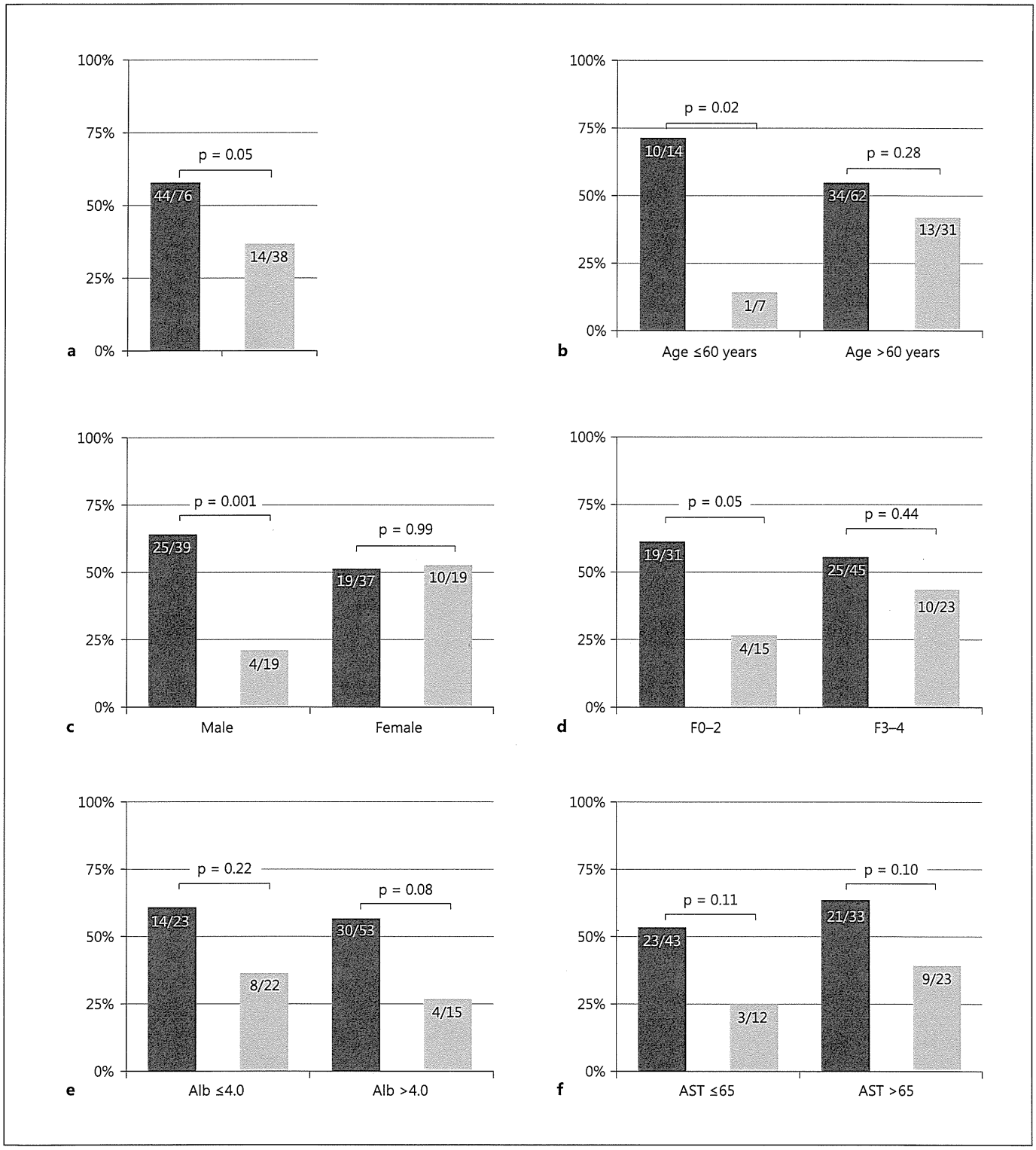

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey bars represent patients with and without HCC, respectively.

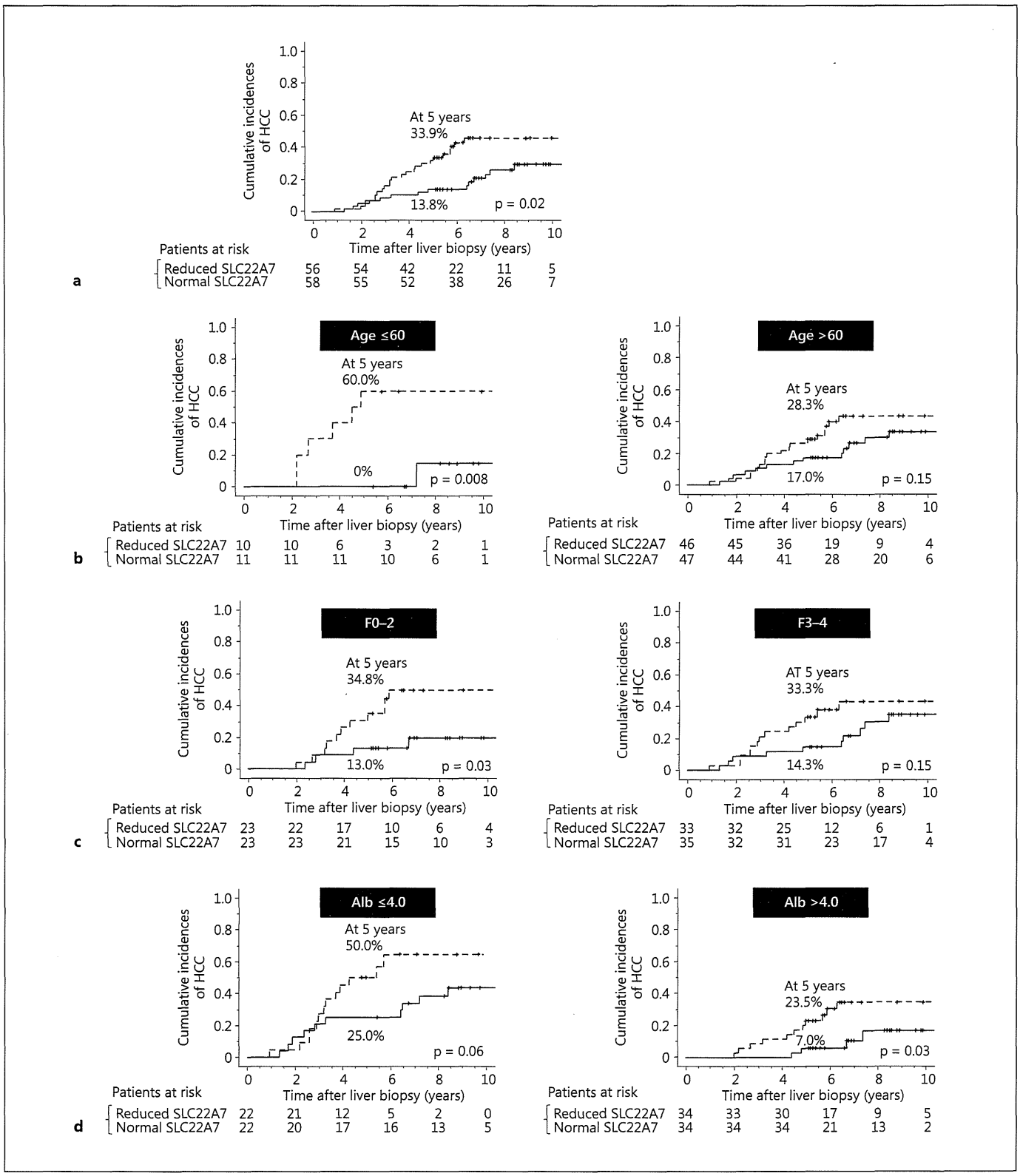

Fig. 6. Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b–d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**) and albumin (Alb) level (**d**), respectively.

tients without advanced fibrosis, SLC22A7 expression can provide an important cost-effective screening tool. Moreover, we confirmed previous knowledge of low serum albumin levels as an independent risk factor for HCC development in patients matched for age, gender and stage of liver fibrosis. Nonetheless, in patients with higher serum albumin levels (≥4.0 g/dl), reduced SLC22A7 expression remained a significant independent risk factor for HCC.

The *SLC22A7* gene encodes OAT2, which is distributed mainly in the liver and kidney. As a protein predominantly expressed in the liver [23], OAT2 transports several antiviral drugs as well as prostaglandins. A recent study in rats showed that OAT2 is responsible for the uptake of orotic acid [24], which reportedly promotes liver carcinogenesis [25, 26]. In the clinical setting, orotic aciduria was also observed in HCC patients without liver cirrhosis [27]. Moreover, a previous study using gene-set enrichment analysis revealed that SLC22A7 expression is significantly correlated with mitochondrial oxidoreductase activity and fatty acid metabolism. Mitochondrial dysfunction and oxidative stress are considered key mechanisms for the development of HCC. Collectively, these studies indicate that reduced SLC22A7 expression promotes hepatic carcinogenesis by increasing the concentration of orotic acid around hepatocytes and promoting oxidative stress and mitochondrial dysfunction. Our study suggests that these microenvironmental changes might occur in patients with chronic HCV in an early stage. As for HCC recurrence after surgical resection, gene expression has been extensively investigated in tissues surrounding HCC [16, 28–30]. However, it remains unknown whether these signatures correlate with multifocal occurrence of HCC. Indeed, the precise mechanisms involved in the association between SLC22A7 expression and HCC development require further investigation.

In this study, personally gifted antibody was used for IHC. Staining performance of our antibody was similar to that of commercially available antibodies (Atlas Antibodies, Stockholm, Sweden) by a small pilot study (unpubl. data).

Our retrospective study design and low patient numbers must be acknowledged as limitations, particularly in the first study. However, this first study confirmed that our biopsy specimens were feasible for IHC analysis of SLC22A7, and we could therefore proceed to the larger matched-control study. To improve reproducibility, we conducted a propensity score matched study and only included patients who were HCV-positive and had not achieved SVR with interferon therapy, so our results may not pertain to chronic HCV patients who achieve SVR or patients with other chronic diseases of the liver. A larger prospective study will be required to confirm our results.

In conclusion, our study showed the importance of IHC staining for SLC22A7 as a predictive tool for HCC. We propose that patients with reduced SLC22A7 expression and lower serum albumin levels are candidates for intensive HCC surveillance, even if they do not exhibit other known risk factors.

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Original Article

Serum granulysin levels as a predictor of serious telaprevir-induced dermatological reactions

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Aim: Telaprevir-based therapy for chronic hepatitis C patients is effective; however, the high prevalence of dermatological reactions is an outstanding issue. The mechanism and characteristics of such adverse reactions are unclear; moreover, predictive factors remain unknown. Granulysin was recently reported to be upregulated in the blisters of patients with Stevens–Johnson syndrome (SJS). Therefore, we investigated the risk factors for severe telaprevir-induced dermatological reactions as well as the association between serum granulysin levels and the severity of such reactions.

Methods: A total of 89 patients who received telaprevir-based therapy and had complete clinical information were analyzed. We analyzed the associations between dermatological reactions and clinical factors. Next, we investigated the time-dependent changes in serum granulysin levels in five and 14 patients with grade 3 and non-grade 3 dermatological reactions, respectively.

Results: Of the 89 patients, 57 patients had dermatological reactions, including nine patients with grade 3. Univariate analysis revealed that grade 3 dermatological reactions were significantly associated with male sex. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Three patients with grade 3 dermatological reaction had severe systemic manifestations including SJS, drug-induced hypersensitivity syndrome, and systemic lymphoid swelling and high-grade fever; all were hospitalized. Importantly, among the three patients, two patients' serum granulysin levels exceeded 8 ng/mL at onset and symptoms deteriorated within 6 days.

Conclusion: Male patients are at high risk for severe telaprevir-induced dermatological reactions. Moreover, serum granulysin levels are significantly associated with the severity of dermatological reactions and may be a predictive factor in patients treated with telaprevir-based therapy.

Key words: drug-induced hypersensitivity syndrome, granulysin, hepatitis C virus, telaprevir, toxic epidermal necrolysis

INTRODUCTION

HEPATITIS C IS a major pathogen causing liver cirrhosis and hepatocellular carcinoma worldwide. Until recently, standard therapies for chronic hepatitis C virus (HCV) genotype 1 infection were based on the combination of pegylated interferon (PEG IFN) and ribavirin (RBV); these combination therapies yield a sustained virological response (SVR) rate of approximately 50%.[1] Several classes of novel direct-acting antivirals

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(DAA) were recently developed and tested in clinical trials. Two first-generation HCV NS3/4A protease inhibitors, boceprevir[2,3] and telaprevir,[4–6] have been approved for the treatment of genotype 1 HCV infection. The inclusion of these agents in HCV treatment regimens has led to large improvements in treatment success rates.

Telaprevir, the first DAA, is administrated in combination with PEG IFN and RBV for 24 weeks, resulting in SVR rates up to 70–80%.[4,6–8] Although the telaprevir combination regimen is highly effective, the high frequency and severity of adverse events are outstanding issues limiting its use. Dermatological reactions are particularly prevalent, developing in 56–84.6% of patients treated with telaprevir, PEG IFN and RBV combination therapy.[9,10] Moreover, the prevalence of severe dermatological reactions including Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and drug-induced hypersensitivity syndrome (DIHS) are substantially higher in patients treated with telaprevir-based therapy than PEG IFN and RBV combination therapy.[8,10] McHutchison *et al.* reported that 7% of patients treated with telaprevir, PEG IFN and RBV combination therapy discontinue therapy because of rash or pruritus in contrast to only 1% of patients treated with PEG IFN and RBV.[8] In some patients, serious skin reactions persist even after stopping all drugs.[10] However, the pathogenesis and clinical predictors of these adverse reactions are poorly understood.

Granulysin is a 15-kDa cationic cytolytic protein released by cytotoxic T lymphocytes and natural killer cells that induces apoptosis in target cells and has antimicrobial activities.[11] Serum levels of granulysin are elevated in primary virus infections including Epstein–Barr virus and parvovirus B19.[12] It was recently reported that serum granulysin levels are significantly elevated in patients with several types of severe dermatological lesions including SJS/TEN, which is the characteristic serious adverse event in telaprevir-containing regimens.[13,14]

Accordingly, the present study determined the risk factors for severe dermatological reactions in patients receiving telaprevir, PEG IFN and RBV combination therapy as well as the association between serum levels of granulysin and severe dermatological reactions.

METHODS

Patients and methods

IN THIS RETROSPECTIVE case–control study, at Hokkaido University Hospital and associated hospitals in the NORTE Study Group, between December 2011 and November 2013, a total of 123 patients positive for HCV genotype 1 with high serum HCV RNA titer (>5 log IU/mL) received PEG IFN, RBV and telaprevir combination therapy. Patients were excluded if they required hemodialysis or had a positive test result for serum hepatitis B surface antigen, co-infection with other HCV genotypes or HIV, evidence of autoimmune hepatitis or alcoholic hepatitis, or malignancy. Serum granulysin levels were analyzed in five healthy volunteers with no HCV, HIV or hepatitis B virus infection or any inflammatory diseases.

Written informed consent according to the process approved by the hospital's ethics committee was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of each participating hospital.

Study design and treatment regimen

Telaprevir 500 or 750 mg was typically administrated every 8 h after meals for 12 weeks. PEG IFN-α-2b (PegIntron; MSD, Tokyo, Japan) 1.5 IU/kg was administrated s.c. once per week for 24 weeks. RBV (Rebetol; MSD) was administrated for 24 weeks in two divided daily doses according to bodyweight: 600, 800 and 1000 mg for patients with bodyweights of less than 60, 60–80 and more than 80 kg, respectively. The doses of PEG IFN-α-2b, RBV and telaprevir were reduced at the attending physician's discretion on the basis of hemoglobin levels, decreased white blood cell or platelet counts, or adverse events.

During treatment, patients were assessed as outpatients at weeks 1, 2, 4, 6 and 8, and then every 4 weeks thereafter for the duration of treatment. Physical examinations and blood tests were performed at all time points.

Outcomes

The primary end-point was SVR, which was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. The secondary end-points were end-of-treatment virological responses (HCV RNA undetectable in serum) and rapid virological response (RVR), which was defined as undetectable serum HCV RNA at 4 weeks after the start of treatment. Dermatological reactions were classified according to severity in the same manner as in phase III trials in Japan.[10]

Serum granulysin measurement

To evaluate serum granulysin levels in chronic hepatitis C, we first measured serum granulysin levels in five

healthy volunteers and compared them with those of 20 chronic hepatitis C patients before treatment. Serum granulysin levels were measured at the onset of dermatological reactions (within 3 days of onset); if the symptoms worsened, the time when worsening occurred was adopted. Meanwhile, in patients with no dermatological reactions, the highest serum granulysin level during treatment was adopted.

Serum granulysin levels were measured by a sandwich enzyme-linked immunosorbent assay as described previously.[12,14,15] Briefly, plates coated with 5 mg/mL mouse antibody against human granulysin, RB1 antibody, were washed with phosphate-buffered saline containing 0.1% Tween-20. Next, they were blocked with 10% fetal bovine serum in washing buffer at room temperature for 2 h. The samples and standards (Recombinant Granulysin; R&D Systems, Minneapolis, MN, USA) were incubated for 2 h at room temperature. Next, they were reacted with 0.1 mg/mL biotinylated mouse antibody against human granulysin, RC8 antibody. The plates were subsequently treated with horseradish peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland). The plates were then incubated with tetramethyl-benzidine substrate (Sigma, St Louis, MO, USA), and 1 M sulfuric acid was then added. The optical density was measured at 450 nm using a microplate reader.

Diagnosis of dermatological reactions

Dermatological reactions were investigated throughout the 24-week administration period in the telaprevir-based combination therapy. Dermatological reactions were classified according to severity as follows. Grade 1 was defined as involvement of less than 50% of the body surface and no evidence of systemic symptoms. Grade 2 was defined as involvement of less than 50% of the body surface but with multiple or diffuse lesions or rashes with characteristic mild systemic symptoms or mucous membrane involvement with no ulceration/erosion. Grade 3 was defined as a generalized rash involving 50% or more of the body surface or a rash with any new significant systemic symptoms and considered to be related to the onset and/or progression of the rash. Life-threatening reactions included SJS, TEN, drug rash with eosinophilia and systemic symptoms (DRESS)/DIHS, erythema multiforme and other life-threatening symptoms, or patients presenting with features of serious disease.

When adverse skin reactions were detected, the attending physician classified the degree of severity and referred the patients to a dermatologist as needed. In principal, when grade 3 dermatological reactions occurred, the attending physician referred the patient to a dermatologist and discontinued telaprevir. When severe dermatological reactions including SJS/TEN and DRESS/DIHS were suspected, all drugs were discontinued immediately. SJS/TEN and DIHS were diagnosed by skin biopsy and according to disease criteria, respectively.

Statistical analysis

Categorical and continuous variables were analyzed by the χ^2-test and the unpaired Mann–Whitney *U*-test, respectively. All *P*-values were two-tailed, and the level of significance was set at $P < 0.05$. Multivariate logistic regression analysis with stepwise forward selection included variables showing $P < 0.05$ in univariate analyses.

The association between dermatological reactions and serum granulysin levels were evaluated by one-way ANOVA followed by Tukey's honestly significant difference test. All statistical analyses were performed using SPSS version 21.0 (IBM Japan, Tokyo, Japan).

RESULTS

Patients

WE INCLUDED 123 chronic hepatitis C patients who received telaprevir-based triple therapy. Of these, 89 patients who had proper information of dermatological adverse events were included. The baseline characteristics of patients are shown in Table 1.

Of these 89 patients, time-dependent changes of serum granulysin concentrations were measured in 20 who had had conserved serum, at least, at the pretreatment point, 1 and 2 weeks after commencement of therapy, 1 and 2 months after commencement of therapy, the onset point of dermatological adverse reaction and the worsening point if symptoms became worse.

Among the 89 patients, 64% (57/89) developed dermatological reactions, including nine with grade 3 reactions (Table 2). The characteristics of dermatological reactions by grade are shown in Table 2. Non-grade 3 dermatological reactions tended to occur early during treatment compared to grade 3 dermatological reactions.

Association between dermatological reactions and treatment outcomes

First, we determined whether dermatological reactions were associated with final treatment outcomes.

Table 1 Baseline characteristics of the participating patients

Total number	89
HCV genotype 1b (1b/others)	89/0
Age (years)†	60.0 (19–73)
Sex (male/female)	48/41
Bodyweight (kg)†	63.0 (32–97)
Baseline white blood cell count (/μL)†	4800 (1500–9800)
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)
Baseline platelet count ($\times 10^3$)†	15.9 (6.6–86)
Baseline ALT level (IU/L)†	40 (15–300)
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.5 (3.2–7.6)
Initial telaprevir dose (1500/2250 mg)	20/89
Initial PEG IFN dose (1.5/<1.5 μg/kg)	775/14
Initial RBV dose (mg/kg)†	9.8 (2.2–15.5)
IL28B gene (rs8099917) (TT/non-TT/ ND)	51/22/16
HCV 70 core mutation (wild/mutant/ND)	43/24/22
Previous treatment (naïve/relapse/NVR)	40/38/11

†Data are shown as median (range) values.
ALT, alanine transaminase; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; PEG IFN, pegylated interferon; RBV, ribavirin.

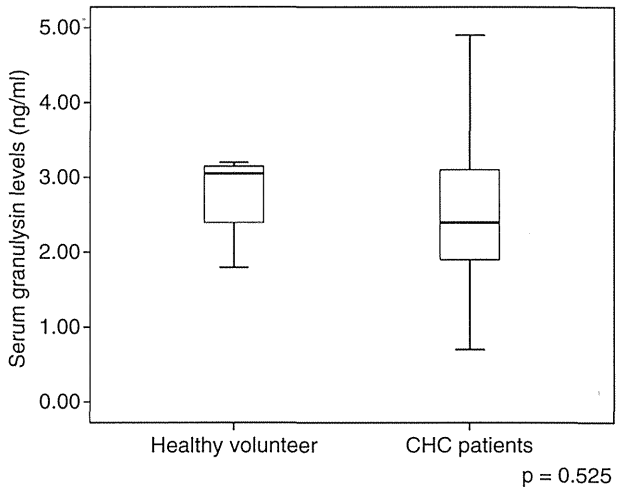

Figure 1 Serum granulysin levels of healthy volunteers and chronic hepatitis C patients. Serum granulysin levels were compared between five healthy volunteers and untreated 20 chronic hepatitis C patients. $P < 0.05$, Mann–Whitney *U*-test.

Univariate analyses identified baseline white blood cell and platelet counts, RVR, and non-grade 3 dermatological reactions significantly associated with SVR (Table 3). Among the nine patients with grade 3 dermatological reactions, three discontinued all treatment and six discontinued telaprevir administration; SVR was achieved in zero of the three (0%) and two of the six (33%), respectively.

Multivariate analysis showed that RVR and non-grade 3 dermatological reactions were significantly associated with SVR (Table 3).

Analysis of risk factors for telaprevir-induced dermatological reactions

Next, we analyzed the association between severe (i.e. grade 3) dermatological reactions and clinical parameters (Table 4). Univariate analysis showed that only sex was significantly associated with the grade 3 dermatological reactions ($P = 0.03$).

Serum granulysin levels in healthy subjects and chronic hepatitis C patients

As shown in Figure 1, serum granulysin levels did not differ significantly between healthy volunteers and chronic hepatitis C patients. Next, we evaluated the association between the severity of dermatological reactions and serum peak granulysin levels in 20 patients including five, four, five and six with grades 1, 2 and 3, and no dermatological events, respectively. One-way ANOVA showed that serum granulysin level was significantly associated with the severity of dermatological reactions ($P = 0.036$); in addition, Tukey's honestly significant difference test revealed that the serum

Table 2 Characteristics of the patients with each dermatological adverse event grade

	n	Age†	Sex (male/female)	Initial telaprevir dose (2250/1500)	Onset of DAR (days)
No DAR	32	61 (28–72)	15/17	26/6	
Grade 1	32	58 (19–73)	15/17	24/8	7 (3–50)
Grade 2	16	61 (44–73)	10/6	12/4	3.5 (1–56)
Grade 3	9	61 (48–65)	8/1	8/1	22 (1–60)

†Data are shown as median range) values.
DAR, dermatological adverse reaction

Table 3 Comparison of the clinical and laboratory characteristics of the patients with HCV infection based on therapeutic response

All patients	SVR	Non-SVR	Univariate analysis	Multivariate analysis		
$n = 89$	$n = 68$	$n = 21$	P	OR	95% CI	P
Age (years)†	60 (19–73)	62 (28–73)	0.402			
Sex (male/female)	37/31	11/10	0.870			
Bodyweight (kg)†	62 (39–97)	64 (32–87)	0.761			
Baseline white blood cells (/μL)†	5135 (1500–9800)	4200 (2490–7200)	0.048	0.492	(0.121–1.993)	0.320
Baseline hemoglobin level (g/dL)†	13.5 (10.5–16.7)	12.1 (9.9–15.4)	0.862			
Baseline platelet count ($\times 10^3$)†	16.7 (6.6–31.5)	12.8 (7.2–86)	0.025	0.388	(0.093–1.614)	0.193
Baseline ALT level (IU/L)†	37 (15–300)	53 (23–159)	0.070			
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.7 (3.2–7.6)	6.4 (5.7–7.3)	0.812			
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.7 (0.5–0.9)	0.433			
Initial telaprevir dose (1500/2250 mg)	52/16	17/4	0.460			
Initial PEG IFN dose (1.5/<1.5 μg/kg)	58/10	17/4	0.430			
Initial RBV dose (mg/kg)†	9.9 (2.2–15.5)	9.5 (4.4–12.5)	0.546			
IL28B gene (rs8099917) (TT/non-TT/ND)	43/15/10	8/7/6	0.107			
Core 70 a.a. mutation (wild/mutant/ND)	36/16/16	7/8/6	0.108			
Previous treatment (naive/relapse/NVR)	34/28/6	6/10/5	0.095			
Rapid virological response (+/−)	60/8	10/11	<0.001	10.89	(2.838–41.83)	0.001
Grade 3 DAR (−/+)	66/2	14/7	<0.001	27.44	(3.718–202.5)	0.001

†Data are shown as median (range) values.
a.a., amino acid; ALT, alanine transaminase; CI, confidence interval; Cr, creatinine; DAR, dermatological adverse reaction; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; NVR, non-virological response; OR, odds ratio; PEG IFN, pegylated interferon; SVR, sustained virological response; RBV, ribavirin.

granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of patients with grade 1 or no dermatological reactions (both $P < 0.05$, Fig. 2).

Time-dependent changes in serum granulysin levels

We investigated the time-dependent changes in serum granulysin levels in five and 15 patients with grade 3 and non-grade 3 dermatological reactions, respectively (Fig. 3). Serum granulysin levels of patients with non-grade 3 dermatological reactions never exceeded 10 ng/ml. Of the five patients with grade 3 reactions, three had severe systemic manifestations that necessitated hospital admission: one each had SJS, DIHS, and systemic lymphoid swelling and high fever (>39 °C). All patients with grade 3 dermatological reactions with systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL; importantly, the serum granulysin levels of two patients already exceeding 8 ng/mL at the onset of the reactions worsened within 6 days.

DISCUSSION

THE PRESENT STUDY demonstrates a significant association between telaprevir-induced dermatological reactions and elevated serum granulysin levels for the first time. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Thus, the results indicate that serum granulysin level seems to be a useful predictor of telaprevir-induced dermatological reactions. Because the emergence of grade 3 dermatological reactions was significantly associated with non-SVR (Table 3), probably associated with high rate of treatment discontinuation, it is important to predict dermatological events in the early stage to achieve good treatment outcomes.

Table 4 Comparison of the clinical and laboratory characteristics of the patients based on the presence or absence of at least a grade 3 dermatological adverse event

All patients $n = 89$	Non-grade 3 $n = 80$	Grade ≥3 $n = 9$	Univariate analysis P
Age (years)†	60 (19–73)	61 (48–65)	0.453
Sex (male/female)	40/40	8/1	0.027
Bodyweight (kg)†	62 (32–97)	64 (51–87)	0.593
Baseline white blood cell count (/μL)†	4900 (1500–9800)	4700 (3000–7000)	0.876
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)	14.4 (12.1–15.4)	0.196
Baseline platelet count ($\times 10^3$)†	16.0 (6.6–86.0)	13.5 (10.4–22.5)	0.605
Baseline ALT level (IU/L)†	40(15–300)	37 (23–87)	0.765
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.8 (0.6–0.9)	0.123
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.6 (3.2–7.6)	6.4 (5.7–7.1)	0.465
Initial telaprevir dose (1500/2250 mg)	62/18	7/2	0.675
Initial telaprevir/bodyweight (mg/kg)	33.7 (20–71.4)	30.0 (23.6–44.1)	0.563
Initial PEG IFN dose (1.5/<1.5 μg/kg)	66/14	9/0	0.198
Initial RBV dose (mg/kg)†	9.7 (2.2–15.5)	10.7 (7.7–12.9)	0.161
IL28B gene (rs8099917) (TT/non-TT/ND)	47/19/14	4/3/2	0.353
Core 70 a.a. mutation (wild/mutant/ND)	38/22/20	5/2/2	0.511
Previous treatment (naïve/relapse/NVR)	35/36/9	5/2/2	0.972
Onset of dermatological AE (days)	5 (1–75)	22 (1–60)	0.352

†Data are shown as median (range) values.
a.a., amino acid; AE, adverse event; ALT, alanine transaminase; Cr, creatinine; HCV, hepatitis C virus; IL28B, interleukin 28B; NVR, non-virological response; PEG IFN, pegylated interferon; RBV, ribavirin.

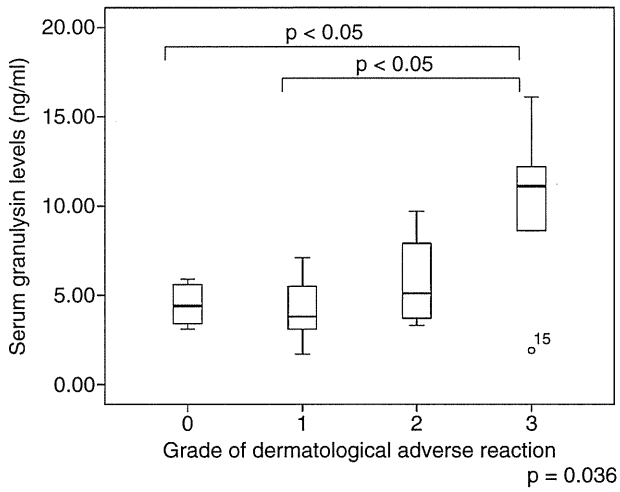

Figure 2 Association between dermatological adverse reaction severity and serum granulysin level. Serum granulysin levels were measured at the onset of dermatological reactions (i.e. within 3 days of onset); if the symptoms worsened, the time of worsening was adopted. In patients with no dermatological events, the highest serum granulysin level during treatment was adopted. $P < 0.05$, one-way ANOVA.

Recent genome-wide association studies have identified that genetic polymorphisms around the IL28B gene locus significantly associated with the outcome of PEG IFN and RBV combination therapy in HCV patients. Thus, PEG IFN and RBV combination therapy is ineffective in a subset of HCV-infected patients who have IL28B TG or GG genotypes, limiting the use of this therapy.[16] Therefore, novel drugs with different antiviral mechanisms were required. Accordingly, DAA were developed; they are mainly classified as NS3/4A protease inhibitors, or NS5B or NS5A inhibitors.[17] The NS3/4A serine protease inhibitor telaprevir, in combination with PEG IFN and RBV, has demonstrated the most promising results.[6–8] However, adverse events, especially severe dermatological reactions, develop more frequently in patients treated with telaprevir than those treated with only PEG IFN and RBV.

Little is known about the mechanisms of telaprevir-induced dermatological reactions. Reactions develop in patients treated with PEG IFN and RBV combination therapy[18,19] as well as telaprevir monotherapy.[20,21] It should be noted that the dermatological reactions in telaprevir monotherapy or PEG IFN and RBV therapy alone are generally mild.[7,8,20] However, dermatological

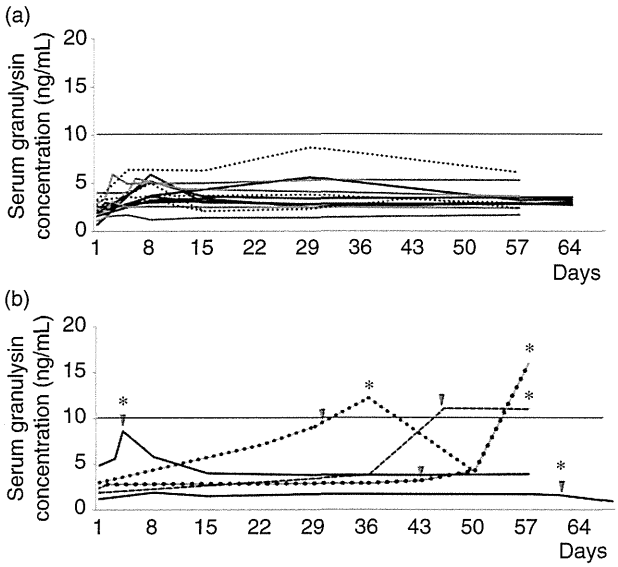

Figure 3 Association between time-dependent changes in serum granulysin levels and severe telaprevir-induced dermatological adverse reactions. (a) Time-dependent changes in serum granulysin levels patients with non-grade 3 dermatological reactions (three, five and six with grade 2, grade 1 and no reactions, respectively). The dashed line, gray line and black line indicate grade 2, grade 1 and no reaction, respectively. (b) Time-dependent changes in serum granulysin levels of five patients with grade 3 dermatological events. The dashed line indicates patients with severe systemic manifestations. Arrowheads indicate the onset of dermatological events and asterisks indicate the onset of grade 3 dermatological events.

reactions in telaprevir and PEG IFN/RBV combination therapy may be severe, indicating a synergistic effect. Severe dermatological events including SJS/TEN and DIHS have been reported in telaprevir-based triple therapy; these are life-threatening, and fatal cases have been reported.

The onset of grade 3 dermatological reactions tended to be later than non-grade 3 reactions, the same as in the study of Torii *et al.*[10] Taken together with the finding that male sex is a clinical risk factor, the results indicate that late-onset dermatological reactions in male patients treated with telaprevir-based triple therapy require more attention.

Roujeau *et al.* analyzed the risk factors for telaprevir-induced eczematous dermatitis and report that the incidence of telaprevir-related dermatitis was significantly higher age of more than 45 years, body mass index of less than 30 (kg/m^2), Caucasian ethnicity and treatment-naïve status.[9] While they analyzed the risk factors for telaprevir-induced eczematous dermatitis, the present study focused on the risk factors for severe telaprevir-induced dermatological reactions, because such reactions can affect treatment outcome (Table 2) and can be fatal. As mentioned above, male sex was significantly associated with grade 3 dermatological reactions. Sex is reported to be associated with the prevalence of some kinds of severe drug-induced dermatological events, although the underlying mechanism remains unknown.[22]

Fujita *et al.* report that serum granulysin levels are significantly elevated in SJS/TEN patients and thus may be a good predictive factor.[14] Therefore, we hypothesized that in telaprevir-based triple therapy for chronic hepatitis C patients, serum granulysin levels are associated with the severity of dermatological reactions and may thus be a predictive biomarker. However, Ogawa *et al.* report that serum granulysin levels also increase as a result of primary virus infections such as Epstein–Barr virus or parvovirus B19.[12] Thus, it remains unclear whether and how chronic viral infections, especially HCV, affect serum granulysin levels. In the present study, we compared serum granulysin levels between healthy volunteers and chronic hepatitis C patients; the results show that chronic HCV infection was not associated with serum granulysin levels (Fig. 1).

Chung *et al.* have reported that granulysin is the most highly expressed cytotoxic molecule in blisters of SJS/TEN and that massive keratinocyte death was induced by granulysin.[11] Fujita *et al.* reported that serum granulysin levels increased in the early stage of SJS/TEN caused by drugs including carbamazepine, imatinib and phenytoin.[14] Taken together with our results, we speculate that granulysin may be involved in the pathogenesis of early stage telaprevir-mediated dermatological adverse reactions possibly through induction of keratinocyte death.

Of five patients with grade 3 reactions, two patients without severe systemic manifestations did not have elevated serum granulysin of more than 10 ng/mL or did not have elevated levels before symptoms worsened. On the contrary, three patients with severe systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL, and the symptoms of two patients with serum granulysin levels already exceeding 8 ng/mL at onset and within 6 days worsened. Therefore, serum granulysin tests may predict grade 3 dermatological adverse reaction with systemic manifestations. Furthermore, if serum granulysin levels elevate more than 8 ng/mL, more attention should be paid.

In Western countries, the prevalence of dermatological reactions in patients treated with telaprevir-based and

PEG IFN/RBV therapy are reported to be approximately 55% and 33%, respectively;[9,23] meanwhile, in Japanese patients, the respective rates are 74.9% and 58.7%. Moreover, approximately 4% and 9% of patients in Western and Japanese patients develop grade 3 reactions, respectively;[10] this is almost the same as that in the present study (10%). The difference may be due to genetic or ethnic variation. Therefore, genome-wide association studies may have identified a gene locus associated with telaprevir-induced severe dermatological reactions.

A limitation of this study is that the number of patients with grade 3 dermatological reactions is relatively small. However, the serum granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of other patients. Also, in two of the three patients with severe dermatological reactions, the serum granulysin level elevated before symptoms worsened, which are novel findings. Further study is required.

Triple therapy with the second-generation protease inhibitor simeprevir is reported to result in a similar prevalence of adverse reactions as PEG IFN and RBV combination therapy.[24,25] However, simeprevir is not approved worldwide. Although simeprevir-based triple therapy is effective, only 36–53% of prior non-responders achieve SVR.[24] Shimada *et al.* recently reported that by extending PEG IFN and RBV therapy from 24 to 48 weeks, telaprevir-based triple therapy improves the SVR to up to 68% in prior null responders.[26] Thus, telaprevir is a therapeutic option for prior null responders.

In conclusion, the present study suggests that male sex is a significant risk factor for severe telaprevir-induced dermatological reactions. In addition, serum granulysin levels are significantly associated with the severity of dermatological reactions and thus may be a good predictor of severe dermatological reactions with systemic manifestations in patients treated with telaprevir-based triple therapy.

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PLOS ONE

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* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; 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 ($P = 1.55 \times 10^{-7}$; 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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