A genome-wide association study identifies polymorphisms in the HLA-DR region associated with non-response to hepatitis B vaccination in Chinese Han populations

Liping Pan1,†, Li Zhang3,†, Wei Zhang4, Xiaopan Wu1, Yuanfeng Li5, Bingyu Yan3, Xilin Zhu1, Xing Liu1, Chao Yang1, Jianfeng Xu6, Gangqiao Zhou5, Aiqiang Xu3, Hui Li2 and Ying Liu1,*

1National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and 2Department of Epidemiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, China, 3Shandong Center for Disease Control and Prevention, Jinan, China, 4Beijing Center for Disease Control and Prevention, Beijing, China, 5State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing, China and 6Center for Cancer Genomics, Wake Forest School of Medicine, Winston-Salem, NC, USA

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Vaccination against hepatitis B virus is an effective and routine practice that can prevent infection. However, 5–10% of healthy adults fail to produce protective levels of antibody against the hepatitis B vaccination. It has been reported that host genetic variants might affect the immune response to hepatitis B vaccination. Here, we reported a genome-wide association study in a Chinese Han population consisting of 108 primary high-responders and 77 booster non-responders to hepatitis B vaccination using the Illumina HumanOmniExpress Beadchip. We identified 21 SNPs at 6p21.32 were significantly associated with non-response to booster hepatitis B vaccination (P-value < 1310^−2). The most significant SNP in the region was rs477515, located ∼12 kb upstream of the HLA-DRB1 gene. Its P-value (4.81310^−8) exceeded the Bonferroni-corrected genome-wide significance threshold. Four tagging SNPs (rs477515, rs28366298, rs3763316 and rs13204672) that capture genetic information of these 21 SNPs were validated in three additional Chinese Han populations, consisting of 1336 primary high-responders and 420 primary non-responders. The four SNPs continued to show significant associations with non-response to hepatitis B vaccination (P-combined = 3.9810^−13 – 1.4210^−8). Further analysis showed that the rs477515 was independently associated with non-response to hepatitis B vaccination with correction for other three SNPs in our GWAS and the known hepatitis B vaccine immunity associated SNP in previous GWAS. Our findings suggest that the rs477515 was an independent marker associated with non-response to hepatitis B vaccination and HLA-DR region might be a critical susceptibility locus of hepatitis B vaccine-induced immunity.

INTRODUCTION

Infection with hepatitis B virus (HBV) is a public health problem that seriously threatens human life. Approximately more than 400 million people worldwide are chronically infected with HBV and more than half a million people die each year from HBV-related liver disease (1,2). HBV infection shows a marked regional diversity and is prevalent in the Asia and Sub-Saharan Africa (3). In China, ∼93 million people are carriers of HBV, and 300 000 deaths occur each year as a result of HBV infection
Recombinant vaccines have been proven to be effective in preventing HBV infection and have been used widely for >30 years. However, 5–10% of healthy adults fail to produce protective levels of antibody (non-responders) after standard three doses of hepatitis B vaccination (5). Furthermore, a subset of primary non-responders could not elicit protective antibodies yet, even though they are administered booster vaccination. These individuals remain at high risk for HBV infection.

Several epidemiological factors such as older age, male gender, higher body mass index (BMI) and a history of smoking are associated with a decreased antibody response to hepatitis B vaccination (6–9). Different injection methods could also affect the efficacy of hepatitis B vaccine (10). It has been reported that parts of primary non-responders might develop to responders after booster vaccination (another one dose or revaccinated with another standard three doses of hepatitis B vaccine) (11,12). In addition, a twin study indicated that host genetic background account for 77% of all factors influencing immune response to hepatitis B vaccination (13). Although candidate-gene-based case–control studies have implicated that genetic variants in interleukins (IL)1b, IL4, IL10, IL12b, CD3Z, and several HLA loci were associated with variable immune response to hepatitis B vaccination (14–20), none of these associations was conclusive.

Genome-wide association study (GWAS) has been developed to systematically investigate the associations between polymorphisms and polygenic inheritance disorders (21). Recently, a GWAS reported that genetic variants in HLA-DR, HLA-DP and HLA-III loci were strongly associated with postvaccination antibody titers of hepatitis B vaccination in an Indonesian population (22). In this study, they grouped the subjects into three groups on the basis of their antibody titers and focused on investigating the association between polymorphisms and the post-vaccination antibody titers, rather than the non-responses. In fact, World Health Organization reported that persons with antibody ≥10 mIU/ml could prevent HBV infection, namely, the non-responders whose antibody titers <10 mIU/ml are susceptible and at a high risk of infection with HBV (23). Therefore, it is clinically meaningful to identify genetic variants contributing to non-response to hepatitis B vaccination. Until now, only one candidate-gene-based case–control study has paid attention to non-responders to hepatitis B vaccination in Chinese Han population, but the sample size and the number of candidate SNPs were limited (24 non-responders and 46 responders, 51 SNPs) (14). Therefore, we performed a multistage GWAS consisting of 498 non-responders and 1449 high-responders in four Chinese Han populations to identify the susceptible loci of non-response to hepatitis B vaccination. Our findings might contribute to a better understanding of the genetic variants that were associated with the non-response to hepatitis B vaccination, and facilitating to investigate detailed mechanisms of non-response to hepatitis B vaccination. Furthermore, the results might be helpful to identify specific genes as targets in the development of novel and effective vaccines.

RESULTS

In the GWAS stage, we genotyped 731 442 SNPs across the genome in 113 primary high-responders (cases) and 78 booster non-responders (controls). After quality control, a total of 588 026 SNPs in 108 cases and 77 controls were remained for further analysis. Principal component analysis (PCA) showed that cases and controls had similar distributions of the top three eigenvectors (Supplementary material, Fig. S1). Consistent with this result, we found no evidence of population stratification, with a genomic inflation factor (λ) of 1.03 (Supplementary material, Fig. S2). We also performed a logistic regression model to adjust for the top five principal components (generated in EIGENSTRAT) and derived a similar genomic inflation factor (λ = 1.03). In addition, the direct comparisons showed that the association results of top 1000 SNPs from the analysis with or without adjustment for top five principal components were generally similar. All these results indicated that the population stratification in the remaining samples had a minimal impact on the association results.

The SNPs showing strongest association were on chromosome 6p21.32 (Fig. 1; Supplementary materials, Table S1). The lead SNP in this region was rs477515 (P = 4.81 × 10^-8, OR = 3.59), which exceeded the Bonferroni-corrected genome-wide significance threshold (0.05/588 026 = 8.50 × 10^-8). A detailed examination of this region revealed that a total of 21 SNPs were significantly associated with non-response to the vaccination (P-values < 1 × 10^-6; Table 1 and Fig. 2). The lead SNP (rs477515) locates ~12 kb upstream of the HLA-DRB1 gene, and these 21 SNPs overlapped with four known genes; C6orf10, BTN12, HLA-DRA and HLA-DRB1.

Linkage disequilibrium (LD) analysis identified four tagging SNPs (rs477515, rs28366298, rs3763316 and rs13204672) that capture genetic information of these 21 SNPs (Supplementary material, Table S1). Recombinant vaccines have been proven to be effective in preventing HBV infection and have been used widely for >30 years. However, 5–10% of healthy adults fail to produce protective levels of antibody (non-responders) after standard three doses of hepatitis B vaccination (5). Furthermore, a subset of primary non-responders could not elicit protective antibodies yet, even though they are administered booster vaccination. These individuals remain at high risk for HBV infection.
The rs3135363 was replicated consistently in both GWAS ($P$-value $= 0.0007$) and confirmation Ia set ($P$-values $= 0.0002$), but it showed a weaker association than the identified four SNPs in our GWAS. Neither rs9277535 nor rs9267665 showed significant associations with non-response to hepatitis B vaccination in both GWAS and confirmation sets ($P$-values $> 0.05$). Because the known rs3135363 was replicated in our GWAS and confirmation sets, and it was located near the identified SNPs in our study, we next investigated whether the identified four SNPs might simply be from the tracking of rs3135363. LD between rs3135363 and each of the identified four SNPs (rs477515, rs28366298, rs3763316 and rs13204672) were performed firstly. The results indicated that no tight LDs were observed in the analysis ($r^2 \leq 0.120$, Supplementary Materials, Table S3). Furthermore, the identified four SNPs were strongly associated with non-response to hepatitis B vaccination even after stratification with rs3135363 in GWAS and confirmation sets, using a forward stepwise conditional logistic regression analysis (Supplementary material, Table S4). These results together implicated the independent effects of the identified four SNPs on immune response to hepatitis B vaccination.

To further test whether these four SNPs were independently associated with response to the hepatitis B vaccination, we fit a logistic regression model using a forward selection procedure, starting with the most significant SNP rs477515 in all study subjects (GWAS and three confirmation stages). Only rs477515 showed independent association with non-response to hepatitis B vaccination, while none of the other three SNPs was significant, suggesting the observed associations of these four SNPs (and perhaps 21 SNPs) at 6p21.32 with response to the vaccination are dependent and likely due to a common variant near or at rs477515 ($P$-value $= 1.64 \times 10^{-17}$, OR $= 2.00$, 95% CI $= 1.71–2.35$).

The HLA-DRB1 region was also genotyped using direct sequencing method among the 185 samples in the GWAS stage. In line with previous studies, we also found significant

Figure 1. Manhattan plot of testing associations with booster non-response to hepatitis B vaccination in GWAS. Note: The genome-wide $P$ values of the allelic frequency model from 588 026 SNPs in 77 cases and 108 controls are presented by chromosome. The x-axis represents genomic position, and the y-axis shows the $-\log_{10}(P)$. 

(Supplementary materials, Table S2). The rs3135363 was replicated consistently in both GWAS ($P$-value $= 0.0007$) and confirmation Ia set ($P$-values $= 0.0002$), but it showed a weaker association than the identified four SNPs in our GWAS. Neither rs9277535 nor rs9267665 showed significant associations with non-response to hepatitis B vaccination in both GWAS and confirmation sets ($P$-values $> 0.05$). Because the known rs3135363 was replicated in our GWAS and confirmation sets, and it was located near the identified SNPs in our study, we next investigated whether the identified four SNPs might simply be from the tracking of rs3135363. LD between rs3135363 and each of the identified four SNPs (rs477515, rs28366298, rs3763316 and rs13204672) were performed firstly. The results indicated that no tight LDs were observed in the analysis ($r^2 \leq 0.120$, Supplementary Materials, Table S3). Furthermore, the identified four SNPs were strongly associated with non-response to hepatitis B vaccination even after stratification with rs3135363 in GWAS and confirmation sets, using a forward stepwise conditional logistic regression analysis (Supplementary material, Table S4). These results together implicated the independent effects of the identified four SNPs on immune response to hepatitis B vaccination.

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Table 1. The top 21 SNPs with $P$-values of $<1 \times 10^{-6}$ in the GWAS stage

<table>
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<tr>
<th>Chr.</th>
<th>SNP</th>
<th>Position</th>
<th>Gene/nearby gene</th>
<th>Location</th>
<th>Alleles</th>
<th>MAF</th>
<th>$P$-unadjusted OR (95% CI)</th>
<th>OR (95% CI)</th>
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The SNPs that were not detected in previous published GWAS were emphasized in bold.

\[ \text{MAF, minor allele frequency; HR, high responder to primary vaccination (anti-HBs} \geq 1000 \text{mIU/ml after three doses); NR, non-responder to booster vaccination (anti-HBs} < 10 \text{mIU/ml after six doses).} \]
association of HLA-DRB1*0701 allele with non-response to hepatitis B vaccination \( (P\text{-value} = 1.50e^{-06}, \text{OR} = 3.69; \text{Supplementary Material, Table S5}) \). Further LD analysis indicated that there was a strong LD between identified rs13204672 and HLA-DRB1*0701 \( (r^2 = 0.982) \), while other three SNPs showed weaker LD with HLA-DRB1*0701 \( (r^2 < 0.548) \).

To further increase genome coverage, we carried out an imputation analysis to infer the genotypes of additional common SNPs using the genotype data from 1000 Genomes Project Han Chinese in Beijing (CHB) and Han Chinese South (CHS) (March 2012 release) as a reference. For all over 38 million auto-some imputed SNPs, \( \sim 5.7 \) million imputed SNPs were retained for the association analysis after rigorous quality control. The SNPs showing strongest association were also located on chromosome 6p21.32 (Supplementary materials, Fig. S4, Table S6). The top associated SNPs with chromosome 6p21.32 (Supplementary materials, Fig. S4, Table S6). The top associated SNPs with \( P\text{-values} < 1 \times 10^{-6} \) were located near the identified four SNPs in our GWAS \( (< \sim 340 \text{ kb}) \). No novel region associated with non-response to hepatitis B vaccination was found in the imputation.

Of the total 588,026 SNPs, 288,743 SNPs were mapped to 17,432 genes within 5 kb upstream and downstream, which were assigned to 104 pathways. Pathway enrichment analysis using the improved gene-set-enrichment analysis approach (i-GSEA4GWAS) have identified that 11 pathways were significantly enriched with association signals with FDR \( \leq 0.05 \) (Supplementary material, Table S7). The top one significant pathway is antigen processing and presentation, which is consistent of several genes in HLA region and is directly involved in immune response. The rs477515 nearby gene, HLA-DRB1, was also included in this significantly enriched gene sets in the pathway analysis. We also performed a pathway analysis with omitting the SNPs in HLA region, and found that no significant pathway was enriched after omitting the HLA associations.

### DISCUSSION

Hepatitis B vaccine-induced immunity is a complex process that is controlled by numerous factors. In addition to environmental factors and host-related physical factors, genetic variations also play an important role in regulating the immune response to hepatitis B vaccination \( (13,24) \). Previous candidate-gene studies have suggested that polymorphisms in the HLA-DR region were associated with immune response to hepatitis B vaccination \( (15) \), and HLA-DRB1*0701 allele was consistently validated as a risk factor for immune response to hepatitis B vaccination \( (18,25) \). Recently, a GWAS focused on postvaccination antibody titer has reported that rs3135363 in the HLA-DR region was associated with differed postvaccination antibody titer \( (22) \). In our study, a GWAS and subsequent confirmation focused on non-response to hepatitis B vaccination has also detected that polymorphisms in the HLA-DR region did show strong associations with non-response to hepatitis B vaccination.

Therefore, previous studies and our study have together implicated that the HLA-DR region is very likely the key loci associated with immune response to hepatitis B vaccination.

Hepatitis B vaccine is an exogenous antigen. It cannot be presented to the CD4+ T lymphocyte unless it is degraded into peptides and combined with HLA class II molecules \( (26,27) \). HLA-DR, a subtype of the HLA class II molecule, is consisted of two subunits HLA-DRA and HLA-DRB \( (16) \). HLA-DR genes are highly polymorphic, especially the HLA-DRB genes. It has been reported that allelic differences of HLA-DR genes would affect the T-cell recognition of MHC-peptides complexes in hepatitis B vaccine-triggered immunity, and then affect the outcomes of immune responses \( (28) \). Furthermore, HLA-DR genes are in high LD with numerous genes involved in immunity. Although polymorphisms that located in non-coding sequences of HLA-DR genes looks like no direct effect, it

<table>
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<tr>
<th>SNPs</th>
<th>Alleles</th>
<th>Near gene</th>
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<th>HR</th>
<th>NR</th>
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<td>GWAS</td>
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<tr>
<td></td>
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<td></td>
<td>Confirmation Ib</td>
<td>0.190</td>
<td>0.302</td>
<td></td>
<td>6.30e-04</td>
<td>1.85 (1.30–2.63)</td>
</tr>
<tr>
<td></td>
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<td>Confirmation II</td>
<td>0.175</td>
<td>0.267</td>
<td></td>
<td>0.048</td>
<td>1.72 (1.00–2.94)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Combined*</td>
<td>0.375</td>
<td>0.191</td>
<td></td>
<td>3.75e-03</td>
<td>1.84 (1.56–2.17)</td>
</tr>
<tr>
<td>rs13204672</td>
<td>G/A</td>
<td>HLA-DRB1</td>
<td>GWAS</td>
<td>0.102</td>
<td>0.305</td>
<td></td>
<td>7.42e-07</td>
<td>3.87 (2.22–6.77)</td>
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<tr>
<td></td>
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<td>Confirmation Ia</td>
<td>0.119</td>
<td>0.194</td>
<td></td>
<td>1.30e-05</td>
<td>1.79 (1.37–2.33)</td>
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<td></td>
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<td>Confirmation Ib</td>
<td>0.143</td>
<td>0.207</td>
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<td>0.026</td>
<td>1.56 (1.05–2.33)</td>
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<td>Confirmation II</td>
<td>0.110</td>
<td>0.228</td>
<td></td>
<td>2.00e-03</td>
<td>2.39 (1.34–4.23)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Combined*</td>
<td>0.145</td>
<td>0.103</td>
<td></td>
<td>2.01e-03</td>
<td>2.01 (1.67–2.43)</td>
</tr>
</tbody>
</table>

HR, high-responder to primary vaccination (anti-HBs \( \geq 1000 \) mIU/ml after three doses); NR, non-responder to booster vaccination (anti-HBs < 10 mIU/ml after six doses); NR, non-responder to primary vaccination (anti-HBs < 10 mIU/ml after three doses); *the combined analysis of GWAS and confirmation stages.
cannot rule out that they might link to other causal loci affecting the quality of immune response. Here, we detected that the rs477515, which located in the upstream of HLA-DRB1 gene, was shown to be an independent and the strongest signal associated with non-response to hepatitis B vaccination. We suspected that it might be a genetic marker in HLA-DR region and in high LD with other function-related SNPs that affect the T-cell recognition of MHC–peptide complexes on immune cells, and then result in weak or no immune response to hepatitis B vaccination.

Previous twin study has reported that non-HLA account for 60% of genetic factors that influence the immune response to hepatitis B vaccination (24). Furthermore, several case–control studies have detected polymorphisms of genes involved in immune response were associated with hepatitis B-induced immunity (14,17,18,20). However, the two GWA studies have verified that the strongest association signals were located within HLA region, and other SNPs located in non-HLA region were shown to be weaker association signals. Immune response elicited by a vaccine is a cumulative result of interactions driven by genes in the immune response network, where HLA and other immunity-relevant genes are both indispensable (29). In the present study, genome-wide complex trait analysis (GCTA) showed that heritability of response to hepatitis B vaccination due to the whole-genome SNPs was estimated to be 56.7%, while 5.62% for the associated 21 SNPs within HLA region. This result implicated that the polymorphisms of HLA alone do not explain all variations in the immune response to vaccines, and the polymorphic contributions appear to be the norm (30).

It is conceivable that the effect of HLA polymorphisms might be principal, and the effect of polymorphisms of non-HLA genes themselves might be limited, or linked with HLA alleles (31). Thus, our results did not deny the effect of non-HLA genes, but implicated that antigen presentation and recognition of T-cell receptor repertoire relying on the HLA molecules might play a critical role in immune response triggered by hepatitis B vaccine.

To the best of our knowledge, this study is the first GWA study focusing on non-response to hepatitis B vaccination in Chinese Han population. In the present study, we replicated the previously reported rs3135363 within HLA-DR region in our GWSA and confirmation Ia sets (22). However, we found that rs477515 within the HLA-DR region was the strongest association signal and was independently associated with non-response to hepatitis B vaccination even after adjustment of rs3135363 within the HLA-DR region, which implicated the independent effects of rs477515 on immune response to hepatitis B vaccination. The different associated SNPs in the same HLA-DR loci that were detected in the two GWAS might be due to the different ethnic populations in the two GWAS. Furthermore, in previous GWAS, they focused on postvaccination antibody titer and assigned the subjects to three subgroups on the basis of antibody titers, rather than analyzing as a binary trait. In contrast, we used the international standard that the subjects with antibody <10 mIU/ml was defined as non-responders and the subjects with antibody ≥1000 mIU/ml was defined as high-responders (15,32). In addition, the non-responders in our GWAS stage were administrated two rounds of standard three dose of hepatitis B vaccine, which represented a more precise and true non-response status. Since booster non-responders are the persons who have no protective antibody titers after revaccination and remain high risk of infection with HBV, studies focused on these subjects will be helpful and meaningful to clarify the genuine polymorphisms associated with non-response to hepatitis B vaccination and find the really useful loci to improve the efficacy of vaccine. The previously reported SNP rs9277535 within HLA-DP region and rs9267665 within HLA-III region failed to show significant association with non-response to hepatitis B vaccination in both our GWAS and following confirmation stage. Together the GWAS and confirmation sets, our study had >85% statistical power to detect these two SNPs with an OR >2.0 at a significance level of 0.05; hence, the sample size might not be the critical reason for failed replication, while these different results might owing to ancestry difference or different grouping patterns in the two studies.

We also investigated the known HBV infection associated SNPs (rs3077, rs9277535, rs9277542, rs2856718, rs7453920, rs652888 and rs1419881 within HLA region) (33–36) and hepatitis B-related hepatocellular carcinoma (HCC)-associated SNPs (rs17401966 within 1p36.22, rs7574865 within 21q21.3, and rs9275319, rs9272105 within HLA region) (37–39) with non-response to hepatitis B vaccination in our GWAS discovery analysis. Due to the lack of rs9277542, rs17401966 and rs9275319 in our GWAS data, their linkage SNPs (rs9277533 instead of rs9277542, r² = 1.0; rs3748578 instead of rs17401966, r² = 0.847; and rs9275371 instead of rs9275319, r² = 0.847.) were investigated in the GWAS analysis. LD analysis identified that there is no evidence of strong LD between rs477515 and each of the abovementioned SNPs (r² ≤ 0.27). We found the evidence of the association at rs3077 (P-value = 9.8 × 10⁻³, OR = 0.56), rs3748578 (P-value = 8 × 10⁻³, OR = 1.84) and rs9272105 (P-value = 3.6 × 10⁻⁴, OR = 0.45) with non-response to hepatitis B vaccination. The rs3077 showed both protective effects for response to hepatitis B vaccination and for HBV infection, suggesting the similar genetic background in these two phenotypes. These results were consistent with previous report that the vaccine response may provide a useful experimental model of a natural infection for genetic study of infection, and the vaccine is least effective in those who need it most (22). However, the minor allele of the shard rs3748578 and rs9272105 between response to hepatitis B vaccination and HCC showed protective effects on response to hepatitis B vaccination, while as a risk effect on development of HCC. Further studies will be needed to demonstrate whether the opposite associations of response to hepatitis B and HCC at these two SNPs are due to different causal variants.

In previous GWAS and candidate studies, a total of 103 SNPs have been reported to be significantly associated with immune response to hepatitis B vaccination. In our study, we also evaluated the genetic effects of these SNPs in our GWAS stage (Supplementary materials, Table S8). Of these 103 SNPs, the genotype data of 96 SNPs could be got from our GWAS array or the imputation analysis. Among the 96 SNPs, 41 SNPs showed significant association with non-response to hepatitis B vaccination (P-values < 0.05), while there is no association between other 55 SNPs and non-response to hepatitis B vaccination. Given the frequencies of occurrence of the risk factors of these 103 SNPs ranged from 1.4 to 54.2%, the present GWA
that GWAS was also replicated, as well as the consistent validation rs3135363 within previously reported SNPs were validated in our GWAS, and the decreased the statistical power. However, almost half of previously reported SNPs were validated in our GWAS and therefore the true associations that show modest effects, which might be the reason why some of previously reported associations were not replicated in our study. Furthermore, due to the small impact of the associated 21 SNPs on heritability (5.62%) of non-response to hepatitis B vaccination, further studies focusing on whole-genome SNP results deserve to be performed. In the present study, the confirmation studies were not strict repetitions for the GWAS, because primary non-responders were included in the confirmation stage and the subjects in confirmation Ia and Ib sets, as well as the subjects in GWAS stage, were coming from the same population. However, comparing the frequencies of risk allele of each associated SNPs (Table 2), we found that the order of frequency of risk allele was booster non-responders > primary non-responders > primary high-responders. These results were plausible and well-founded, because following parts of the primary non-responders convert to high or normal responders after booster vaccination (12), the frequency of risk allele in booster non-response group must be increased. Once they were deviated from this order, the associations might be suspicious. In the present study, all the four SNPs were demonstrated to associate with booster non-response as well as primary non-response to hepatitis B vaccination and were in accordance with this order concurrently, suggesting that the four SNPs were the true association signals and implicating that it was plausible in some extent to use primary non-responders to confirm the associations obtained in using booster non-responders. In our GWAS, although there are two kinds of doses (10/20 μg) in the first round of standard vaccination, the primary non-responders were all revaccinated with another round of standard vaccination; therefore the different kinds of doses were unremarkable. Since the sample size in our GWAS was not large, it is necessary to replicate this association in a larger cohort. Furthermore, given that the genetic structures are different among different geographical-origin populations, further validations should be performed in other ethnics.

In conclusion, we have detected that a polymorphism (rs477515) within the HLA-DR region was strongly associated with non-response to hepatitis B vaccination, and this association was independent from rs3135363 that was reported previously. Considering the importance of HLA region in immune response to hepatitis B vaccination, our results implicate that antigen presentation and recognition of T-cell receptor repertoire on HLA-DR molecules might be critical in total process of immune response triggered by hepatitis B vaccination. Further in-depth analysis focusing on HLA-DR region might be helpful to understand the mechanisms of immune response to hepatitis B vaccination and detect novel loci for developing more effective hepatitis B vaccine.

MATERIALS AND METHODS

Subjects

Participants in the GWAS stage, together with confirmation Ia and Ib stages were recruited from a large hepatitis B vaccination campaign in Shandong province in 2009. Participants in the confirmation II stage were recruited from among healthy volunteers in the community in Beijing in 2007. Written informed consent and completed questionnaires including demographic information, smoking history, vaccination history, chronic disease and immunosuppressive disease/medications were obtained from all participants. All individuals were tested for five markers of hepatitis B using an Abbott i2000 detection kit (Abbott Laboratory, Chicago, IL, USA). Individuals who were negative for the five markers of hepatitis B were tested further for HBV DNA and for anti-HCV and anti-HIV. Participants were excluded if: (i) they were positive for HBV DNA, hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBs, anti-HBc, anti-HBe, anti-HCV and/or anti-HIV; (ii) they had been vaccinated previously with any hepatitis B vaccine; (iii) they had a chronic disease, such as diabetes, cancer or cardio-cerebrovascular disease, or were undergoing renal dialysis; (iv) they had any immunosuppressive disease or were taking any immunosuppressive medication; (v) they were not of Han ethnicity; (vi) they were <18 years.

A total of 3985 qualifying individuals from Shandong province were administrated three doses of 10 or 20 μg recombinant hepatitis B vaccines according to standard 0-1-6 schedule (20 μg recombinant hepatitis B vaccine, GlaxoSmithKline Investment Co., Ltd, UK; North China Pharmaceutical Co., Ltd, Beijing, 10 μg recombinant hepatitis B vaccine, Dalian Hissen Biopharmaceutical Co., Ltd, Dalian). Levels of anti-HBs were measured at 1 month after the final dose using the Abbott i2000 detection kit. Four hundred and twenty-two primary non-responders (anti-HBs < 10 mIU/ml after three doses immunization) were revaccinated with another standard three doses of hepatitis B vaccines (20 μg recombinant hepatitis B vaccine, GlaxoSmithKline Investment Co., Ltd, UK). Levels of anti-HBs were measured once again at 1 month after the final dose using the Abbott i2000 detection kit. After completing the revaccination schedule, 78 vaccines with an antibody titer < 10 mIU/ml were booster non-responders and included in case group in the GWAS stage. One hundred and thirteen primary high responders (anti-HBs ≥ 1000 mIU/ml after three dose immunization) were randomly selected as control group to match for vaccine...
doses, age and gender ratio against case group. The remaining 374 primary non-responders, together with 1122 age and gender matched primary high-responders were assigned to confirmation Ia and Ib cohorts (confirmation Ia: 20 μg group, 263 primary non-responders and 825 primary high-responders; confirmation Ib: 10 μg group, 111 primary non-responders and 297 primary high-responders).

A total of 599 qualifying individuals from Beijing were administered three doses of 10 μg recombinant hepatitis B vaccine according to standard 0-1-6 schedule (North China Pharmaceutical Co., Ltd, Beijing, China). Levels of anti-HBs were measured at 1 month after the final dose using the Abbott i2000 detection kit. Forty-six primary non-responders and 214 primary high-responders were assigned to confirmation II stage.

The demographic characteristics and clinical features of subjects in the GWAS stage and the confirmation stages are summarized in Supplementary Materials, Table S9.

The study was performed in accordance with the guidelines of the Helsinki Declaration and was approved by the Ethics Committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Science.

SNP genotyping and quality control

Genomic DNA was extracted from peripheral blood using the phenol–chloroform method. In the GWAS stage, 191 samples (78 cases and 113 controls) were genotyped for 731,442 SNPs using Illumina HumanOmniExpress BeadChip (Illumina, CA, USA). The SNP with call rate <95% (n = 13,567), minor allele frequency <0.01 in both control and case group (n = 123,224) and/or P values of Hardy–Weinberg equilibrium (HWE) test <1 × 10−5 in control group (n = 8,454) were excluded. The 191 samples were subsequently assessed for population stratification using a PCA, and six genetic outlier samples (one case and five controls) were excluded.

After quality control, 588,026 SNPs in 185 samples (77 cases and 108 controls) were remained for the subsequent association analyses. The association analysis was performed with and without adjusting for the top five principal components (generated in EIGENSTRAT). A direct comparison was also performed between the association results of the top 100 SNPs (rs28366298, rs3763316 and rs13204672) were in high LD with the top 21 SNPs (P-values < 1 × 10−6) were further analyzed. LD analysis based on tagger pairwise method using Haploview software showed that four SNPs (rs477515, rs28366298, rs3763316 and rs13204672) were in high LD with other SNPs (r2 > 0.8), respectively. Hence, the four SNPs were further genotyped in the subsequent confirmation studies. The previously reported three SNPs (rs3135363, rs9277535 and rs9267665) were also genotyped in our GWAS and confirmation sets. All of these seven SNPs were genotyped using the TaqMan-MGB (Genecore Biotech Co., Ltd, Shanghai, China) or TaqMan-BHQ (Sangon Biotech Co., Ltd, Shanghai, China) probe-based real-time polymerase chain reaction (PCR). The primer and probe sequences that were used to genotype each SNP are shown in Supplementary Material, Table S10. Amplification and detection were conducted using a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

To confirm the genotyping results in the GWAS stage, 20% of the samples from GWAS stage were selected randomly and were replicated with the TaqMan-MGB or TaqMan-BHQ probes that were used in the confirmation stages, and we obtained 100% identical results.

HLA-DRB1 genotyping

HLA-DRB1 region was genotyped using the direct sequence-based genotyping method (PCR-SBT), among the 108 high-responders and 77 booster non-responders in the GWAS stage.

Imputation

To generate additional genotypes, we performed imputation using SHAPEIT [Shape-IT: new rapid and accurate algorithm for haplotype inference] and IMPUTE2 [a flexible and accurate genotype imputation method for the next generation of genome-wide association studies] using the genotype data from CHB and CHS samples of the 1000 Genomes Project as reference. For all datasets, cases and controls were imputed together. We performed quality control for all 38,043,533 imputed SNPs. In detail, we removed SNPs with low imputation quality (info score <0.8 for IMPUTE2) as suggested. We also removed SNPs which showed MAF <0.01 or showed call rate of <95% or significant deviation from HWE in controls (the same as for the genotyped SNPs). Many of the imputed SNPs were removed from further analyses because of low call rate and deviation from HWE, probably owing to low LD between genotyped and imputed SNPs. Association test of non-response to hepatitis B vaccination risk with each of these imputed SNPs was carried out using PLINK software (version 1.07).

Statistical analysis

A χ2 goodness-of-fit test was used to examine whether genotype distributions of each SNP were conformed to HWE in both case and control group. A PCA was performed to check for signs of population stratification in GWAS stage using the EIGENSTRAT software. The statistical significance of the association with each SNP was assessed with and without correction for the top five principal components (generated in EIGENSTRAT), using a logistic regression model. These calculations were conducted with PLINK software (version 1.07). Regional association plot was performed by the online software LocusZoom (43). Pathway analysis was performed to explore the most biologically relevant pathways impacted by a list of input SNPs in the GWAS stage, using the improved gene-set-enrichment analysis approach (i-GSEA4GWAS). GCTA (GCTA software, version 1.04) was used to estimate the phenotypic variance explained by the 21 statistically significant SNPs or whole-genome-wide SNPs in the GWAS stage with correction for the top five principal components (generated in EIGENSTRAT) (44). LD test was conducted using Haploview software (version 4.2). The allele frequencies for each SNP were compared between case and control groups using the χ2-test in the confirmation stages, and the calculation was conducted using the online software SHEsis (45). Logistic regression analysis was also used to adjust for confounding factors and the previous reported top SNP rs3135363 in the HLA-DR region (22). The
independent test for the four SNPs was also performed by a logistic regression model using forward selection procedure. These analyses were performed using SPSS software (version 11.0).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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We thank all the subjects who participated in this study.

Conflict of Interest statement. None declared.

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