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Key *HLA-DRB1-DQB1* Haplotypes and Role of the *BTNL2* Gene for Response to a Hepatitis B Vaccine

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Approximately 5-10% of individuals who are vaccinated with a hepatitis B (HB) vaccine designed based on the hepatitis B virus (HBV) genotype C fail to acquire protective levels of antibodies. Here, host genetic factors behind low immune response to this HB vaccine were investigated by a genome-wide association study (GWAS) and Human Leukocyte Antigen *(HLA)* association tests. The GWAS and *HLA* association tests were carried out using a total of 1,193 Japanese individuals including 107 low responders, 351 intermediate responders, and 735 high responders. Classical *HLA* class II alleles were statistically imputed using the genome-wide SNP typing data. The GWAS identified independent associations of *HLA-DRB1-DQB1*, *HLA-DPB1* and *BTNL2* genes with immune response to a HB vaccine designed based on the HBV genotype C. Five *HLA-DRB1-DQB1* haplotypes and two *DPB1* alleles showed significant associations with response to the HB vaccine in a comparison of three groups of 1,193 HB vaccinated individuals. When frequencies of *DRB1-DQB1* haplotypes and *DPB1* alleles were compared between low immune responders and HBV patients, significant associations were identified for three *DRB1-DQB1* haplotypes, and no association was identified for any of the *DPB1* alleles. In contrast, no association was identified for *DRB1-DQB1* haplotypes and *DPB1* alleles in a comparison between high immune responders and healthy individuals. Conclusion: The findings in this study clearly show the importance of *HLA-DR-DQ* (i.e., recognition of a vaccine related HB surface antigen (HBsAg) by specific *DR-DQ* haplotypes) and BTNL2 molecules (i.e., high immune response to HB vaccine) for response to a HB vaccine designed based on the HBV genotype C. (Hepatology 2018; 68:848-858).

Hepatitis B (HB) is one of the most common infectious diseases, with 350 million chronic hepatitis B virus (HBV) carriers worldwide. In Japan, an estimated 1.1-1.4 million individuals (about 1% of the country's population) are infected with HBV; most of these infections were caused by mother-to-child transmission before the start of a nationwide HB immunization program initiated by the Japanese government in 1986. About 80% of the HBV-infected patients in mainland Japan were HBV genotype C.[(1)] Genome-wide association studies (GWASs) have identified several susceptibility loci with the risk of chronic hepatitis B (CHB) infection in Asian and Arabian populations, including *HLA* class

Abbreviations: CHB, chronic hepatitis B; GWAS, genome-wide association study; HB, hepatitis B; HBcAb, hepatitis B core antibody; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HLA, Human Leukocyte Antigen; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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II, *EHMT2*, *TCF19*, *FDX1*, *HLA-C*, *UBE2L3*, and *VARS2-SFTA2*.[2-8] Association tests of *HLA* class II with CHB infection showed that two *HLA-DRB1-DQB1* haplotypes (i.e., *DRB1*15:02-DQB1*06:01* and *DRB1*13:02-DQB1*06:04*) and three *DPB1* alleles (i.e., *DPB1*02:01*, **04:02*, and **05:01*) were independently associated with CHB infection in the Japanese population.[9]

Universal HB universal vaccination programs have now implemented in over 180 countries worldwide. In Taiwan, a universal HB vaccination program was launched as early as 1984 to prevent HBV carriage from perinatal mother-to-infant infection.[10] Although a selective HBV vaccination program continued until 2016 in Japan, the Japanese government started a universal HB vaccination program in October 2016. Several HB vaccines have been developed to prevent HBV infection corresponding to HBV genotypes (i.e., genotype A [Heptavax-II], genotype A2 [Engerix-B; Recombivax HB], or genotype C [Bimmugen]). The immune response to HB vaccination differs among individuals, with 5-10% of healthy individuals failing to acquire protective levels of antibodies. This result suggests the involvement of host genetic factors in the response to vaccination. Indeed, associations of SNPs in *HLA* class II region with response to HB vaccines have been identified in Asian and European populations.[11-14] In these previous reports, the studied individuals were vaccinated with HB vaccine designed based on the HBV genotype A2. We describe here a GWAS to identify host genetic factors associated with response to a HB vaccine designed based on the HBV genotype C. We know SNP-based GWAS do not necessarily detect the primary susceptibility locus in the *HLA* region[9]; therefore, we carried out association tests of *HLA* class II alleles in comparisons between HB vaccinated individuals (low responders and high responders), healthy individuals, individuals with spontaneous HBV clearance and HBV patients who carried HBV genotype C, to identify commonality and heterogeneity between these groups.

Participants and Methods

Ethics approval

This study was approved by the Ethics Committee of The University of Tokyo and by all of the following institutes and hospitals throughout Japan that participated in this collaborative study: National Center for Global Health and Medicine; Kawasaki Medical School; University of Tsukuba; Iwate Medical University; and Chiba University. All participants provided written informed consent for participation in this study and the methods were carried out in accordance with the approved guidelines.

Samples and clinical data

All 1,193 Japanese genomic DNA samples used in this study were obtained from healthy adult volunteers ($\geq$18-years-old) who were vaccinated in three doses (0.5 ml) at 0, 1, and 6 months with a recombinant absorbed HB vaccine (Bimmugen, Kaketsuken, Kumamoto, Japan). Individuals who were vaccinated with the Heptavax-II vaccine (MSD KK, Tokyo, Japan) were not included in this study. Serum anti-HBV surface antibody (HBsAb) and serum anti-HBV core antibody (HBcAb) were tested before the vaccination and at 1 month after final inoculation, using the anti-HBs kit and the anti-HBc II kit, respectively, with a fully automated chemiluminescent enzyme immunoassay system

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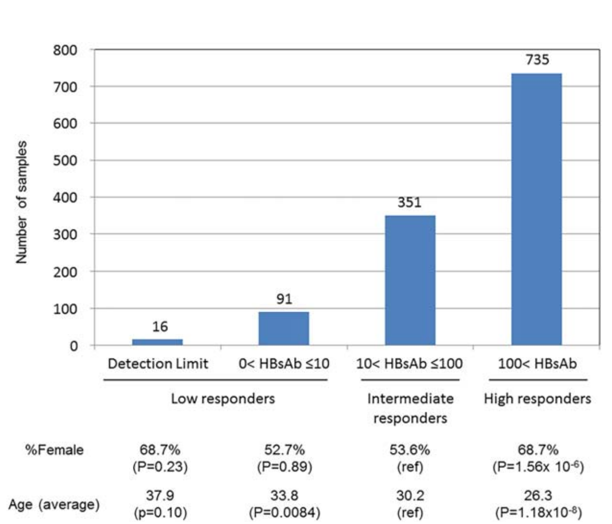

FIG. 1. Distribution of HBsAb levels among 1,193 Japanese individuals. P values were calculated using a chi-quared test and Welch's t-test for percentage of female and average age, respectively.

using the Architect i2000SR analyzer (Abbott Japan, Tokyo, Japan). Individuals who were HBcAb-positive (>1.0 S/CO) were not included in this study. In this study, we categorized 1,193 individuals into three groups: group_0, low responders, HBsAb ≤10 mIU/mL; group_1, intermediate responders, 10 mIU/mL< HBsAb ≤100 mIU/mL; group_2, high responders, HBsAb >100 mIU/mL. The clinical information of 1,193 individuals is summarized for each group in Supporting Table S1. The distribution of HBsAb levels among the 1,193 Japanese individuals is summarized in Fig. 1. Percentage of female participants and average age between groups were tested using group 1 (intermediate responders) as reference by a chi-squared test and Welch's t-test, respectively. Data of age and number of times of past vaccination with Bimmugen were collected from all 1,193 individuals in the writing of the questionnaire. Data of computed gender for the 1,193 individuals were acquired from the genome-wide SNP genotyping data of the Affymetrix AXIOM genome-Wide ASI 1 array acquired in this study.

Genome-wide SNP genotyping and data cleaning

For the GWAS, the 1,193 Japanese genomic DNA samples were genotyped using the Affymetrix Axiom Genome-Wide ASI 1 Array, according to the manufacturer's instructions. All samples had an overall call rate of more than 96%; the average overall call rate was 99.23% (96.77-99.88) and passed a heterozygosity check. No related individual (PI ≥0.1) was identified in identity-by-descent testing. Principal component analysis was carried out to check the genetic background in the studied 1,193 samples together with HapMap samples (43 JPT, 40 CHB, 91 YRI, and 91 CEU samples). This analysis showed that all 1,193 samples formed the same cluster using the first and second components, indicating that the effect of population stratification was negligible in the studied samples (Supporting Fig. S1).

A total of 1,193 individuals were categorized into three groups: group_0, low responders, HBsAb ≤10 mIU/mL (n=107); group_1, intermediate responders, 10 mIU/mL< HBsAb ≤100 mIU/mL (n=351); group_2, high responders, HBsAb >100 mIU/mL (n=735). Genome-wide multiple regression analysis was carried out using age and sex as covariates, where the above-mentioned group (i.e., 0, 1, or 2) was used as a dependent variable. The following thresholds were then applied for SNP quality control during the data cleaning: SNP call rate ≥ 95%; minor allele frequency ≥5%; and Hardy-Weinberg Equilibrium *P*-value ≥0.001. All cluster plots for SNPs with a $P < 0.0001$ based on a chi-square test of the allele frequency model were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded. Of the SNPs on autosomal chromosomes, 427,664 SNPs finally passed the quality control filters and were used for the association analysis (Fig. 2). To avoid false positives due to multiple testing, the significance levels for α were set at α=0.05/427,664. The 194 SNPs with P < 0.0001 in the GWAS are listed except for the *HLA* class II region (*HLA-DRA* to *HLA-DPA3*; Chr6: 32,377,284-33,099,120) in Supporting Table S2. Supporting Fig. S2 shows the regional Manhattan plot of the *HLA* class II region. The 212 SNPs with P < 0.0001 in the GWAS are listed for the *HLA* class II region in Supporting Table S3.

HLA imputation

SNP data from 1,193 samples were extracted from an extended MHC (xMHC) region ranging from 25759242 to 33534827 bp based on the hg19 position. We conducted 2-field *HLA* genotype imputation for four class II *HLA* genes using the HIBAG R package.[15] For *HLA-DRB1*, *DQA1*, *DQB1* and *DPB1*, our in-house Japanese imputation reference was used for *HLA* genotype imputation.[16] We applied post-

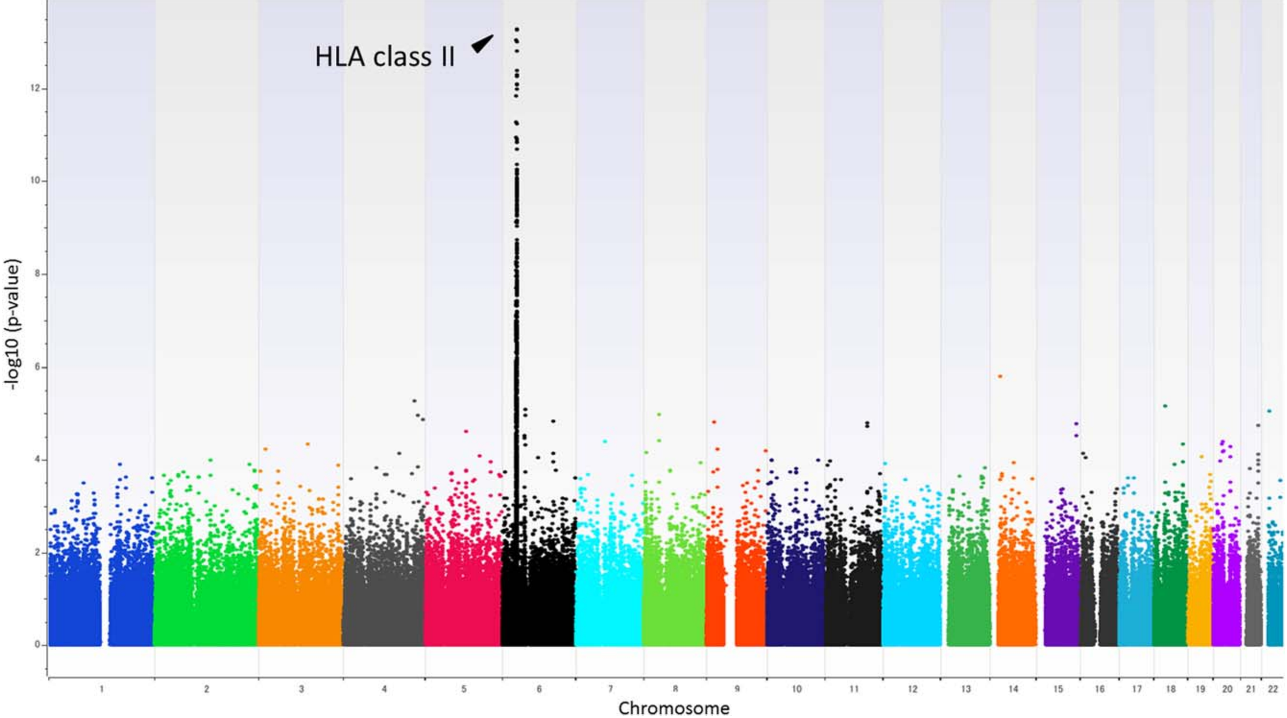

FIG. 2. Genome-wide association results applying a regression analysis with age and sex as covariates. From a total of 1,193 Japanese individuals who received HB vaccination, P values were calculated for 427,664 SNPs by multiple regression analysis in three responder groups (group_0, low responder, HBsAb ≤10; group_1, intermediate responder, 10< HBsAb ≤100; group_2, high responder, HBsAb >100).

imputation quality control using call-threshold (CT >0.5). A total of 1,103 samples showed four estimated *HLA* genotypes, all of which met the threshold. In total, we imputed 25 *HLA-DRB1*, 14 *HLA-DQA1*, 14 *HLA-DQB1*, and 11 *HLA-DPB1* genotypes for *HLA* class II genes.

Haplotype estimation

The phased haplotypes consisting of three *HLA* class II loci (*HLA-DRB1*, *-DQB1*, and *-DPB1*) were estimated by using the PHASE program version 2.1.[17,18] The estimated 3-locus haplotypes were further used for the estimation of haplotypes of *HLA-DRB1* and *DQB1* loci (i.e., the collapsing method was applied to the phased data for three *HLA* loci).

Pairwise linkage disequilibrium between *HLA* class II alleles

The pairwise linkage disequilibrium (LD) parameters, r^2 and D',[19] between alleles at different class II *HLA* loci were calculated based on the haplotype frequencies estimated by using the expectation maximization (EM) algorithm.[20] Here, each *HLA* allele was assumed to be one of the alleles at a bi-allelic locus, and the other *HLA* alleles at the same locus were assumed to be the other allele. For example, the *DRB1*01:01* allele and the other *DRB1* alleles were designated as the “A allele” and the “B allele”, respectively. Accordingly, the EM algorithm for the estimation of haplotype frequencies for two loci each with two alleles could be applied to two *HLA* alleles at different loci.[21]

HLA association test

To assess the association of *HLA* allele or haplotype with response to HB vaccination, P values were calculated using the Cochran-Armitage trend test in three responder groups (group_0, low responder, HBsAb ≤10; group_1, intermediate responder, 10< HBsAb ≤100; group_2, high responder, HBsAb >100). To avoid false positives due to multiple testing, the

significance levels for α were set at $\alpha=0.05/50$ for beta chain of *HLA* class II alleles (including *DRB1*, *DQB1*, and *DPB1*), $\alpha=0.05/14$ for *HLA-DQA1* and $\alpha=0.05/31$ for *HLA DRB1-DQB1* haplotypes.

Web resources

GWAS data in this study will be submitted to the public database "NBDC Human Database" in Japan. The URL for data presented herein is as follows:

NBDC Human Database, https://humandbs.biosciencedbc.jp/en/

Results

All 1,193 Japanese healthy individuals in this study were vaccinated with the same recombinant absorbed HB vaccine, following standard operating procedures (see Participants and Methods for details). Serum and genomic DNA were collected from the 1,193 individuals, and the presence of serum anti-HBV surface antibody (HBsAb) and serum anti-HBV core antibody (HBcAb) was then tested at 1 month after the final dose. Because individuals who are HBcAb-positive are prone to be low responders (HBsAb $\leq$10 mIU/mL) to HB vaccination, HBcAb-positive individuals were excluded from this study. In this study, we categorized the 1,193 individuals into three groups: group_0, low responders, HBsAb $\leq$10 mIU/mL (n=107); group_1, intermediate responders, 10 mIU/mL< HBsAb $\leq$100 mIU/mL (n=351); and group_2, high responders, HBsAb >100 mIU/mL (n=735). Among the 1,193 samples, 16 individuals who did not reach the detection limit of the HBsAb level were categorized into group_0 (i.e., low responders). A comparison of percentage of female and average age between the three groups indicated that the frequencies of younger individuals and females were significantly increased in high responders (Fig. 1). The significant associations of sex and age with HBsAb levels were also identified by simple linear regression analysis ($P = 4.04\times10^{-6}$ and $P = 1.75\times10^{-12}$, respectively). In contrast, the number of past vaccinations with Bimmugen showed no association in simple linear regression analysis (P = 0.056).

Genome-wide multiple regression analysis was carried out, in which HBsAb levels were categorized into three groups as a dependent variable, and information of age and sex were used as covariates. The top hit SNP rs2395179 was identified in a *HLA* class II region, showing $P = 5.27\times10^{-14}$ (Fig. 2). The regional Manhattan plot of a *HLA* class II region (from *HLA-DRA* to *HLA-DPA3*; Chr6: 32,377,284-33,099,120, GRCh37 hg19) showed three peaks (rs2395179 for *HLA-DRA*, rs34039593 for *HLA-DRB1*, and rs9277549 for *HLA-DPB1*) (Supporting Fig. S2). To investigate the relationship between these three variants and HBsAb levels, we performed regression analysis using various combinations of the three associated SNPs as covariates (Supporting Fig. S3). When the regression analysis was performed using the two SNPs located in the *HLA-DR-DQ* region (i.e., rs2395179 and rs34039593, Supporting Fig. S3A), the associations of a number of other SNPs located around these two SNPs were weakened, whereas those for the SNPs in the *HLA-DP* region were not. Similarly, in the regression analysis using rs9277549 located in the *HLA-DP* region, associations of surrounding *HLA-DP* SNPs weakened, whereas associations for SNPs in *HLA-DR-DQ* region were not weakened (Supporting Fig. S3B). In the regression analysis using the three representative SNPs as covariates, associations of a number of other SNPs located in the *HLA* class II region were weakened (Supporting Fig. S3C). These results indicated that although SNPs in the *HLA* class II region were in strong LD with each other, *HLA-DR-DQ* and *HLA-DP* regions were independently associated with immune response to the HB vaccine. When a GWAS was carried out using individuals under the age of 30 years, which included 56 low-responders, 226 intermediate-responders, and 582 high-responders, a significant association of SNP in the *HLA* class II region was identified as similar to the result using a total of 1,193 individuals (rs9268657, $P = 4.08\times10^{-9}$) (Supporting Fig. S4). Among individuals who were categorized into intermediate responders to the HB vaccine, there may exist ambiguous individual(s) who need to be re-categorized into low or high responders. We therefore carried out a GWAS that compared low responders and high responders, by applying a logistic regression analysis with age and sex as covariates. This GWAS identified a significant association of the *BTNL2* gene (rs4248166, $P = 5.51\times10^{-12}$), which is located in a *HLA* class III gene-rich region adjacent to a *HLA* class II region, with response to HB vaccination (Supporting Fig. S5).

Next, we performed statistical imputation of classical *HLA* alleles for four *HLA* loci including *HLA-DRB1*, *DQA1*, *DQB1*, and *DPB1* using 1,193 genome wide SNP typing data as established in our previous report.[16] After removing the defect data to compare P values and odds ratios (ORs) of each *HLA* allele, associations of each *HLA* allele with HBsAb levels were assessed by calculating P values using the Cochran-

TABLE 1. Associations of *HLA* class II alleles with response to the HB vaccine

Allele	p-value*	Frequencies in Group_0 (2n = 188)	Frequencies in Group_1 (2n = 646)	Frequencies in Group_2 (2n = 1,372)	Allele Frequency	Poor Responder (1) / Responder (0)
DRB1*01:01	**8.89E-05**	**1.6**	**3.7**	**7.1**	**5.6**	**0**
DRB1*04:05	**2.61E-09**	**28.2**	**17.3**	**12.1**	**15.0**	**1**
DRB1*08:03	**2.75E-06**	**2.1**	**5.6**	**10.1**	**8.1**	**0**
*DRB1*09:01*	0.04748	18.1	15.5	13.3	14.4	1
*DRB1*13:02*	0.03048	4.8	4.6	7.2	6.3	0
DRB1*15:01	**0.0002494**	**1.6**	**6.2**	**8.7**	**7.3**	**0**
*DRB1*15:02*	0.1919	11.2	12.8	10.1	11.0	0
*DQB1*03:01*	0.4503	12.8	10.4	10.3	10.6	1
*DQB1*03:02*	0.1264	10.1	10.5	8.2	9.1	0
*DQB1*03:03*	0.1728	18.1	16.1	14.7	15.4	1
DQB1*04:01	**5.01E-09**	**28.2**	**17.5**	**12.3**	**15.2**	**1**
DQB1*05:01	**4.47E-05**	**1.6**	**3.9**	**7.4**	**5.9**	**0**
*DQB1*05:03*	0.3598	5.3	5.9	4.7	5.1	0
*DQB1*06:01*	0.02667	13.3	18.4	20.3	19.1	0
DQB1*06:02	**0.0003225**	**1.6**	**6.0**	**8.5**	**7.2**	**0**
*DQB1*06:04*	0.01879	3.7	4.5	6.8	5.8	0
*DPB1*02:01*	0.05939	22.9	19.7	24.9	23.2	0
*DPB1*03:01*	0.9037	3.7	5.9	5.0	5.2	0
DPB1*04:02	**7.58E-08**	**1.6**	**6.0**	**11.4**	**9.0**	**0**
DPB1*05:01	**4.76E-09**	**57.4**	**47.7**	**38.3**	**42.7**	**1**
*DPB1*09:01*	0.1832	10.6	11.8	9.3	10.2	0

The allele frequencies over 5.0% in a total of 1,103 Japanese individuals are shown in the table.
*P values were calculated using Cochran-Armitage trend test to test a trend in three groups (group_0, low responder, HBsAb $\leq$10; group_1, intermediate responder, 10$<$ HBsAb $\leq$100; group_2, high responder, HBsAb $>$100). P values, statistically significant after correction of the significance level ($P < 0.05/50$), are indicated in bold.

Armitage trend test to test for a trend in the three groups (i.e., group_0 [n=94], group_1 [n=323], and group_2 [n=686]). Significant associations after correction for the total number of the observed alleles ($P < 0.05/50$) were detected for nine *HLA* class II alleles (Table 1). The strongest association was observed for *HLA-DRB1*04:05* with a poor response to the HB vaccine ($P = 2.61 \times 10^{-9}$). On the other hand, among *HLA* class II alleles, *HLA-DPB1*04:02* showed the strongest association with response to the HB vaccine ($P = 7.58 \times 10^{-8}$). Because strong LD between *DRB1* and *DQB1* alleles have been reported in many populations including the Japanese population,[22-24] we then estimated *HLA-DRB1-DQB1* haplotypes in 1,103 studied individuals. This haplotype analysis identified significant associations after correction of the significance level ($P < 0.05/31$) for a total of five haplotypes (Table 2). Among the estimated haplotypes whose frequency was over 2.0% in the 1,103 studied individuals, two significant *DRB1-DQB1* haplotypes, *DRB1* 04:05-DQB1*04:01* and *DRB1*14:06-DQB1*03:01*, were associated with a poor response to the HB vaccine, and the remaining three significant *DRB1-DQB1* haplotypes, *DRB1* 01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:01-DQB1* 06:02,* were associated with response to the HB vaccine.

Frequencies of *HLA-DRB1-DQB1* haplotypes and *HLA-DPB1* alleles were then compared between HB vaccinated individuals and HBV patients described in our previous report.[9] Here, the associations of *HLA* class II genes with chronic HBV infection were recalculated, because *HLA* imputation was carried out again using our in-house Japanese imputation reference (Supporting Table S4). In a comparison between low responders to the HB vaccine and HBV patients, three *DRB1-DQB1* haplotypes, *DRB1*04:05-DQB1*04:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*14:06-DQB1* 03:01,* showed significant associations, while no association of *DPB1* alleles was identified (Table 3). There was no significant association of any haplotype or allele in a comparison between high responders to HB vaccine and healthy individuals (Supporting Table S5). Moreover, frequencies of *HLA-DRB1-DQB1* haplotypes and *DPB1* alleles were compared between low responders to the HB vaccine and spontaneous HBV clearance individuals described in our previous paper.[4] A similar result was obtained, as shown in the comparison between low responders and high responders, because frequencies of *HLA* alleles showed very similar

TABLE 2. Associations of *HLA-DRB1-DQB1* haplotypes with response to the HB vaccine

Haplotype (*DRB1-DQB1*)	p-value	Frequencies in group_0 (2n = 188)	Frequencies in Group_1 (2n = 646)	Frequencies in Group_2 (2n = 1,372)	Allele Frequency	Poor Responder (1)/ Responder (0)
01:01-05:01	**8.89E-05**	**1.6**	**3.7**	**7.1**	**5.6**	**0**
04:03-03:02	0.02484	4.3	3.7	2.2	2.8	1
04:05-04:01	**2.61E-09**	**28.2**	**17.3**	**12.1**	**15.0**	**1**
04:06-03:02	0.2962	4.3	3.6	3.0	3.3	1
08:02-03:02	0.32	1.1	3.3	3.1	2.9	0
08:02-04:02	0.03955	0.5	1.5	2.5	2.0	0
08:03-06:01	**2.75E-06**	**2.1**	**5.6**	**10.1**	**8.1**	**0**
09:01-03:03	0.04748	18.1	15.5	13.3	14.4	1
11:01-03:01	0.05902	1.1	1.7	2.8	2.3	0
12:01-03:01	0.2003	1.1	2.3	2.7	2.4	0
13:02-06:04	0.01879	3.7	4.5	6.8	5.8	0
14:05-05:03	0.5282	2.7	2.6	2.2	2.4	1
14:06-03:01	**0.0006397**	**5.3**	**2.8**	**1.5**	**2.2**	**1**
14:54-05:03	0.4548	2.7	3.3	2.4	2.7	0
15:01-06:02	**0.0003225**	**1.6**	**6.0**	**8.5**	**7.2**	**0**
15:02-06:01	0.1919	11.2	12.8	10.1	11.0	0

The estimated haplotype frequencies over 2.0% in a total of 1,103 Japanese individuals are shown in the table.
*P values were calculated using Cochran-Armitage trend test to test a trend in three groups (group_0, low responder, HBsAb $\leq$10; group_1, intermediate responder, 10< HBsAb $\leq$100; group_2, high responder, HBsAb >100). P values, statistically significant after correction of the significance level (P < 0.05/31), are indicated in bold.

both in spontaneous HBV clearance individuals and high responders (Supporting Table S6).

In general, the beta chain of *HLA* class II molecule shows higher level of polymorphism than the alpha chain and is considered to be better marker for risk prediction. Moreover, it is possible to estimate the *DRA1/DQA1* allele from the *DRB1/DQB1* allele because a strong LD exists between *DRA1/DQA1* and *DRB1/DQB1* loci. Here, we carried out *HLA* imputation for *HLA-DQA1*. In the comparison of *HLA-DQA1* frequencies between low responders and high responders, three *DQA1* alleles showed significant associations after correction for the total number of the observed alleles (P < 0.05/14) (Supporting Table S7).

Discussion

Among a total of 1,193 studied individuals who were vaccinated with a HB vaccine designed based on HBV genotype C (i.e., Bimmugen), 107 individuals (8.9%) showed a low response to this HB vaccine (HBsAb $\leq$10 mIU/mL). The distribution of low responders in the studied individuals is in concordance with past empirical data, with 5-10% of healthy individuals failing to acquire protective levels of antibodies. Although there has been a report of a HBV-vaccinated donor showing a HBsAb value of 96 IU per liter who was found to be positive for HBV DNA,[25] we set levels of antibodies against HBV surface antigen as 10 IU per liter or more to be considered as having immunity against HBV, following the World Health Organization recommendation. In this study, we categorized 1,193 individuals into three groups of vaccine responders and identified that the frequencies of younger individuals and females were significantly increased in high responders.

Significant associations of SNPs located in the *HLA* class II region with vaccine response were identified in a genome-wide multiple regression analysis using age and sex as covariates. Moreover, *HLA-DRB1-DQB1* and *DPB1* regions showed independent associations with response to the HB vaccine when regression analysis was applied using three variants (rs2395179 for *HLA-DRA*, rs34039593 for *HLA-DRB1*, and rs9277549 for *HLA-DPB1*) as covariates. There was a total of 194 SNPs outside and 212 SNPs inside the *HLA* class II region that had a P value lower than 0.0001 in the GWAS. Among these 194 SNPs, SNPs located in the *BTNL2* gene showed a significant association with response to HB vaccination (rs4248166, P=1.49×10^{-12}). Regression analysis in which the three SNPs (rs2395179, rs34039593, and rs9277549) were applied as covariates showed that a *BTNL2* SNP (rs4248166) was independently associated with a response to HB vaccine (P=0.006). This is the same SNP (rs4248166) that was significantly identified in the GWAS in which regression analysis was applied with age and sex as covariates, in a comparison between 107 low responders and 735 high responders

TABLE 3. Comparison of *DRB1-DQB1* haplotypes and *DPB1* alleles between low responders to the HB vaccine and HBV patients

Allele/Haplotype	Group_0 (2n = 188)		HBV Patients (2n = 1,630)		P-value	OR	95% CI	
	count	%	count	%			Lower	Upper
*DRB1*01:01-DQB1*05:01*	3	1.6	44	2.7	3.67E-01	0.58	0.18	1.90
*DRB1*04:03-DQB1*03:02*	8	4.3	24	1.5	6.00E-03	2.97	1.32	6.72
DRB1*04:05-DQB1*04:01	**53**	**28.2**	**212**	**13.0**	**2.31E-08**	**2.63**	**1.85**	**3.72**
*DRB1*04:06-DQB1*03:02*	8	4.3	28	1.7	1.80E-02	2.54	1.14	5.66
*DRB1*04:10-DQB1*04:02*	3	1.6	22	1.3	7.84E-01	1.19	0.35	4.00
DRB1*08:03-DQB1*06:01	**4**	**2.1**	**179**	**11.0**	**1.33E-04**	**0.18**	**0.06**	**0.48**
*DRB1*09:01-DQB1*03:03*	34	18.1	306	18.8	8.19E-01	0.96	0.65	1.41
*DRB1*13:02-DQB1*06:04*	7	3.7	37	2.3	2.19E-01	1.67	0.73	3.79
*DRB1*14:03-DQB1*03:01*	5	2.7	9	0.6	1.75E-03	4.92	1.63	14.84
*DRB1*14:05-DQB1*05:03*	5	2.7	33	2.0	5.64E-01	1.32	0.51	3.43
DRB1*14:06-DQB1*03:01	**10**	**5.3**	**2**	**0.1**	**7.98E-17**	**45.73**	**9.94**	**210.36**
*DRB1*14:54-DQB1*05:03*	5	2.7	30	1.8	4.39E-01	1.46	0.56	3.80
*DRB1*15:01-DQB1*06:02*	3	1.6	113	6.9	4.58E-03	0.22	0.07	0.69
*DRB1*15:02-DQB1*06:01*	21	11.2	306	18.8	1.02E-02	0.54	0.34	0.87
*DPB1*02:01*	43	22.9	301	18.5	1.44E-01	1.31	0.91	1.88
*DPB1*03:01*	7	3.7	85	5.2	3.77E-01	0.70	0.32	1.54
*DPB1*04:01*	2	1.1	36	2.2	2.99E-01	0.48	0.11	1.99
*DPB1*04:02*	3	1.6	89	5.5	2.21E-02	0.28	0.09	0.90
*DPB1*05:01*	108	57.4	756	46.4	4.01E-03	1.56	1.15	2.12
*DPB1*09:01*	20	10.6	266	16.3	4.28E-02	0.61	0.38	0.99
*DPB1*13:01*	3	1.6	28	1.7	9.03E-01	0.93	0.28	3.08

The estimated *DRB1-DQB1* haplotype frequencies over 1.5%, and *DPB1* allele frequencies over 1.0% in 94 HB vaccine low responders (group_0) are shown in the table. Significance level (α) was adjusted based on the number of observed haplotypes and alleles at each locus. Significance levels for *DRB1-DQB1* haplotypes and *DPB1* alleles were set at $\alpha = 0.05/31$ and 0.05/50, respectively. P value and odd ratio (OR) were calculated by Pearson's chi-square test in presence vs. absence of each allele. P values and OR, statistically significant after correction of the significance level, are indicated in bold.

(OR=0.20, P = 5.51×10^{-12}). In previous GWASs in Indonesian and Chinese populations,[11,13] associations of SNPs located in *HLA-DRB1* and *BTNL2* genes with response to a HB vaccine designed based on the HBV genotype A2 were identified. Because the current HBV-A2 vaccines are known to have cross-reactivity with, and confer cross-protection against non-A2 HBV genotypes,[26] genetic backgrounds behind response to the HBV-A2 and the HBV-C vaccines may be very similar.

The *BTNL2* gene has been reported to have an important function in the regulation of T cell activation,[27] which has implications for a variety of immune diseases, such as sarcoidosis[28] and inflammatory bowel disease,[29] as well as for immunotherapy.[30] Moreover, the resolution of woodchuck hepatitis virus (WHV) infection was reported to be accompanied by high level expression of inhibitory T cell receptors PD-1 (PDCD1) and BTNL2.[31] WHV was the first of the mammalian and avian hepadnaviruses described after discovery of the HBV, and known to develop progressively severe hepatitis and hepatocellular carcinoma. An SNP rs9277534 in the 3′ UTR region of *HLA-DPB1*, which showed a strong LD (r^2=0.99) with rs9277535 in the Japanese individuals, was reported to be associated with HBV recovery in both European- and African-American populations.[32] Moreover, the GG genotype at rs9277534 was revealed to be associated with higher levels of HLA-DP surface protein and transcript expression in European- and African-American cohort.[32] These observations designate the importance of functional analysis of HLA expression level in hepatocytes in the Japanese population. The *DPB1*04:02* and *DPB1*05:01* alleles, which showed the higher and lower response to the HB vaccine in this study, have A and G nucleotides at rs9277534, respectively. Although the possible contribution of HLA-DP expression levels on response to the HB vaccine needs further investigation on the other DP alleles in the Japanese population, the expression level could also affect the response to the HB vaccine. These results imply that both recognition of HBV surface antigens (HBsAg) by HLA class II molecules and regulation of T cell activation by BTNL2 molecules are key mechanisms for response to a HB vaccine.

Here, functional variant(s) located in *HLA* class II and *BTNL2* genes were predicted using the

bioinformatic tool SNP Function Prediction (FuncPred; http://snpinfo.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi) and the database GTEx portal (http://gtexportal.org/home/). Two hundred ten and 58 SNPs, having LD >0.2 with rs2395179 (top hit SNP in *HLA* class II) and rs4248166 (top hit SNP in *BTNL2*) in the Japanese population, respectively, were selected. SNP function predictions were then carried out including nonsynonymous SNP (nsSNP), splicing regulation, stop codon, polyphen prediction, SNPs3D prediction, TFBS prediction, miRNA binding site prediction, regulatory potential score, conservation score, and nearby genes. Six SNPs for *HLA* class II gene and 11 SNPs for *BTNL2* gene were finally selected to be predicted as transcription binding site(s) or nsSNP (Supporting Table S8 and Supporting Table S9). All six SNPs for *HLA* class II and 10 out of 11 SNPs for *BTNL2* showed a significant association with HLA-DRB1 mRNA expression in both whole blood and lung (Supporting Fig. S6), and with BTNL2 mRNA expression in small intestine (Supporting Fig. S7).

To further understand the association of *HLA* class II genes with response to the HB vaccine, we carried out *HLA* association tests by applying statistical imputation of *HLA* alleles using genome-wide SNP typing data. Nine *HLA* class II alleles showed significant associations with response to the HB vaccine; three of these alleles were associated with a poor response, and the remaining six were associated with response to the HB vaccine. Among these nine *HLA* class II alleles, the association of *DQB1*04:01* with non-responsiveness to the HBsAg,[33] and the association of five other alleles with vaccine response (*DRB1*01:01*, *DRB1*08:03*, *DQB1*05:01*, and *DPB1*04:02* with sufficient response to the HB vaccine and *DPB1*05:01* with a poor response to the HB vaccine)[34] were clearly replicated the results of previous studies in the Japanese population. Here, an epitope prediction for the nine associated *HLA* class II alleles was carried out using the immune epitope database (IEDB) 3.0, showing predicted core peptides within vaccinated HBsAg region for each *HLA* allele (Supporting Table S10). The half maximal inhibitory concentration (IC50) for the predicted core peptide showed smaller in *HLA-DQ* and *DP* allele products which were associated with responder than the ones which are associated with poor responder. In this study, we carried out haplotype analysis for *HLA-DRB1* and *DQB1* loci, and then associations with vaccine response were analyzed using the Cochran-Armitage trend test to test for a trend in three responder groups. Five *HLA-DRB1-DQB1* haplotypes showed significant associations with response to the HB vaccine. When frequencies of *DRB1-DQB1* haplotypes and *DPB1* alleles were compared between low responders to the HB vaccine and HBV patients, three out of the five *DRB1-DQB1* haplotypes showed a significant association; however, no association was observed for *HLA-DPB1* alleles.

TABLE 4. Associations of genes and alleles with response to HB vaccine, CHB infection and spontaneous HBV clearance.

Response to HB vaccine*	*HLA-DRB1/-DQB1/-DPB1*	*DPB1*04:02* *DPB1*05:01* *DRB1*01:01-DQB1*05:01* *DRB1*04:05-DQB1*04:01* *DRB1*08:03-DQB1*06:01* *DRB1*14:06-DQB1*03:01* *DRB1*15:01-DQB1*06:02*
	BTNL2	rs4248166 C/T
Chronic hepatitis B infection	*HLA-DRB1/-DQB1/-DPB1*†	*DPB1*02:01* *DPB1*04:01* *DPB1*05:01* *DRB1*15:02-DQB1*06:01* *DRB1*13:02-DQB1*06:04*
	HLA-DPA1/-DPB1‡	rs3077 C/T rs9277535 A/G
Spontaneous HBV clearance	*HLA-DPA1/-DPB1*‡	rs3077 C/T rs9277535 A/G
	HLA-DPB1§	rs9277534 A/G

*Our current study
†Reference 9
‡Reference 4
§Reference 32

These three *DRB1-DQB1* haplotypes may specifically recognize a HBsAg peptide derived from the HB vaccine, which might then lead to activation of humoral immune responses. The remaining two *DRB1-DQB1* haplotypes and *DPB1* alleles may have an important role in the development of chronic HBV infection via cellular immune responses against the HBsAg, although one of the remaining two *DRB1-DQB1* haplotypes, *DRB1*15:01-DQB1*06:02*, showed a borderline association in the comparison. Two associated *HLA-DPB1* alleles, *DPB1*04:02* (high response to HB vaccine) and **05:01* (low response to HB vaccine), indeed showed to be protective against and susceptible to CHB infection, respectively.

On the other hand, in a comparison of frequencies of *DRB1-DQB1* haplotypes and *DPB1* alleles between high responders to the HB vaccine, and both healthy individuals and spontaneous HBV clearance individuals, no association with vaccine response was identified. This result indicated that *HLA* class II genes have no association with high response to the HB vaccine (i.e., HBsAb >100 mIU/mL). Together with the fact that the *BTNL2* gene showed a significant association in the comparison between high responders and low responders to the HB vaccine, the *BTNL2* gene may have T cell or B cell mediated functions for high response to the HB vaccine.

Table 4 summarizes associations of genes and alleles with response to the HB vaccine, CHB infection and spontaneous HBV clearance. The findings in this study clearly show the importance of *HLA-DRB1-DQB1* haplotypes (i.e. recognition of a HB vaccine related HBsAg by specific DR-DQ haplotypes) and BTNL2 molecules (i.e., high immune response to the HB vaccine) for response to a HB vaccine designed based on the HBV genotype C. The future functional analysis including analysis of specific binding interactions between HLA class II molecules and HBsAg peptides, HLA expression in human hepatocytes, T helper cell responses to HB vaccination, and HBsAb production in specific-HLA expressing cells, may lead to clarification of the mechanism behind the development of chronic HBV infection.

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Supporting Information

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