS) (Kamatani et al. 2009). A second GWAS using 2667 Japanese patients with persistent HBV infection and 6496 controls revealed an independent effect of CHB susceptibility of SNPs rs2856718 and rs7453920 in the *HLA-DQ* gene cluster, which is located adjacent to *HLA-DP* (Mbarek et al. 2011a). Those positive associations have been replicated in several studies with populations of Chinese, Korean, and Saudi Arabian origin (Al-Qahtani et al. 2014; Nishida et al. 2012; Li et al. 2011; Hu et al. 2012). In addition, a study of a Caucasian population revealed a significant association of rs3077 in *HLA-DPA1*, but not rs9277535 in *HLA-DPB1*, with HBV infection (Vermehren et al. 2012).

Moreover, there exists a significant association of SNPs rs2856718 and rs9275319 in *HLA-DQ* as well as rs3077 in *HLA-DP* with the progression of CHB to HCC (Hu et al. 2012; Jiang et al. 2013; Chen et al. 2013). In contrast, no significant association was revealed between SNPs rs3077 and rs9277542 in *HLA-DP* in a Korean sample and rs2856718, rs7453920 and rs9275572 in *HLA-DQ*, and rs3077 and rs9277535 in *HLA-DP* in the Saudi Arabian sample of patients with HC or HCC (Al-Qahtani et al. 2014; Nishida et al. 2012). Also, for the progression of HBV carriers to CHB, no association of SNPs rs2395309 and rs9277535 in *HLA-DP/DQ* was revealed in studies of Caucasian and Chinese subjects (Vermehren et al. 2012; Li et al. 2011). Thus, more studies are greatly needed to determine whether the variants in this broad and important genomic region contribute to HBV infection and HBV-related diseases (Jiang et al. 2014).

Considering that previously reported studies examined only limited SNPs within this broad region containing the gene-rich HLA complex (180 protein-coding genes in a span of 4 Mb) (Strachan and Read 2010), in this study, we conducted a comprehensive fine mapping analysis of this region, especially in the *HLA-DP/DQ* gene clusters, with the primary goals of identifying: (1) which subset of SNPs in this region affect the susceptibility to HBV infection; (2) whether these involvements are dependent or independent; and (3) whether the variants in this regions are associated with the progression of HBV-related diseases.

Subjects and methods

Subject recruitment and demographic characteristics

A total of 1657 patients were recruited from the Outpatient Department of Infectious Diseases, First Affiliated Hospital of Zhejiang University, between February 2011 and August 2012. Within the same period, based on the age and sex proportion of the case group, 1456 control subjects were recruited from among healthy volunteers during the annual physical examination at the First Affiliated Hospital of Zhejiang University. All subjects were examined by a series of laboratory tests including assays for hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), hepatitis B *e* antigen (HBeAg), hepatitis B *e* antibody (HBeAb), hepatitis B surface antibody (HbsAb), hepatitis C surface antibody (HCVAb), serum alanine aminotransferase (ALT), and HBV DNA. Subjects who were co-infected with hepatitis A, hepatitis C, or hepatitis E virus were excluded from this study. In addition, subjects who were pregnant, had any type of cancer or autoimmune disease, or were positive for human immunodeficiency virus (HIV) infection were excluded. Finally, those subjects with an HBV-vaccination history were excluded. All subjects were unrelated to each other and had Chinese Han ethnicity. Informed written consent was obtained from all the participants, and the project was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

The case subjects were selected on the basis of laboratory testing results as being “HBsAg positive”, “HBV DNA positive” or “both positive”. The subgroups of all subjects were further classified according to the diagnostic criteria published by the Chinese health authority (Chinese Society of and Chinese Society of Infectious Diseases 2011) as: (1) inactive HBV carrier: seropositive for HBsAg or HBV DNA, persistent normal concentration of ALT, or self-reported as not infected; (2) CHB: seropositive for

Table 1 Demographic characteristics of samples

	Controls		Total	Cases				Total
	HBV clearance	Healthy control		HBV carrier	CHB	LC	HCC	
Sample size	667	749	1440	394	878	301	75	1648
Age (SD)	39.4 (11.3)	37.7 (12.4)	38.1 (12.1)	36.7 (11.5)	39.1 (11.3)	47.8 (9.8)	51.7 (11.4)	40.7 (12.0)
Sex (males/females)	437/230	450/299	906/534	256/138	663/215	238/63	12/63	1220/428
Female (%)	34.48	39.92	37.10	35.03	24.49	20.93	16.00	26.00

HBsAg or HBV DNA, persistent or recurrently elevated concentration of ALT for >6 months; (3) hepatic cirrhosis: determined on the basis of medical records of each patient with typical symptoms and signs of ascites, hypersplenism, evaluated ALT, and typical result of imaging examination; or (4) HCC: determined on the basis of medical records of each patient with typical symptoms and signs of evaluated α-fetoprotein and typical result of imaging examination. The control group was defined as "both of HBsAg and HBV DNA negative" and had not received HBV vaccination. This group was separated into two subgroups: (1) healthy control: HBsAg, HBcAb, and HBsAb negative; and (2) spontaneous HBV clearance: HBsAg negative but HBcAb or HBsAb positive, as defined in a previously reported study on the same category (Guo et al. 2011). On the basis of these criteria, a total of 1657 cases and 1456 controls were included for the current study.

Genotyping and quality control

Genomic DNA was extracted from 3 ml of EDTA-treated peripheral venous blood using the Qiagen DNA purification kit according to the manufacturer's instruction. All SNPs selected from the *HLA* region on chromosome 6 were genotyped using an Illumina iSelect custom genotyping array according to the Illumina Infinium HD Assay Ultra Manual. Following genotyping, quality control analyses were carried out on all genotyped SNPs, which excluded 68 SNPs with a call rate <97 % and 44 SNPs with *P* values for Hardy–Weinberg equilibrium (HWE) $<10^{-5}$ in all samples. Finally, a total of 140 SNPs were included for the association analyses reported in this communication, which included 62 SNPs in *HLA-DP*, 76 SNPs in *HLA-DQ*, and 2 SNPs in *HLA-DRB1*. Additional 96 SNPs selected from different chromosomes by the manufacturer were also included in the iSelect array and used for population structure analysis of all samples examined in this study.

Quality control of each subject sample was further determined by the call rate of all genotyped SNPs. Under this QC step, we removed 9 case and 16 control samples with a call rate of <97 %. Next, we re-checked the clinical information on each subject and excluded 24 control subjects who were born after the year 1992, when the national vaccine project was initiated, and who had received vaccination for HBV.

Finally, a total of 1648 case and 1416 control samples remained with various HBV-related phenotypes for the 140 SNPs. A detailed description of those subjects and their corresponding demographic characteristics is shown in Table 1, which included 394 HBV carriers, 878 CHB patients, 301 HC patients, and 75 HCC patients for the case group and 667 persons with spontaneous HBV clearance and 749 healthy individuals for the control group.

Genetic association analysis

For individual SNP-based association analysis, we used the PLINK program (v. 1.07) (Purcell et al. 2007) to perform multiple logistic regression under the additive genetic model with sex, age, and the first three PCs (to control for population stratification) as covariates. Pair-wise LD values as well as haplotypes and LD blocks were calculated using Haploview (v. 4.2) with the default parameters (Barrett et al. 2005). By computing score statistics under the same genetic model and covariates, we calculated haplotype *P* values as well as the score for each haplotype and a global *P* value for each LD block using the R package "haplo.stats" (v. 1.6.8) function "haplo.score" (Schaid et al. 2002). We used "haplo.stats" function "haplo.cc" to calculate odds ratios and 95 % confidence intervals for each haplotype, with the most common haplotype within each block as the baseline. For the most frequent one, we took the second most frequent haplotype as the baseline, as reported (Mbarek et al. 2011a). Only statistically significant results of individual SNPs and major haplotypes with a frequency of ≥5 % after Bonferroni correction for multiple testing are reported in this communication.

To determine the relations among detected genetic associations between LD blocks, we performed conditional analysis by testing all block-included SNPs conditional on each other (Dixon et al. 2014; Mbarek et al. 2011a), which yielded a matrix of conditional *P* values. To visualize the interaction patterns for associations, we constructed an ideograph with color indicating the independent effect of the blocks and SNPs.

To determine whether these SNPs are associated with the progression of HBV-related diseases, we conducted further association analysis for the following subgroups: HBV carriers vs. CHB and CHB vs. HC. Because of the relative small sample ($N = 75$), the HCC subgroup was excluded from this part of the analysis. Further, we conducted association analysis for the following subgroups: (a) HBV clearance vs. HBV carriers; (b) HBV clearance vs. CHB; and (c) HBV clearance vs. HC.

Both the conditional analysis and subgroup-based association analyses were performed using PLINK under the additive genetic model with sex, age, and the first three PCs as covariates.

Results

Individual SNP-based association analysis

Of 140 SNPs analyzed in this study, 76 showed significant association with HBV infection after Bonferroni correction for multiple testing (i.e., the adjusted P value is $0.05/140 = 3.57 \times 10^{-4}$) (Table 2). We not only confirmed significant associations of 12 SNPs in the HLA region (i.e., rs3179779, rs3180554, rs3180553, rs1042190, rs2301220, rs9277341, rs3135021, rs7770370, rs9277533, rs9277534, rs9277535, and rs10484569), which were reported to be significant in the discovery stage of the previous GWAS on HBV (Kim et al. 2013; Kamatani et al. 2009; Mbarek et al. 2011b), but also found 41 novel SNPs in *HLA-DP* and 23 novel SNPs in *HLA-DQ* to be significantly associated with HBV infection. Of the 76 detected significant SNPs, 55 reached genome-wide significance ($P < 5.0 \times 10^{-8}$) (Dudbridge and Gusnanto 2008; Gao et al. 2008; Pe'er et al. 2008).

Interestingly, we found that 7 of the 11 SNPs with the most significant associations are located in the 3′-UTR region of *HLA-DPB1* (i.e., rs931, rs928, rs9277532, rs9277533, rs9277534, rs9277535, and rs9277536; see block 5 of Table 2 for details), with P values of 2.06×10^{-16} to 5.49×10^{-18}. Moreover, these seven SNPs were also found to be significant in subsequent association analyses between the viral-clearance subgroup from the control group and subgroups of HBV carriers, CHB, or HC from the case samples. Detailed association results among these subgroups analysis are shown in Supplementary Table 1.

Also, in the *HLA-DP* gene cluster, we found four nonsynonymous SNPs to be significantly associated with HBV infection (Table 2). This includes SNPs rs1042190 ($P = 7.77 \times 10^{-11}$) and rs1042178 ($P = 2.30 \times 10^{-11}$) in *HLA-DPA1*, encoding the HLA-DP α1 chain of class II histocompatibility antigen, and rs1042169 ($P = 3.87 \times 10^{-15}$) and rs14362 ($P = 3.98 \times 10^{-5}$) in *HLA-DPA1/B1* and *HLA-DPB1*, encoding the HLA-DP β1 chain of HLA class II histocompatibility antigen. Of the four nonsynonymous SNPs, three were predicted by the PolyPhen-2 program (Adzhubei et al. 2010) to be "probably damaging," suggesting they are likely to be functional (Table 3).

Haplotype-based association analysis

By using Haploview (Gabriel et al. 2002), we found five LD blocks for 66 of the 76 SNPs significantly associated with HBV infection (Table 4; Fig. 2). The corresponding position of each block with its included SNPs and gene locations is shown in Table 2 and Fig. 1. As shown in Table 4, there are two LD blocks in the *HLA-DQ* cluster, with Block 1 consisting of 9 SNPs in *HLA-DQA1/B1* and Block 2 consisting of 12 SNPs in *HLA-DQA2/B2*, whereas in the *HLA-DP* cluster, there are three blocks, with Block 3 consisting of 35 SNPs in *HLA-DPA1*, Block 4 consisting of 2 SNPs in the *HLA-DPA1/B1* overlapping region, and Block 5 consisting of 8 SNPs in *HLA-DPB1*.

Following determination of LD blocks and major haplotypes (defined as a frequency $\geq$5 %) within each block, we performed association analysis using the Haplo.stats program to determine their associations with HBV infection (Table 4). All five LD blocks were significantly associated with HBV infection, with a global P value of 4.51×10^{-21}, 4.0×10^{-9}, 3.42×10^{-8}, 8.86×10^{-16}, and 1.75×10^{-16} for Blocks 1 to 5, respectively. On the basis of the positive haplotype scores, we identified one risky major haplotype for HBV infection from each block: GGGCAAAAA in Block 1 (haplotype score 5.968; $P = 2.40 \times 10^{-9}$), AAGTAGAAGCGG in Block 2 (haplotype score 6.454; $P = 1.09 \times 10^{-10}$), CACGAGAAAAGAAGATGGCCCGAGGGAGAGAGAGG in Block 3 (haplotype score 5.675; $P = 1.39 \times 10^{-8}$, AG in Block 4 (haplotype score 8.13; $P = 4.31 \times 10^{-16}$), and CACAAGGA in Block 5 (haplotype score 7.613; $P = 2.67 \times 10^{-14}$). In contrast, we detected at least one major haplotype from each block that showed a protective effect against HBV infection: GGAAAGGAG (haplotype score −7.176; $P = 7.19 \times 10^{-13}$) and AGGCAAAGG (haplotype score −5.683; $P = 1.32 \times 10^{-8}$) in Block 1, GGAAGAGGAGAA (haplotype score −6.371; $P = 1.88 \times 10^{-10}$) in Block 2, ATAAGAGCGGATGATAAAGGAACAAAGCGAGAGCA (haplotype score −5.591; $P = 2.25 \times 10^{-8}$) in Block 3, CA (haplotype score −7.881; $P = 3.25 \times 10^{-15}$) in Block 4, and CGGCGAAG (haplotype score −7.45; $P = 9.34 \times 10^{-14}$) in Block 5 (Table 4). Detailed haplotype-based association results and their corresponding frequency <0.05 are presented in Supplementary Table 2.

Table 2 Significantly associated SNPs (adjusted $P < 3.57 \times 10^{-4}$) in HLA-DP/DQ gene cluster on chromosome 6 with HBV infection under an additive genetics model

Gene	Block	No.	dbSNP ID	Chr 6 position**	Alleles	SNP location	OR (95 % CI)	*P* value
HLA-DQA1	Block 1	1	rs9272131	32600671	C/T	Unknown	0.53 (0.30-0.75)	3.15E−08
		2	rs28383345	32605234	A/G	5′UTR	0.66 (0.45-0.88)	1.65E−04
HLA-DQB1		3	rs9273440	32627561	C/T	3′UTR	0.45 (0.23-0.67)	1.43E−12
		4	rs9273442	32627582	G/T	3′UTR	0.47 (0.26-0.67)	6.39E−13
		5*	rs1049225	32627747	C/T	3′UTR	0.46 (0.25-0.67)	6.62E−13
		6	rs1049122	32627807	A/C/T	3′UTR	0.62 (0.36-0.87)	1.90E−04
		7	rs1049055	32634387	A/G	3′UTR	0.47 (0.26-0.67)	5.25E−13
		8	rs1049053	32634405	A/G	5′UTR	0.47 (0.26-0.67)	5.25E−13
		9	rs9275319	32666295	A/G	Unknown	0.72 (0.56-0.88)	3.60E−05
		10	rs2856718	32670255	A/G	Unknown	0.80 (0.70-0.90)	1.20E−05
HLA-DQA2	Block 2	11*	rs9276401	32709204	C/G	5′UTR	0.39 (0.06-0.72)	2.03E−08
		12	rs2071798	32714592	C/T	3′UTR	0.61 (0.45-0.76)	2.79E−10
		13	rs28693951	32714732	C/T	Downstream 500B	0.61 (0.45-0.76)	2.44E−10
		14	rs28420297	32714760	A/G	Downstream 500B	0.61 (0.45-0.76)	2.44E−10
		15	rs9276440	32714783	A/T	Downstream 500B	0.60 (0.44-0.76)	2.19E−10
HLA-DQB2		16	rs7756516	32723917	C/T	3′UTR	0.61 (0.46-0.77)	4.96E−10
		17	rs9276558	32724061	A/G	3′UTR	0.61 (0.45-0.77)	6.97E−10
		18	rs6457646	32724143	A/G	3′UTR	0.61 (0.45-0.76)	2.95E−10
		19	rs3213490	32724258	A/G	Intron	0.61 (0.45-0.76)	3.94E−10
		20	rs3213489	32724305	A/G	Intron	0.61 (0.45-0.77)	8.79E−10
		21	rs3213488	32724312	C/G	Intron	0.61 (0.46-0.77)	8.88E−10
		22	rs3213486	32724324	A/G	Intron	0.61 (0.45-0.77)	8.79E−10
		23	rs3213485	32724719	A/G	Intron	0.60 (0.44-0.76)	8.97E−10
HLA-DPA1	Block 3	24	rs17220927	33032347	G/T	3′UTR	0.67 (0.56-0.79)	1.64E−11
		25	rs17220934	33032358	A/T	3′UTR	0.68 (0.56-0.80)	7.19E−11
		26	rs17214555	33032430	A/C	3′UTR	0.67 (0.56-0.79)	1.64E−11
		27	rs17214567	33032600	A/G	3′UTR	0.67 (0.56-0.79)	1.77E−11
		28	rs17220968	33032663	A/G	3′UTR	0.67 (0.56-0.79)	1.77E−11
		29	rs17214573	33032668	A/G	3′UTR	0.67 (0.56-0.79)	1.64E−11
		30	rs17214580	33032692	C/T	3′UTR	0.67 (0.56-0.79)	1.64E−11
		31	rs17214587	33032698	G/T	3′UTR	0.67 (0.55-0.78)	7.65E−12
		32	rs1042926	33032822	C/T	3′UTR	0.67 (0.56-0.79)	1.41E−11
		33	rs1042920	33032831	C/T	3′UTR	0.67 (0.55-0.78)	6.80E−12
		34	rs1042872	33032905	A/G	3′UTR	0.68 (0.57-0.80)	1.05E−10
		35	rs1042688	33033055	A/T	3′UTR	0.67 (0.56-0.79)	1.17E−11
		36*	rs73400037	33035445	C/T	Intron	0.73 (0.63-0.83)	2.13E−09
		37	rs73400039	33035616	A/G	Intron	0.71 (0.61-0.82)	1.73E−10
		38	rs1054025	33035771	A/G	Intron	0.71 (0.61-0.82)	1.71E−10
		39	rs72870109	33035855	A/T	Intron	0.67 (0.56-0.79)	2.30E−11
		40	rs1062658	33035925	A/T	Intron	0.67 (0.56-0.79)	2.30E−11
		41	rs3179779	33035969	C/T	Intron	0.68 (0.56-0.79)	2.52E−11
		42	rs3179778	33035974	C/T	Intron	0.67 (0.56-0.79)	2.30E−11
		43	rs3179776	33036003	C/G	Intron	0.67 (0.56-0.79)	2.30E−11
		44	rs67818943	33036085	C/G	Intron	0.68 (0.56-0.80)	5.84E−11
		45	rs3180555	33036116	A/C	Intron	0.69 (0.58-0.81)	7.38E−10
		46	rs3180554	33036177	C/T	Intron	0.67 (0.56-0.79)	2.30E−11
		47	rs3180553	33036210	A/C	Intron	0.67 (0.56-0.79)	2.02E−11
		48	rs67516795	33036233	A/G	Intron	0.68 (0.56-0.79)	2.68E−11
		49	rs67476989	33036242	C/T	Intron	0.67 (0.56-0.79)	2.30E−11
		50	rs72879609	33036312	A/G	Intron	0.67 (0.56-0.79)	2.30E−11

Table 2 continued

Gene	Block	No.	dbSNP ID	Chr 6 position**	Alleles	SNP location	OR (95 % CI)	*P* value
		51	rs68071176	33036388	C/T	Intron	0.67 (0.55-0.79)	1.10E−11
		52	rs1042434	33036505	C/G	Synonymous codon	0.68 (0.56-0.79)	3.26E−11
		53	rs2308929	33036959	C/T	Synonymous codon	0.65 (0.52-0.77)	1.47E−11
		54*	rs2308927	33036977	C/T	Synonymous codon	0.72 (0.62-0.83)	6.12E−10
		55	rs1042190	33036999	A/G	Missense	0.69 (0.58-0.80)	7.77E−11
		56*	rs1126544	33037061	C/G	Synonymous codon	0.73 (0.63-0.83)	1.69E−09
		57	rs1126543	33037419	C/T	Synonymous codon	0.67 (0.56-0.79)	1.74E−11
		58	rs1042178	33037522	A/G	Missense	0.67 (0.56-0.79)	2.30E−11
		59	rs1062481	33037611	A/C/G	Synonymous codon	0.71 (0.60-0.81)	1.51E−10
		60	rs1042174	33037626	C/G	Synonymous codon	0.70 (0.58-0.81)	1.23E−10
		61*	rs2301220	33038766	A/G	Intron	0.69 (0.58-0.80)	2.93E−11
		62	rs9277341	33039625	C/T	Intron	0.63 (0.48-0.78)	8.21E−10
		63*	rs3135021	33045558	A/G	Intron	0.78 (0.66-0.90)	8.19E−05
HLA-DPA1	Block 4	64	rs1042169	33048686	A/G/T	Upstream 2 KB; missense	0.64 (0.53-0.75)	3.87E−15
HLA-DPB1		65	rs7770370	33048921	A/G	Upstream 2 KB; intron	0.65 (0.55-0.76)	1.39E−15
		66*	rs7770501	33048937	C/G	Upstream 2 KB; Intron	0.64 (0.54-0.75)	2.34E−17
HLA-DPB1	Block 5	67	rs14362	33052981	A/C	Missense	0.50 (0.17-0.83)	3.98E−05
		68*	rs1071597	33052986	C/T	Synonymous codon	0.64 (0.53-0.75)	1.09E−15
		69	rs931	33054550	G/A	3′UTR	0.62 (0.51-0.73)	5.49E−18
		70	rs928	33054552	C/G	3′UTR	0.63 (0.52-0.74)	1.94E−17
		71	rs9277532	33054711	G/T	3′UTR	0.62 (0.51-0.73)	5.49E−18
		72	rs9277533	33054721	C/T	3′UTR	0.62 (0.51-0.73)	5.49E−18
		73	rs9277534	33054807	A/G	3′UTR	0.62 (0.51-0.73)	5.49E−18
		74	rs9277535	33054861	A/G	3′UTR	0.62 (0.51-0.73)	3.88E−18
		75*	rs9277536	33054890	C/T	3′UTR	0.64 (0.54-0.75)	2.06E−16
		76	rs10484569	33058952	A/G	Unknown	1.48 (1.38-1.58)	3.19E−14

* SNP is not included in the block

** Assembly GRCh37/hg19

Conditional association analysis

To further investigate the relations among the five LD blocks and determine whether the significant associations with HBV infection detected in the *HLA-DP/DQ* gene clusters were attributable to certain SNP(s) or block(s), we conducted a conditional analysis on the 66 initial significant SNPs from the five LD blocks using these SNPs themselves as covariates. The results from such analyses are presented in Fig. 3, where we use the horizontal signal set as covariates and the vertical signal set as the adjusted results.

To determine whether variants in the *HLA-DP* and *HLA-DQ* clusters contribute to HBV infection jointly or independently, we analyzed the *P* value matrix at the gene cluster level (Fig. 3a). By using the SNPs in *HLA-DQ* as covariates (horizontal signal), we found it had no impact on the association of SNPs in *HLA-DP* with HBV infection (vertical signal; presented as white). Likewise, the association of SNPs in *HLA-DQ* with HBV infection remained significant even after adjustment for the SNPs in the *HLA-DP* cluster. This clearly indicates that our detected significant associations of SNPs in *HLA-DQ* and *HLA-DP* with HBV infection are independent. In other words, the SNPs in these two gene clusters independently contribute to HBV infection.

Next, within each gene cluster, we wanted to determine which LD block contributes most to the detected association signal (Fig. 3b, c). As stated earlier, in the *HLA-DP* locus, there are Block 3 (in *HLA-DPA1*), Block 4 (in *HLA-DPA1/B1* overlapping region), and Block 5 (in *HLA-DPB1*) (Fig. 3b). When the SNPs in Block 3 were considered as covariates, the association of SNPs in Blocks 4 and 5 with HBV infection remained significant (shown in white in the figure). When we used SNPs in Block 4 as covariates, SNPs in Block 5 remained significantly associated with HBV infection (white), but not for the SNPs in Block 3 (dark gray). Further, we detected no significant association between SNPs in Block 4 or Block 3 with HBV infection when SNPs in Block 5 were used as covariates (dark grey). From these analyses, we thus concluded that the variants in Block 5 within *HLA-DPB1* contribute to the most

Table 3 Protein function prediction of non-synonymous SNPs with PolyPhen-2 program

Gene	dbSNP ID	Alleles	Protein name	Prediction
HLA-DPA1	rs1042190	A/G	HLA class II histocompatibility antigen DP alpha 1 chain (p20036)	Probably damaging
	rs1042178	A/G	HLA class II histocompatibility antigen DP alpha 1 chain (p20036)	Benign
HLA-DPA1	rs1042169	A/G	HLA class II histocompatibility antigen DP beta 1 chain (p04440)	Benign
HLA-DPB1		A/T		Possibly damaging
HLA-DPB1	rs14362	A/C	HLA class II histocompatibility antigen DP beta 1 chain (p04440)	Probably damaging

significant effect on the association of variants in this locus with HBV infection.

With the same approach, we analyzed the relation between Block 1 (i.e., *HLA-DQA1/B1*) and Block 2 (*HLA-DQA2/B2*) in the *HLA-DQ* cluster, which revealed a significant association of variants in Block 1 with HBV infection regardless of whether we adjusted for SNPs in Block 2 or vice versa (Fig. 3c). This indicates that the variants in Blocks 1 and 2 contribute independently to the detected association of *HLA-DQ* locus with HBV infection.

To validate these conditional analysis results, we used the same approach to analyze all individual SNPs within each LD block and found that none of the SNPs showed a significant association with HBV infection when conditioned on other SNPs for any given LD block (Fig. 3d). This confirms that the SNPs within the same LD block are in strong LD with each other and that these LD blocks represent the primary genetic unit of this region in determining the susceptibility of the *HLA-DQ/DP* locus to HBV infection.

Further association analysis among different subgroups

To determine whether SNPs in the *HLA-DP* and *HLA-DQ* clusters contribute to the progression of HBV-related diseases, we conducted association analysis of HBV carriers vs. CHB and CHB vs. HC, which revealed that none of the analyzed SNPs in the *HLA-DP* and *HLA-DQ* clusters was significant (data not shown). Thus, we conclude that the SNPs in the *HLA-DQ/DP* clusters are unlikely to influence the progression of HBV-related diseases, at least in our current investigated samples.

Further, we performed association analysis between the viral-clearance subgroup from the control sample and the subgroups of CHB, LC, or IHBV carriers from the case sample. These analyses revealed that a great number of SNPs between the HBV clearance and nine SNPs (located primarily in the 3′-UTR region of *HLA-DPB1*) between the HBV clearance and LC groups were significant, and no SNP was significant between the HBV clearance and IHBV carriers (see Supplementary Table 1).

Discussion

In this study, we not only confirmed but also greatly expanded the association of SNPs in the *HLA-DP/HLA-DQ* gene clusters on chromosome 6 with HBV infection by genotyping and analyzing 140 SNPs from the region in 1648 hepatitis cases and 1416 controls. With conditional analysis, we demonstrated that the variants in the *HLA-DP* and *HLA-DQ* clusters contribute independently to HBV infection and the variants in *HLA-DPB1* play a leading role in HBV infection. Furthermore, we conducted association analysis between HBV carriers vs. CHB subjects and CHB vs. HC subjects and found that the variants in this genomic region were not significantly associated with the progression of HBV-related diseases except for HBV infection.

During the past several years, both GWAS and candidate gene-based association studies of the HLA region on chromosome 6 have been conducted with different ethnic populations (Al-Qahtani et al. 2014; Nishida et al. 2012; Li et al. 2011; Hu et al. 2012; Vermehren et al. 2012; Mbarek et al. 2011a; Kamatani et al. 2009). One of the most convincing findings was identification of SNPs in the *HLA-DP* and *HLA-DQ* clusters as susceptibility loci for HBV infection (Jiang et al. 2014). However, within this complex genomic region, almost all previous studies examined only a limited number of SNPs, and the detailed relation between the effects of those SNPs in each cluster with HBV infection has not been fully investigated. For example, to the best of our knowledge, whether the variants in the *HLA-DP* and *HLA-DQ* clusters contribute to HBV infection either jointly or independently has rarely been addressed in the literature. However, determination of the effects of the two gene clusters on HBV infection is highly significant for understanding the mechanisms underlying such infection.

Significant associations of SNPs in the *HLA-DP* cluster with HBV infection have been replicated by several studies with different samples, which include SNPs rs3077 in *HLA-DPA1* and rs9277535 in *HLA-DPB1* (Jiang et al. 2014). In the current study, we not only confirmed the association of rs9277535 with HBV infection, but also revealed additional novel SNPs that are significantly associated with

Table 4 Association analysis of major haplotypes (frequency ≥0.05) in HLA-DP/DQ gene cluster on chromosome 6 with HBV infection under additive genetics model

Gene cluster	Block	Haplotype*	Control frequency	Case frequency	OR (95 % CI)**	Haplotype score	Haplotype *P* value	Global *P* value
HLA-DQ	Block 1 (*A1/B1*)	GGAAAGGAG	0.089	0.043	0.41 (0.33–0.51)	−7.176	7.19E−13	4.51E−21
		AGGCAAAGG	0.076	0.040	0.47 (0.37–0.59)	−5.683	1.32E−08	
		GGGCAAAGG	0.063	0.060	0.83 (0.67–1.03)	−0.571	0.568	
		GGGCAAAAG	0.214	0.237	0.96 (0.85–1.1)	2.266	0.0235	
		GGGCAAAAA	0.486	0.570	1.04 (0.91–1.18)	5.968	2.40E−09	
	Block 2 (*A2/B2*)	GGAAGAGGAGAA	0.141	0.086	0.59 (0.5–0.69)	−6.371	1.88E−10	4.00E−09
		AAGTAGAAGCGG	0.855	0.911	1.69 (1.44–1.99)	6.454	1.09E−10	
HLA-DP	Block 3 (*A1*)	ATAAGAGCGGATGATAAAGGAACAAAGCGAGAGCA	0.102	0.151	0.58 (0.49–0.68)	−5.591	2.25E−08	3.42E−08
		ATAAGAGCGGATGATAAAGGAACAAAGCGAGAGCG	0.123	0.123	0.74 (0.63–0.88)	−2.010	0.0440	
		CACGAGAAAAGAGAATGGCCCGAGGGAGAGAGGGG	0.105	0.105	0.82 (0.69–0.98)	−0.752	0.452	
		CACGAGAAAAGAAGATGGCCCGAGGGAGAGAGAGG	0.577	0.654	1.74 (1.47–2.06)	5.675	1.39E−08	
	Block 4 (*A1/B1*)	CA	0.362	0.266	0.62 (0.55–0.69)	−7.881	3.25E−15	8.86E−16
		AA	0.091	0.082	0.76 (0.64–0.91)	−1.108	0.268	
		AG	0.546	0.652	1.62 (1.45–1.81)	8.130	4.31E−16	
	Block 5 (*B1*)	CGGCGAAG	0.356	0.264	0.61 (0.54–0.68)	−7.450	9.34E−14	1.75E−16
		CACAAGGG	0.182	0.189	0.84 (0.73–0.96)	0.908	0.364	
		CACAAGGA	0.420	0.526	1.65 (1.47–1.85)	7.613	2.67E−14	

* SNP IDs corresponding to each SNP of the blocks listed in Table 2, which are given in sequential order

** Odds ratios of each haplotype calculated using the most frequent haplotype as baseline. The OR of the most frequent one in each block was calculated with the second frequent haplotype as baseline. *P* values, haplotype frequency and scores, odds ratios, and 95 % confidence intervals were calculated as described in "Subjects and methods"

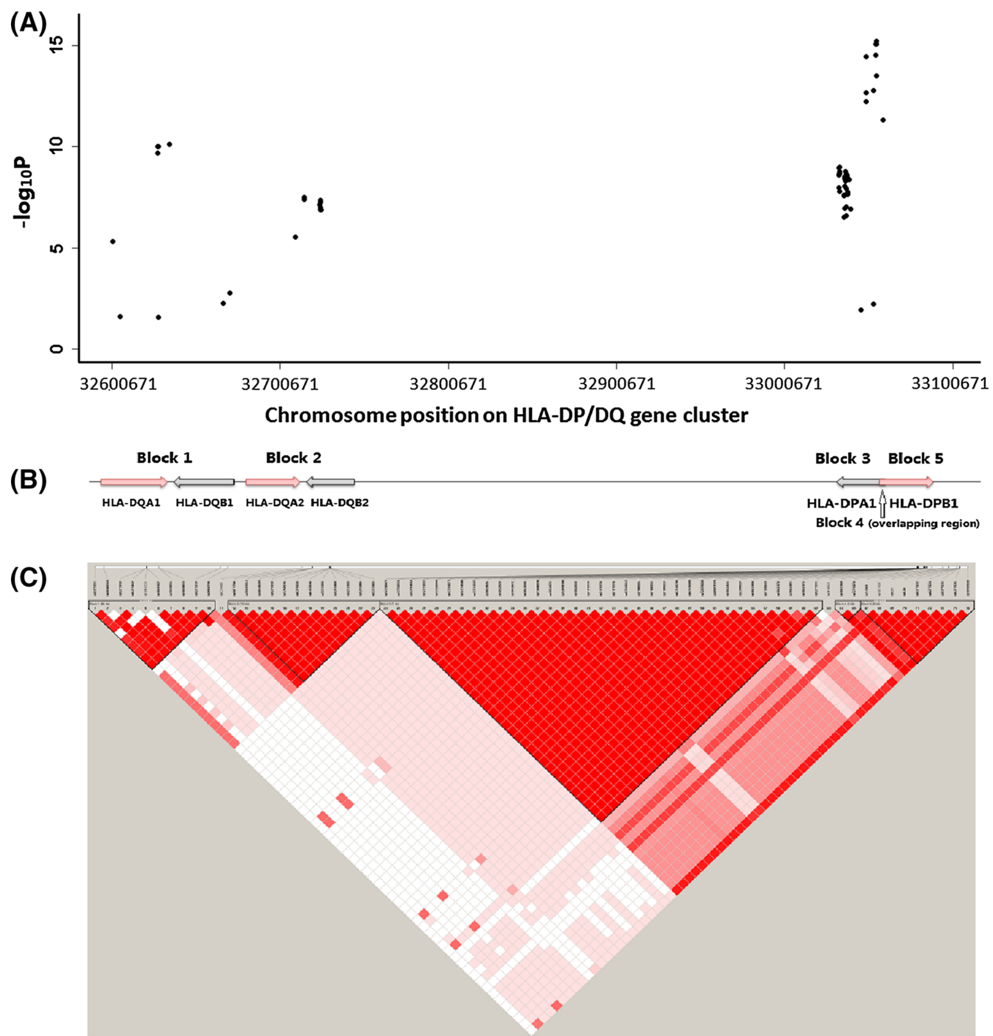

Fig. 1 **a** Scatter plot of the 76 SNPs associated significantly with HBV infection; **b** relation of block and gene locus; **c** LD structures for 76 SNPs in the *HLA-DP/DQ* clusters, which were drawn by Haploview. The details of these SNPs and block information are shown in Supplementary Figure 1 with *D*′ in each *box*

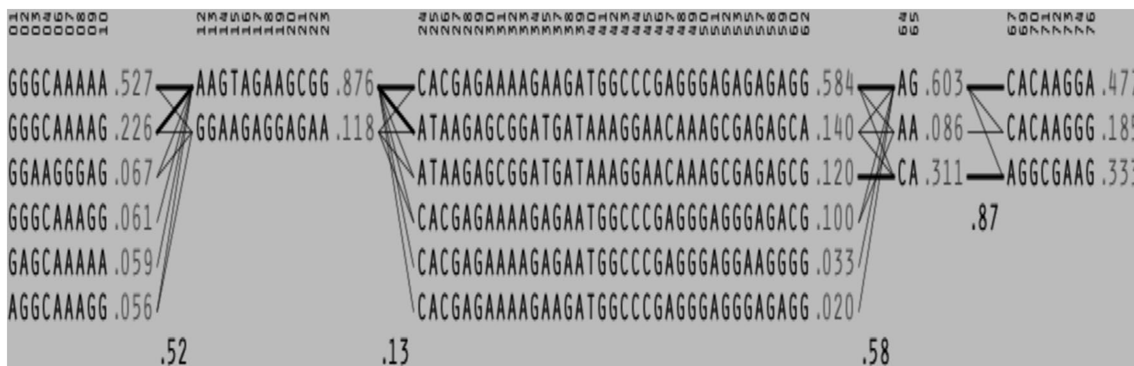

Fig. 2 Five LD block maps based on *D*′ value were constructed by Haploview with the genotypes of all case and control samples included. Blocks 1 and 2 are located in the *HLA-DQ* gene and Blocks 3, 4, and 5 in the *HLA-DP* gene

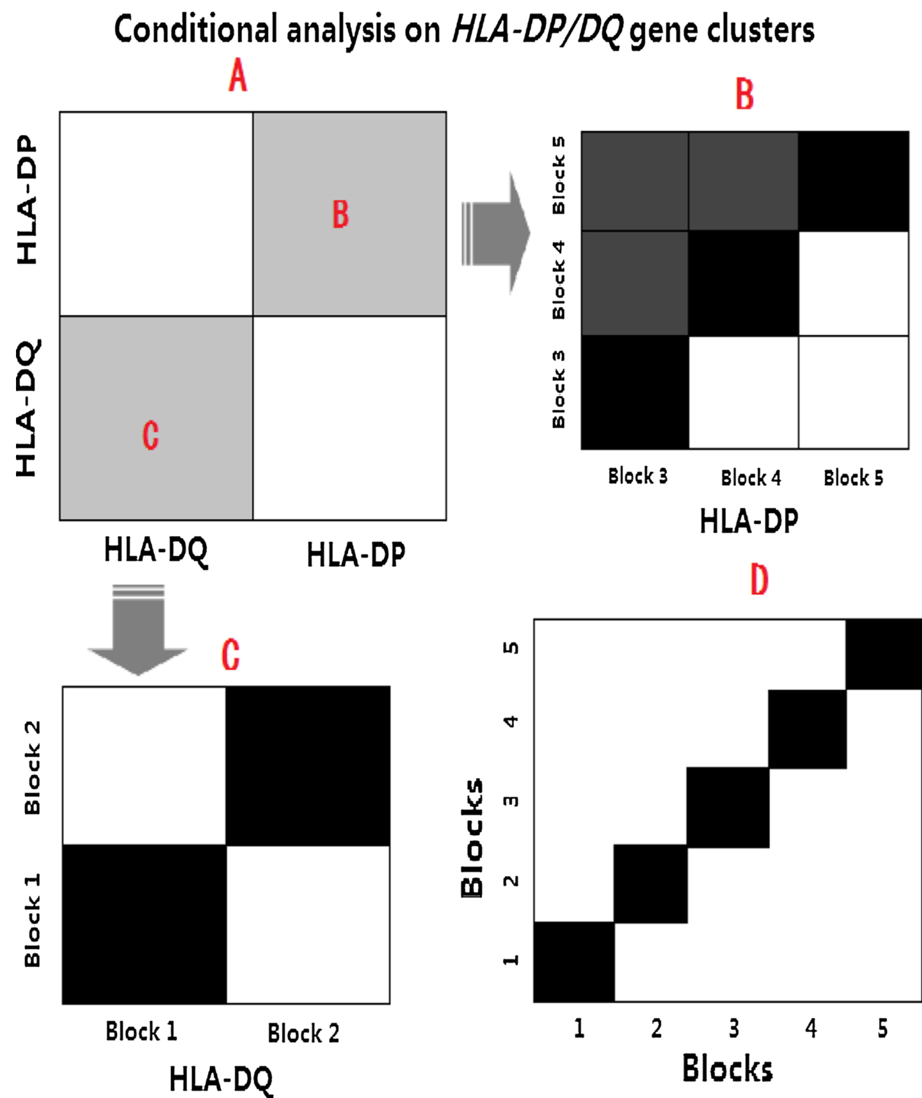

Fig. 3 **a** Pattern of the interaction between gene clusters *HLA-DQ* and *HLA-DP*. **b**, **c** Pattern of the interaction within gene clusters *HLA-DQ* and *HLA-DP*. **d** Pattern of the interaction of the five LD blocks with the horizontal signal set as covariates and the vertical signal set as the result after adjustments. *White* indicates significant association with HBV infection; *light grey* indicates mixed region; *dark grey* indicates no significant association after adjustment by other block(s); *black* indicates no significant association after adjustment by SNPs within the same block

HBV infection. Of the SNPs identified in this study, the seven top SNPs significantly associated with HBV infection are all located in the 3′-UTR region of *HLA-DPB1* gene with *P* values of 10^{-17} to 10^{-18}, which are much smaller than 5.0×10^{-8}, a commonly accepted *P* value for genome-wide significance (Dudbridge and Gusnanto 2008; Gao et al. 2008; Pe'er et al. 2008). In addition, we predict with the PolyPhen-2 program (Adzhubei et al. 2010) that three missense SNPs (i.e., rs1042190, rs1042169, and rs14362) in the *HLA-DP* cluster are likely to be functional, which deserve further investigation in the near future.

Through haplotype-based association analysis and the following conditional analysis, we demonstrated that SNPs in Block 5 within the *HLA-DPB1* locus, which contains Blocks 3–5, play an essential role in determining the observed association of this region with HBV infection. Moreover, significant association of SNPs in Block 5 with HBV infection was confirmed by independent association analysis between the viral-clearance and CHB groups. Thus, our current study not only revealed more SNPs within the *HLA-DP* gene cluster to be associated significantly with HBV infection, but also demonstrated the SNPs in Block 5 (*HLA-DPB1*) as the main susceptibility ones for HBV infection.

Independent of the *HLA-DP* cluster, SNPs rs2856718 and rs7453920 in the *HLA-DQ* cluster have been reported to be associated with HBV infection (Mbarek et al. 2011a; Hu et al. 2012). Another SNP, rs9275319, in the *HLA-DQ* cluster was also found to be associated with HBV infection in a Chinese population (Jiang et al. 2013). In addition to confirming the significant association of the SNPs rs2856718 and rs9275319 in the *HLA-DQ* cluster reported by others (Mbarek et al. 2011a; Al-Qahtani et al. 2014; Nishida et al. 2012; Li et al. 2011; Hu et al. 2012), our fine mapping analysis reported here revealed 21 more novel SNPs to be associated significantly with HBV infection.

Moreover, by using haplotype-based association and conditional analysis, we found highly significant associations between SNPs or haplotypes in the *HLA-DQ* cluster with HBV infection. Together, our results not only validated, but also extended the significant association of SNPs and haplotypes in the *HLA-DQ* gene cluster with HBV infection.

We also investigated the potential association of SNPs in *HLA-DP/DQ* with the progression of HBV-related diseases. By comparing the HBV carrier and CHB groups, our results were in accordance with the Caucasian study or another study with Chinese populations, where no significant association of SNPs in *HLA-DP/DQ* with the progression of HBV-related diseases was detected (Vermehren et al. 2012; Li et al. 2011). There has been controversy regarding the association of the HLA region and the transformation of CHB to HC or HCC according to the reported studies on Chinese populations (Hu et al. 2012; Jiang et al. 2013; Chen et al. 2013) and eastern Asian or Saudi Arab populations (Al-Qahtani et al. 2014). Specifically, in this study, we observed no significant association of SNPs in the *HLA-DP/DQ* region with the progression of CHB to HC. Because of the small sample size, no association analysis was performed with our HCC samples. Thus, future study with a large HCC sample is greatly needed to determine whether there exists any significant association between the SNPs in these two gene clusters and the progression from simple HBV infection to HCC.

In sum, there are three main findings from this study. First, by genotyping significantly more SNPs from the *HLA-DP/DQ* gene clusters on chromosome 6 in a Chinese Han population with a large sample and employing various association analysis approaches, we not only validated a significant association of a limited number of SNPs in this region with HBV infection, but also revealed many more SNPs that are significantly associated with HBV infection. Interestingly, we showed that almost all top SNPs associated significantly with HBV infection are all located in the 3′-UTR region of *HLA-DPB1*, indicating this is an important region for future molecular study to define how the *HLA-DP/DQ* clusters are involved in HBV infection at the molecular level. Second, through a series of conditional analyses of associated *P* values of SNPs or haplotypes within this region with HBV infection, we discovered for the first time that the *HLA-DP* and *HLA-DQ* regions are independently involved in the process underlying HBV infection. Finally, our results indicate that the variants in this genomic region are less likely to be associated with the progression of HBV-related diseases. Because of the relative small sample for the HCC group, no association analysis could be performed of SNPs in this genomic region with HCC. Thus, a large sample of patients with HCC is greatly needed to determine whether this region is indeed related to the progression from CHB to HCC. Similarly, more molecular analysis of SNPs in the 3′-UTR of *HLA-DPB1* is necessary to determine how these variants contribute to the process underlying HBV infection.

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Compliance with ethical standards

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Conflict of interest The authors declare no conflict of interest related to this paper.

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