HBsAg levels at the time of discontinuation	Scores	Hepatitis B core-related antigen (HBcrAg) levels at the time of discontinuation	Scores
<1.9 log IU/mL (<80 IU/mL)	0	<3.0 log U/mL	0
1.9–2.9 log IU/mL (80–800 IU/mL)	1	3.0–4.0 log U/mL	1
≥2.9 log IU/mL (≥800 IU/mL)	2	≥4.0 log U/mL	2

Relapse risk	Total scores	Percentage of prediction success	Assessment
Low-risk group	0	80–90%	Discontinuation can be considered. It is essential to pay attention to relapse because some patients of low risk may develop hepatitis relapse.
Medium-risk group	1–2	~50%	Discontinuation can be considered depending on the situation. Further consideration is needed about conditions and the way to discontinue in the future.
High-risk group	3–4	10–20%	Continuous treatment is recommended. However, patients under 35 years old show a relatively higher rate of successful discontinuation of 30–40%.

IV. Follow-up method after discontinuation and conditions for retreatment

1. HBV DNA levels (real-time PCR) and ALT levels must be periodically measured after discontinuation of NUC to pay attention to HBV proliferation and hepatitis relapse resulting from proliferation.
2. Relapse after discontinuation is mostly observed within 1 year and then gradually decreases. It is rare to relapse after the first 3 years. Therefore, it is necessary to pay attention to relapse immediately after discontinuation. In particular, patients should be followed up by blood tests every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.
3. Transient abnormalities in ALT levels or HBV DNA levels may be observed in approximately two-thirds of patients who successfully discontinued NUC and would finally achieve the inactive carrier state. Therefore, even if the ALT level or the HBV DNA level shows mild elevations, it is possible to keep following up without retreatment. However, patients who meet the following condition are less likely to finally achieve the inactive carrier state and should be considered for NUC retreatment.

Condition to consider retreatment with NUC
ALT ≥80 IU/L or HBV DNA ≥5.8 log copies/mL after discontinuation

V. Key points and future issues

1. The status differs in each patient. Objectives and significance also differ by patient. Thus, doctors must determine whether NUC should be discontinued or not in consideration of those conditions. In case of considering discontinuation, it is recommended to consult with a specialist of hepatic diseases.
2. In case of retreatment with NUC due to hepatitis relapse after discontinuation, it is unknown whether it results in higher emergence of strains resistant to NUC or not compared with patients without discontinuation.
3. Because HBV carriers rarely experience hepatitis relapse even in the inactive carrier state (HBV DNA <4.0 log copy/mL and ALT <30 IU/L), they must be followed up after successful discontinuation. Liver carcinogenesis also requires follow up.
4. The followings are included in future issues; improvement of accuracy in the criteria for discontinuation of NUC; investigation of the criteria used in these guidelines in a prospective study; and investigation of the way to actively discontinue NUC using sequential treatment with interferon.

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BRIEF COMMUNICATION

Genetic polymorphism in *IFNL4* and response to pegylated interferon-α and ribavirin in Japanese chronic hepatitis C patients

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Key words
hepatitis C virus; interferon-λ 4; IL28B; pegylated interferon

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Abstract

A genetic polymorphism of the newly discovered interferon-λ 4 (*IFNL4*) gene was associated with hepatitis C virus (HCV) clearance in individuals of African ancestry. To assess whether a dinucleotide variant of *IFNL4* (ss469415590) also affected treatment outcome of antiviral therapy in Japan, we genotyped 213 patients with chronic genotype 1 HCV infection and 176 healthy subjects. The ΔG allele was associated with treatment failure [odds ratio (OR) 4.73, $P = 0.019$], as was the IFL3 rs8099917 single nucleotide polymorphism (SNP) (OR 5.06, $P = 0.068$). The correlation between ss469415590 and rs8099917 was high ($r^2 = 0.92$, $D' = 0.98$). Multivariate analysis revealed that the rs8099917 SNP was independently associated with treatment failure (OR 5.28, $P = 0.009$). Therefore, ss469415590 may be another predictive marker of antiviral therapy outcome in the Japanese population.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection often develops into chronic hepatitis, leading to liver cirrhosis and/or hepatocellular carcinoma (HCC) (1, 2). As approximately 70–80% of Japanese HCC patients are infected with HCV (3), the successful eradication of HCV, defined as a sustained virological response (SVR), is considered important to decrease the incidence of HCC. Approximately 50% of Japanese patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon-α (PEG-IFN) and ribavirin (RBV) therapy. Although the addition of a direct-acting antiviral agent to this regimen has increased response rate (4), reliable markers are needed to better predict treatment outcome.

The strongest genetic factors associated with patient response to PEG-IFN and RBV therapy and spontaneous clearance are single nucleotide polymorphisms (SNPs) around *IFNL3* (*IL28B*) [reviewed in (5)]. In particular, the rs8099917 SNP has been shown as a good predictive marker for the response to PEG-IFN and RBV therapy in Japanese patients (6–8). Prokunina-Olsson et al. (9) recently discovered a new transiently induced region that harbors a dinucleotide variant ss469415590 (TT or ΔG) between *IFNL3* and *IFNL2*. ss469415590 (ΔG) is a frameshift variant that

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creates a novel gene, designated as *IFNL4*, that encodes the interferon-λ4 protein (IFNL4) having moderate similarities to IFNL3. *IFNL4* is transcribed and translated in primary hepatocyte cell lines by the double-stranded RNA virus analog polyinosinic:polycytidylic acid, causing upregulation of interferon-stimulated genes in these cells and replication reduction of an HCV subgenomic replicon (10). In addition, ss469415590 is strongly associated with HCV clearance in African-Americans (9), but this polymorphism has not yet been investigated in the Japanese population. The objective of this study was to determine the prevalence of this *IFNL4* SNP among the Japanese and assess whether it influenced the treatment outcome of PEG-IFN and RBV therapy in patients with chronic hepatitis C.

We recruited 213 treatment-naïve patients with chronic hepatitis C and 176 healthy subjects. Patients were seen at Shinshu University Hospital or affiliated hospitals. Controls were hospital staff volunteers who had indicated the absence of any major illness on a standard questionnaire. The racial background of all subjects was Japanese. Diagnosis of chronic hepatitis C was based on the following criteria, as reported previously (11): (1) presence of serum HCV antibodies and detectable viral RNA; (2) absence of detectable hepatitis B surface antigen and antibody in the human immunodeficiency virus; and (3) exclusion of other causes of chronic liver disease. No patient had a history of, or developed, decompensated cirrhosis or HCC. The baseline characteristics of the patients are shown in Table 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine (No. 302) and all participants provided written informed consent.

Antibodies to HCV were measured in serum samples via third-generation Abbott HCV EIA-3 assays (Abbott Laboratories, Abbott Park, IL). Serum levels of HCV RNA were determined using Cobas Amplicor assays (sensitivity: 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). All patients in our test cohort were infected with genotype 1b. Relevant biochemical tests were performed using standard methods (12).

Patients received bodyweight-adjusted doses of PEG-IFNα-2b (PegIntron, MSD K.K., Tokyo, Japan) and RBV (Rebetol, MSD K.K.) for 48 weeks, as reported previously (13). Response to therapy was categorized as follows: an SVR was defined as undetectable serum HCV RNA 24 weeks after completing therapy. Relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/ml at week 12 and detectable HCV RNA during the treatment course.

Genomic DNA was isolated from whole blood samples by phenolic extraction of sodium dodecyl sulfate-lyzed and proteinase K-treated cells, as described previously (14). Genotyping of the rs8099917 SNP (T/G) was performed using an ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) (8). Exon 1 of the *IFNL4* gene was amplified by the polymerase chain reaction (PCR) in the presence of Takara Taq™ (Takara Bio Inc., Otsu, Japan) with the sense primer (5′-CATTGCCTTCCCTGGGATCCTAAC-3′) and the antisense primer (5′-GGACCCCTTGGGACAGGAAC-3′). The sizes of the amplified DNA fragments (333 or 334 bp) were confirmed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. PCR products were directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit using an ABI 3100 DNA sequencer. We assessed the rs4803221 (C/G), ss469415590 (TT/ΔG), rs73555604 (G/A), and rs150891559 (G/C) SNPs in this study.

Statistical analyses were performed using PASW Statistics 21.0J software (IBM, Tokyo, Japan). The Mann–Whitney *U*-test was used to analyze continuous variables, whereas the chi-squared test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was <5, Fisher's exact test was employed. The Hardy–Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel

Table 1 Demographic and clinical characteristics of patients with chronic hepatitis C

Characteristic	All ($n=213$)	VR ($n=162$)	NVR ($n=51$)	*P* value
Age (years)[a]	60 (24–80)	60 (24–80)	59 (39–75)	0.859
Male, *n* (%)	66 (58)	50 (58)	16 (57)	0.926
White blood cell count (/μl)[a]	4300 (1870–8610)	4490 (1870–8610)	4055 (2000–8240)	0.076
Hemoglobin (g/dl)[a]	14.3 (9.0–18.2)	14.4 (9.0–18.2)	13.9 (10.9–16.4)	0.049
Platelets (10^4/μl)[a]	15.4 (7.7–33.6)	15.7 (7.7–33.6)	13.3 (7.7–29.2)	0.236
ALT (IU/l)[a]	46 (14–389)	45 (14–389)	48 (19–323)	0.353
AST (IU/l)[a]	43 (17–246)	41 (17–231)	43 (19–246)	0.297
HCV RNA (10^5 IU/ml)[a]	18 (1.1–51)	19 (1.1–51)	14 (1.5–51)	0.411
rs8099917 allele (TT/TG/GG)	144/64/5	124/36/2	20/28/3	<0.001
ss469415590 allele (TT/TT TT/ΔG ΔGΔG)	142/65/6	122/37/3	20/28/3	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; VR, virological response; NVR, null virological response.
[a]Median (range).

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et al. (15) and were based on a 95% confidence interval (CI) of D' with HAPLOVIEW version 4.2 software (16). We plotted r^2 values. A P value of ≤ 0.05 was considered to be statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% CI.

Of the 213 patients receiving PEG-IFN and RBV therapy, 105 (49%) achieved an SVR. Among the 108 patients not reaching an SVR, 57 relapsed and 51 did not respond to therapy. Pre-treatment values for median hemoglobin were significantly higher in the virological response (VR) group compared with the null virological response (NVR) group (Table 1).

We genotyped the rs8099917 SNP and four other SNPs in *IFNL4* (rs150891559, rs73555604, ss469415590, and rs4803221) in 213 patients with chronic hepatitis C and 176 healthy subjects. As rs150891559 and rs73555604 were homozygous for the major allele in all patients and controls, we focused on the rs8099917, ss469415590, and rs4803221 SNPs in this study. The observed genotype frequencies for patients and controls were all in Hardy–Weinberg equilibrium. Among the 213 patients ($2n = 426$), the three SNPs (rs4803221, ss469415590, and rs8099917) showed strong linkage disequilibrium (LD) ($r^2 = 0.88$–0.96) with each other and exhibited haplotypes as follows: haplotype 1: G/TT/T; $n = 348$ (81.7%); haplotype 2: C/ΔG/G; $n = 73$ (17.1%); haplotype 3: G/ΔG/T; $n = 4$ (0.9%); and haplotype 4: G/TT/G; $n = 1$ (0.2%). Among the 176 healthy subjects ($2n = 352$), haplotypes 1 and 2 were seen in 323 (91.8%) and 29 (8.2%) of cases, respectively. The frequencies of haplotypes 1 and 2 showed significant differences between patients with chronic HCV infection and healthy subjects ($P = 0.049$ and $P = 0.025$, respectively). The statistical power of this study was 0.90 and therefore sufficient for analysis.

The G allele frequency of rs8099917 (33.3% *vs* 12.3%; $P = 0.011$, OR = 3.55) and the ΔG allele frequency of ss469415590 (37.3% *vs* 13.3%; $P = 0.044$, OR = 3.27) were significantly higher in the NVR group than in virological responders (Table 2). The overall frequency of the TG or GG genotype for rs8099917 was 60.8% and more common in subjects with an NVR than in those showing a virological response (60.8% *vs* 23.5%; $P = 0.068$, OR = 5.06). TT/ΔG or ΔG/ΔG was also significantly associated with an NVR (60.8% *vs* 24.7%; $P = 0.019$, OR = 4.73). Interestingly, 5 of 213 patients had a rare linkage between rs8099917 and ss469415590 (haplotypes 3 and 4), but showed differing responses to PEG-IFN and RBV therapy. Among the four patients with haplotype 3 (T/ΔG/G), three achieved an SVR and one experienced an NVR. The other patient with haplotype 4 (G/TT/C) exhibited an NVR.

We next evaluated several factors apparently associated with an NVR to PEG-IFN and RBV therapy for independence by multivariate analysis. Only the rs8099917 SNP (TG or GG) was found to be an independent risk factor (OR = 5.28, 95% CI: 2.53–11.01, $P = 0.009$).

In this study, we investigated the frequency of the ss469415590 SNP in Japanese patients with type 1 chronic hepatitis C and analyzed its association with the outcome of PEG-IFN and RBV therapy. As it has been suggested that ss469415590 ΔG/TT is in complete linkage disequilibrium with the minor allele [G] of rs8099917 in individuals of Chinese ancestry, our data were able to confirm that the correlation between ss469415590 and rs8099917 was extremely high ($r^2 = 0.92$, $D' = 0.98$), such that only 5 (2.3%) of 213 individuals were ruled out.

Our data showed that the frequency of haplotype 1 (rs4803221, ss469415590, and rs8099917: G/TT/T) was higher in healthy subjects than in those chronic hepatitis C. These frequencies were very similar to those in published (6) and HapMap data from Japan. Since the major IL28B alleles have been associated with spontaneous clearance of HCV infection (17), carriers of haplotype 1 may have a decreased risk of chronic HCV infection.

As proposed by Prokunina-Olsson et al., ss469415590 [ΔG] was highly associated with treatment outcome in Japanese patients (OR = 4.73, $P = 0.019$). We observed a stronger association for rs8099917 (OR = 5.06) than for ss469415590 with an NVR, although this difference did not reach statistical significance. In multivariate analysis of our cohort,

Table 2 Association of rs8099917 and ss469415590 SNPs with response to PEG-IFN and RBV therapy[a]

SNP	NVR ($n = 51$)	VR ($n = 162$)	OR (95% CI)	P	Controls ($n = 176$)
rs8099917					
G allele	33.3	12.3	3.55 (2.09–6.02)	0.011	8.2
T allele	66.7	87.7			91.8
T/G or G/G	60.8	23.5	5.06 (2.59–9.88)	0.068	16.5
T/T	39.2	76.5			83.5
ss469415590					
ΔG allele	33.3	13.3	3.27 (1.94–5.51)	0.044	8.2
TT allele	66.7	86.7			91.8
TT/ΔG or ΔG/ΔG	60.8	24.7	4.73 (2.43–9.20)	0.019	16.5
TT/TT	39.2	75.3			83.5

CI, confidence interval; NVR, null virological response; OR, odds ratio; SNP, single nucleotide polymorphism; VR, virological response.
[a]All values are expressed as percentages.

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rs8099917 [G] was an independent factor related to an NVR in Japanese patients treated with PEG-IFN and RBV therapy, and treatment response was correlated with the rs8099917 SNP, but not ss469415590, in five patients with varying SNPs. Hence, our results indicate that although rs8099917 remains a powerful predictor of PEG-IFN and RBV therapy in the Japanese, ss469415590 may be an effective marker as well.

Although our data support that ss469415590 [ΔG] is strongly associated with treatment failure in chronic hepatitis C, the mechanism remains unknown by which ss469415590 [ΔG] and the IFN-λ4 protein might cause impaired HCV clearance. Prokunina-Olsson et al. (9) showed that IFN-λ4 may pre-active the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and limit further activation by type-I and type-III IFNs. Furthermore, Bibert et al. (18) have attributed the effect of IFNL4ΔG to decreased induction of IFNL3 and IP-10 mRNA, which rely on the ss469415590 genotype.

In summary, this report demonstrates an association between the dinucleotide variant ss469415590 and treatment response to PEG-IFN and RBV therapy for HCV genotype 1 infections in the Japanese population. Further studies on this polymorphism will more clearly elucidate the mechanism of antiviral response.

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Conflict of Interest

Drs TU and ET are currently conducting research sponsored by MSD. All other authors have declared no conflicting interests.

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P=1.36\times10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P=5.22\times10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P=1.55\times10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
* Resolved individuals were HBsAg negative and HBcAb positive.
** 419 of 467 healthy controls were de-identified, without information on age.
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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, 0.05/11, 0.05/12, and 0.05/16, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: $\text{logit}(p) = -3.905+0.083*\text{age}+(-0.929)*\text{sex}+(-0.684)*DPB1*02{:}01+1.814*c1+(-0.478)*c2+0.782*c3$. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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Research Article

STAT4 Gene Polymorphisms Are Associated with Susceptibility and ANA Status in Primary Biliary Cirrhosis

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Recent genome-wide association studies suggest that genetic factors contribute to primary biliary cirrhosis (PBC) susceptibility. Although several reports have demonstrated that the interleukin (IL) 12 signaling pathway is involved in PBC pathogenesis, its precise genetic factors have not been fully clarified. Here, we performed an association analysis between *IL12A*, *IL12RB*, and *signal transducer and activator of transcription 4 (STAT4)* genetic variations and susceptibility to PBC. Single nucleotide polymorphisms (SNPs) were genotyped in 395 PBC patients and 458 healthy subjects of Japanese ethnicity and evaluated for associations with PBC susceptibility, anti-nuclear antibody (ANA) status, and anti-mitochondrial antibody (AMA) status. We detected significant associations with PBC susceptibility for several *STAT4* SNPs (rs10168266; $P = 9.4 \times 10^{-3}$, rs11889341; $P = 1.2 \times 10^{-3}$, rs7574865; $P = 4.0 \times 10^{-4}$, rs8179673; $P = 2.0 \times 10^{-4}$, and rs10181656; $P = 4.2 \times 10^{-5}$). Three risk alleles (rs7574865; $P = 0.040$, rs8179673; $P = 0.032$, and rs10181656; $P = 0.031$) were associated with ANA status, but not with AMA positivity. Our findings confirm that *STAT4* is involved in PBC susceptibility and may play a role in ANA status in the Japanese population.

1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by destruction of intrahepatic bile ducts and development of hepatic fibrosis that often progress to cirrhosis and liver failure [1]. The etiology of PBC remains poorly understood and is considered to be complex, [2–4] whereby a combination of inherited genetic predisposition factors and environmental exposure is likely required for disease development. Several genetic characteristics have specifically been implicated in PBC etiology in the Japanese population, including the *HLA DRB1*08:03-DQB1*06:01* haplotype and single nucleotide polymorphisms (SNPs) in the *cytotoxic T-lymphocyte-associated protein 4* and *ataxin 2-binding protein 1* genes [5–7].

Recent genome-wide association studies (GWAS) have identified a number of HLA and non-HLA loci with possible relevance to the development of PBC. However, these studies often uncovered different loci in the same signaling pathways across ethnicities; [8–12] genetic variants of the *IL12 α-chain*

(*IL12A*) and *IL12RB2* genes were associated with disease susceptibility in Caucasian studies, [8–11] but such associations have not been confirmed in the Japanese [12].

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor belonging to the STAT family [13] that is required for the development of Th1 cells from naïve CD4+ T cells [14] and IFN-γ production in response to IL12 [15]. Two chains of the IL12 receptor form a heterodimer after IL12 binding and activate the receptor-associated JAK kinases JAK2 and TYK2. STAT4 is phosphorylated by these tyrosine kinases, homodimerizes via its src homology 2 (SH2) domain, and then translocates into the nucleus to activate cytokine-responsive gene transcription [16]. While early GWAS initially showed a weak association between *STAT4* polymorphisms and PBC susceptibility, [8–10] recent investigations have confirmed a definite link between the two [11, 12] and have indicated that common pathogenic pathways, such as IL12 signaling, play an essential and nonredundant role in the development of this disease and some of its clinical features.

Anti-mitochondrial antibody (AMA) positivity is the serologic hallmark of PBC. AMA titers tend to be stable over time in individual patients and do not correlate with disease severity or rate of progression [1, 17]. Antinuclear antibodies (ANA) are found in up to 70% of patients with PBC and are suggested to be associated with more rapid disease progression and a poorer prognosis [18]. Positivity for anti-gp210 and anti-centromere antibodies has been related to PBC progression as well [19, 20]. Since the association between genetic polymorphisms and autoantibody production has not yet been elucidated, we investigated whether such polymorphisms contributed to a genetic predisposition to PBC and autoantibody production in the Japanese population.

2. Patients and Methods

2.1. Ethics Statement. This study was approved by the ethics committees of both participating institutions (Shinshu University School of Medicine, Matsumoto, Japan, and the National Hospital Organization Nagasaki Medical Center, Omura, Japan), and written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Subjects. We analyzed a total of 853 subjects (395 PBC patients and 458 sex-matched healthy controls) enrolled at Shinshu University Hospital, Matsumoto, Japan, and the National Hospital Organization Nagasaki Medical Center, Omura, Japan. As the subjects had no direct relatives of non-Japanese ethnicity, their racial background was considered to be uniformly Japanese. A part of this study's participants had been enrolled in previous genetic association studies [5–7, 12, 21–25]. In particular, 298 of 395 patients (75.4%) had been included in an earlier GWAS from Japan and were defined as the GWAS cohort in this analysis [12]. The remaining 97 (24.6%) patients were newly diagnosed as having PBC and were defined as the replication cohort. Newly enrolled control subjects were volunteers from hospital staff who had indicated the absence of any major illnesses in a standard questionnaire and whose racial background was considered to be uniformly Japanese. The sex-matched control group consisted of 384 women and 74 men with no direct familial relations.

Table 1: Demographic and clinical data of patients.

Characteristic	n = 395
Median age, years (range)	58 (28–87)
Female/male	338/57
Autoantibody	
AMA-positive, n (%)	369 (93.4)
ANA-positive, n (%)	271 (67.6)
Cenp positive, n (%)	119/362 (32.9)*
gp210 positive, n (%)	80/260 (30.8)*

AMA: anti-mitochondrial antibody; ANA: anti-nuclear antibody; Cenp: anti-centromere antibody; gp210: gp210 antibody.
*Only patients who were assessed for Cenp and gp210 are reported.

The diagnosis of PBC was determined based on criteria from the American Association for the Study of Liver Diseases [26]. Serum AMA, which is specific for the pyruvate dehydrogenase complex-E2 component, was measured by the enzyme-linked immunosorbent assay (ELISA). An index of greater than 7 was considered to be a positive result. Serum ANA was determined by immunofluorescence using HEp-2 cells, whereby a titer of ≥40 was considered to be positive, as reported previously [27]. Patterns of ANA reactivity were recorded as well. Serum anti-centromere antibody was detected using commercially available ELISA kits (MBL, Nagoya, Japan), for which an index of >5 U/mL was considered to be positive. Serum gp-210 antibody was also measured by ELISA, whereby an index of >10 U/mL was considered to be a positive result, as previously described [19]. All patients were negative for hepatitis B surface antigen and antibodies to the hepatitis C and human immunodeficiency viruses.

2.3. IL-12 Signaling-Related SNP Genotyping. Genomic DNA from patients was isolated by phenolic extraction of sodium dodecyl sulfate-lyzed and proteinase K-treated cells, as described previously [28], and adjusted to a concentration of 10–15 ng/μL.

IL12A (rs574808), *IL12RB* (rs3790567), and *STAT4* (rs7574865) SNPs were selected based on reported PBC susceptibility [8–12]. Since the *STAT4* (rs7574865) SNP was found to be significantly associated with PBC, we genotyped an additional 7 SNPs located in this gene (rs10168266, rs7594501, rs16833239, rs11889341, rs8179673, rs10181656, and rs6752770) that were not evaluated in the earlier Japanese GWAS using an SNP Genotyping Kit (Applied Biosystems, Tokyo, Japan). These SNPs were selected based on previous reports [8–12, 29–31]. Polymerase chain reaction (PCR) was performed with TaqMan Assays (7500 Real Time PCR System; Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions.

Table 2: *IL12A*, *IL12RB*, and *STAT4* SNPs in PBC patients and healthy subjects.

Gene	db SNP	Allele minor/major	GWAS cohort patients (%) (n = 302)	Replication cohort patients (%) (n = 93)	Combined cohort patients (%) (n = 395)	Controls (%) (n = 458)	P	OR	95% CI
IL12A	rs574808	C/T	17.2	18.3	17.5	18.1	0.75		
IL12RB	rs3790567	A/G	26.8	22.0	25.7	22.9	0.18		
STAT4	rs7574865	T/G	40.6	46.8	41.9	33.5	4.0×10^{-4}	1.43	1.17–1.74

IL12: interleukin 12; *STAT4*: signal transducer activator transcription 4; PBC: primary biliary cirrhosis; SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval.

Table 3: *STAT4* SNPs in PBC patients and healthy subjects.

db SNP	Allele minor/major	Patients (%) (n = 395)	Controls (%) (n = 458)	P	P_c	OR	95% CI
rs10168266	A/G	34.5	28.7	9.4×10^{-3}	0.038	1.31	1.07–1.61
rs7594501	A/G	12.7	16.7	0.021			
rs16833239	T/C	13.0	16.4	0.046			
rs11889341	T/C	39.1	31.7	1.2×10^{-3}	9.6×10^{-3}	1.39	1.14–1.69
rs7574865	T/G	41.9	33.5	4.0×10^{-4}	3.2×10^{-3}	1.43	1.17–1.74
rs8179673	G/A	42.1	33.5	2.0×10^{-4}	1.6×10^{-3}	1.44	1.19–1.75
rs10181656	G/C	43.3	33.7	4.2×10^{-5}	3.4×10^{-4}	1.50	1.24–1.83
rs6752770	G/A	15.0	17.8	0.120			

STAT4: signal transducer activator transcription 4; PBC: primary biliary cirrhosis; SNP: single nucleotide polymorphism; OR: odds ratio; P_c: corrected P value; CI: confidence interval.

2.4. Statistical Analysis. All examined SNPs in control groups were in the Hardy-Weinberg equilibrium. The R-software "Haploview" [32] version 4.2 was used to evaluate the haplotype structure of the 8 *STAT4* SNPs. Pairwise linkage disequilibrium (LD) patterns and haplotype frequency analysis for all SNPs in patients and controls were assessed by the block definition established by Gabriel et al. [33]. We assessed the significance of allele distribution between patients and controls using the χ^2 test by means of 2 × 2 comparisons. A P value of less than 0.05 was considered to be statistically significant. We adjusted P values using Bonferroni's correction by multiplying each locus by 8 (P_c). Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Statistical analysis of data was performed using SPSS 21.0 software (IBM, Armonk, New York).

3. Results

3.1. Genotyping of IL12 Signaling-Related SNPs. To clarify the genetic susceptibility to PBC based on previously reported *IL12* signaling, a total of 395 Japanese patients with PBC and 458 healthy Japanese controls were enrolled for an association analysis of *IL12A* (rs574808), *IL12RB* (rs3790567), and *STAT4* (rs7574865) SNPs (Table 2). Whereas the *IL12A* and *IL12RB* SNPs were not associated with PBC, the rs7574865 SNP in *STAT4* showed a positive association with PBC susceptibility (41.9% versus 33.5%; $P = 4.0 \times 10^{-4}$, OR = 1.43, 95% CI = 1.17–1.74). To further examine its role in PBC, we selected an additional 7 SNPs from *STAT4* (rs10168266, rs7594501, rs16833239, rs11889341, rs8179673, rs10181656, and rs6752770) and genotyped them in all patients and controls. The minor allele frequencies of A at rs10168266, T at rs11889341, T at rs7574865, G at rs8179673, and G at rs10181656 were significantly increased in PBC patients as compared with controls ($P = 9.4 \times 10^{-3}$, $P = 1.2 \times 10^{-3}$, $P = 4.0 \times 10^{-4}$, $P = 2.0 \times 10^{-4}$, and $P = 4.0 \times 10^{-5}$, resp.) (Table 3).

3.2. Distribution of STAT4 Haplotypes among PBC Patients and Controls. We firstly defined LD blocks for the 8 SNPs in *STAT4* (Figure 1). The *STAT4* region was divided into two haplotype blocks, with substantial LD among the SNPs in each block. To estimate haplotype frequency and analyze haplotype association with PBC, we selected tag SNPs using the Tagger algorithm from the Haploview program. Three tag SNPs (rs7594501, rs16833239, and rs11889341) in block A and 3 tag SNPs (rs7574865, rs8179673, and rs10181656) in block B were captured from pairwise measures of LD. The top 3 haplotype frequencies in both blocks are shown in Table 4. Haplotype 2 (GCT) in block A was significantly associated with PBC susceptibility (40.3% versus 32.2%; $P = 3.5 \times 10^{-3}$, OR 1.43, 95% CI 1.12–1.81), as was haplotype 5 (TGG) in block B (43.3% versus 33.6%; $P = 6.0 \times 10^{-4}$, OR 1.51, 95% CI 1.19–1.91). In contrast, protective effects were seen for haplotype 4 (GAC) in block B (53.9% versus 65.4%; $P = 5.0 \times 10^{-5}$, OR 0.62, 95% CI 0.49–0.78).

3.3. Associations between STAT4 SNPs, Haplotypes, and Autoantibodies. The PBC patients enrolled in this study were highly positive for disease-specific autoantibodies (Table 1). There were no significant differences with regard to ANA positivity between 250 of 369 AMA-positive patients (67.8%) and 21 of 26 AMA-negative patients (80.8%) (P = 0.245). Among the 8 *STAT4* SNPs, the frequencies of 3 minor alleles

Table 4: *STAT4* haplotypes in PBC patients and healthy subjects.

Block	Haplotype	SNPs			Patients (%) (n^* = 790)	Controls (%) (n^* = 916)	P	OR	95% CI
A		rs7594501	rs16833239	rs11889341					
	1	G	C	C	46.4	50.0	0.222		
	2	G	C	T	40.3	32.2	3.5×10^{-3}	1.43	1.12–1.81
	3	A	T	C	11.9	15.9	0.045		
B		rs7574865	rs8179673	rs10181656					
	4	G	A	C	53.9	65.4	5.0×10^{-5}	0.62	0.49–0.78
	5	T	G	G	43.3	33.6	6.0×10^{-4}	1.51	1.19–1.91
	6	G	A	G	2.1	0.0	3.0×10^{-4}		

STAT4: signal transducer activator transcription 4; PBC: primary biliary cirrhosis; SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval; n^*: values for n^* indicate two times the number of individuals since each person carries two haplotypes.

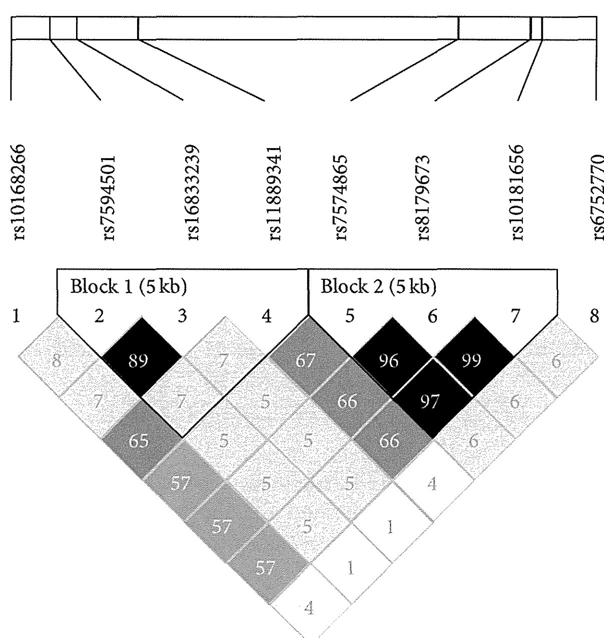

Figure 1: Linkage disequilibrium (LD) plot of 8 SNPs in *STAT4* in 458 healthy subjects. Values of r^2 corresponding to each SNP pair are expressed as a percentage and shown within the respective square.

(T at rs7574865, G at rs8179673, and G at rs10181656) were increased in ANA-positive PBC patients as compared with ANA-negative patients (P = 0.040, P = 0.032, and P = 0.031, resp.) (Table 5), but these statistical significances disappeared after correction. Haplotype 5 (TGG) in block B was significantly correlated with ANA (44.3% versus 36.3%; P = 0.035, OR 1.40, 95% CI 1.02–1.90) (Table 6). Interestingly, haplotype 5 was also significantly associated with the speckled pattern of ANA as compared with the nonspeckled pattern (45.8% versus 36.3%; P = 0.029, OR 1.48, 95% CI 1.04–2.12) (data not shown). No *STAT4* SNPs or haplotypes were associated with other autoantibody positivity or ANA pattern, such as discrete speckled pattern, homogenous pattern, nucleolar pattern, or peripheral pattern.

4. Discussion

In the present study, we investigated the association between *STAT4* SNPs and PBC susceptibility and its clinical significance in the Japanese population. Our key findings were as follows: (1) specific *STAT4* polymorphisms and haplotypes were significantly associated with PBC susceptibility or protection; (2) there were no significant genetic associations between *IL12A* and *IL12RB* SNPs and PBC susceptibility, in contrast to studies of Caucasians; [8–11] and (3) there was a moderate relationship between *STAT4* SNPs and ANA-positive, but not AMA-positive, PBC patients.

STAT4 lies in the signaling pathways of several cytokines, such as IL12, type I interferon, and IL23. This time, a newly diagnosed PBC cohort, albeit small, replicated the previous finding by Japanese GWAS [12] that a *STAT4* SNP at rs7574865 was associated with susceptibility to PBC. To evaluate the power of this study, larger association studies of other ethnicities, including Chinese and Caucasian populations, are required because small sample sizes may lead to false positive or negative results. Moreover, we identified 4 additional SNPs in *STAT4* that conferred susceptibility to PBC and were consistent with findings of GWAS of other ethnicities [11]. Haplotype analysis showed that 3 of the identified risk SNPs, rs7574865, rs8179673, and rs10181656, were located in the same LD block (Figure 1 and Table 4). These SNPs and this haplotype have been linked with several autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus (SLE) [29–31, 34–38]. In particular, it has been reported that SNPs at rs7574865 are associated with numerous other autoimmune diseases [35–38]. As we focused only on *STAT4* polymorphisms, we must concede that our study is rather limited in depth and scope compared with recent immunogenic studies [39, 40] using the Immunochip. However, our findings support the notion that SNPs and haplotypes in *STAT4* may contribute to the development of PBC and other autoimmune diseases.

In addition to our own, two earlier studies from Japan [12, 41] showed that *IL12A* and *IL12RB* SNPs were not associated with PBC, which was in contrast to strong associations found in Caucasian population studies [8–10, 42]. Similarly, GWAS of the Japanese have identified novel significant susceptibility

Table 5: Correlations between *STAT4* SNPs and autoantibody positivity.

db SNP	Allele minor/major	ANA^+ (%) (n^* = 271)	ANA^- (%) (n^* = 124)	P	OR	95% CI	AMA^+ (%) (n^* = 369)	AMA^- (%) (n^* = 26)	P	$Cenp^+$ (%) (n^* = 119)	$Cenp^-$ (%) (n^* = 243)	P	$gp210^+$ (%) (n^* = 80)	$gp210^-$ (%) (n^* = 180)	P
rs10168266	A/G	35.6	33.1	0.50			34.2	42.3	0.24	36.1	35.3	0.83	35.0	32.5	0.58
rs7594501	A/G	11.5	14.9	0.18			12.9	7.7	0.27	13.0	12.6	0.87	11.9	12.8	0.77
rs16833239	T/C	11.6	15.3	0.15			13.1	7.7	0.26	12.6	13.4	0.77	13.1	13.1	0.98
rs11889341	T/C	41.3	34.7	0.076			38.8	46.2	0.29	59.7	59.7	1.00	39.4	37.2	0.64
rs7574865	T/G	44.5	36.7	0.040	1.38	1.01–1.88	41.6	48.1	0.36	42.4	43.6	0.76	43.1	38.3	0.30
rs8179673	G/A	44.8	36.7	0.032	1.40	1.03–1.91	41.9	48.1	0.38	42.9	43.8	0.81	43.1	38.6	0.33
rs10181656	G/C	46.1	37.9	0.031	1.40	1.03–1.91	43.1	50.0	0.33	45.0	44.4	0.90	43.1	38.6	0.33
rs6752770	G/A	15.4	14.1	0.63			15.3	11.5	0.47	13.4	14.7	0.64	15.0	15.8	0.81

STAT4: signal transducer activator transcription 4; PBC: primary biliary cirrhosis; SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval; AMA: anti-mitochondrial antibody; ANA: anti-nuclear antibody; Cenp: anti-centromere antibody; gp210: gp210 antibody; n^*: values for n^* indicate two times the number of individuals since each person carries two haplotypes.

Table 6: Correlations between *STAT4* haplotypes and autoantibody positivity.

Block	Haplotype	SNPs			ANA+ (%) (n^* = 542)	ANA− (%) (n^* = 248)	P	OR	95% CI	AM+ (%) (n^* = 738)	AMA− (%) (n^* = 52)	P	Cenp+ (%) (n^* = 238)	Cenp− (%) (n^* = 486)	P	gp210+ (%) (n^* = 160)	gp210− (%) (n^* = 360)	P
A		rs7594501	rs16833239	rs11889341														
	1	G	C	C	46.7	49.6	0.45			47.7	46.2	0.83	46.6	46.1	0.89	47.5	49.2	0.73
	2	G	C	T	41.3	34.7	0.076			38.8	46.2	0.29	40.3	40.3	1.00	39.4	37.2	0.64
	3	A	T	C	11.1	14.5	0.17			12.5	7.7	0.31	12.6	12.3	0.92	11.9	12.2	0.91
B		rs7574865	rs8179673	rs10181656							0.0							
	4	G	A	C	53.7	61.7	0.035	0.72	0.53–0.98	56.8	48.1	0.22	54.6	55.3	0.85	56.9	61.4	0.33
	5	T	G	G	44.3	36.3	0.035	1.40	1.02–1.90	41.5	46.2	0.51	42.0	43.4	0.72	43.1	38.3	0.30
	6	G	A	G	1.5	1.6	0.88			1.4	3.8	0.16	2.5	0.8	0.066	0.0	0.0	—

STAT4: signal transducer activator transcription 4; PBC: primary biliary cirrhosis; SNP: single nucleotide polymorphism; OR: odds ratio; CI: confidence interval; AMA: anti-mitochondrial antibody; ANA: anti-nuclear antibody; Cenp: anti-centromere antibody; gp210: gp210 antibody; n^*: values for n^* indicate two times the number of individuals since each person carries two haplotypes.

loci for PBC, such as *TNFSF15* and *POU2AF1*, which have not been identified in GWAS of populations of European descent. Meanwhile, Peters et al. found that liver damage severity at clinical presentation is higher among non-Caucasians than Caucasians for PBC [43]. Hence, although the IL12 pathway through STAT4 plays an essential role in PBC etiology, there is evidence of ethnic differences in genetic susceptibility loci. PBC is also concurrent with other autoimmune diseases, including Sjögren syndrome, [44] rheumatoid arthritis, [45] and cutaneous scleroderma, [46] so we cannot exclude the possibility of genetic overlap among these disorders.

Interestingly, our study showed a moderate association between 3 *STAT4* polymorphisms and 2 haplotypes with ANA-positive PBC that was not seen for AMA (Tables 5 and 6). To understand its clinical relevance, we analyzed this association with regard to ANA pattern and found a significant relationship between these SNPs and the speckled pattern of ANA, which was in agreement with a recent meta-analysis showing that the presence of anti-ds-DNA antibodies was associated with rs7574865 within *STAT4* polymorphisms in SLE patients. Our results did not support a relationship between *STAT4* SNPs and gp210 or anti-centromere antibodies, despite these antibodies having been associated with disease progression and prognosis in PBC patients [19]. Taken together, our data implied that *STAT4* SNPs imparted susceptibility to ANA-positive PBC, but for reasons that are still unknown. The mechanisms by which genetic variants are correlated with ANA positivity may be diverse and require further study.

5. Conclusions

Our findings confirm that *STAT4* SNPs and haplotypes contribute to PBC susceptibility and may play a role in mediating ANA status. *STAT4* appears to factor strongly in the pathogenesis of this and other autoimmune diseases and requires continued study.

Abbreviations

PBC: Primary biliary cirrhosis
SNP: Single nucleotide polymorphism
GWAS: Genome-wide association study
IL12: Interleukin12
STAT4: Signal transducer and activator of transcription 4
AMA: Anti-mitochondrial antibody
ANA: Anti-nuclear antibody
PBMCs: Peripheral blood mononuclear cells
LD: Linkage disequilibrium
P_c: Corrected P
OR: Odds ratio
CI: Confidence interval.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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