groups (Fig. 3C). As a result, viral decline was somewhat greater after day 28 in the add-on therapy group but the difference was not significant.

Effect of Amino Acid Substitutions of HCV Core 70 on Viral Response

Add-on therapy was significantly more effective in patients with IL28B minor variation (TG + GG) compared with standard therapy, therefore, we investigated the association between HCV core 70 amino acid mutation and therapeutic outcome. In 27 patients infected with HCV-1b, who had minor variation of IL28B (TG + GG) and were treated with add-on therapy, core 70 amino acid mutation was determined. Sustained virological response was achieved in 10 patients and core 70 mutation (Gln) was found in 6 of the 10 patients (60%). Within the 17 non-sustained virological response patients, the mutation was identified in eight patients (47.1%). Accordingly, within these patients, the core 70 amino acid substitutions did not affect sustained virological response in the add-on therapy.

Predictive Factors Associated With Sustained Virological Response

Among the factors listed in Table III, predictive factors associated with sustained virological response were examined in patients infected with HCV-1b. Univariate analysis identified six parameters that correlated significantly with sustained virological response; age ($P = 0.0038$), fibrotic staging ($P = 0.0012$), γ-glutamyl transpeptidase ($P = 0.0009$), platelet count ($P = 0.0132$), genetic variation of IL28B ($P < 0.0001$) and add-on therapy ($P < 0.0001$; Table III). In multivariate analysis, significant contribution factors for sustained virological response were age (<60 years; OR 3.06, $P = 0.0221$), IL28B (genotype TT; OR 6.69, $P = 0.0019$) and staging (F_{0-1}; OR 5.71, $P = 0.0035$; Table IV). When IL28B was excluded from the factors in multivariate analysis, addition of pitavastatin and EPA (add-on therapy) was also selected as a significant contribution factor for sustained virological response (OR 2.13, $P = 0.0395$).

TABLE IV. Multivariate Analysis for Predictive Factors Associated With Sustained Virological Response

Factors	Category	95% confidence intervals	P
Age (years)	1. ≥60: 1.0		
	2. <60: 3.06	1.20–8.24	0.0221
IL-28B (rs8099917)	1. TG + GG: 1.0		
	2. TT: 6.69	2.17–24.66	0.0019
Staging	1. F_{2-3}: 1.0		
	2. F_{0-1}: 5.71	1.91–20.51	0.0035

Subgenomic HCV Replicon System

Suppression of HCV RNA replication by pitavastatin and/or EPA was examined in Huh7/Rep-Feo-1b cells by luciferase assay. The concentrations of pitavastatin and EPA for the following experiments were determined according to previous studies [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006] and our pilot study for cytotoxicity and luciferase assay (data not shown). Huh7/Rep-Feo-1b cells were incubated with or without 10 μM pitavastatin and/or 20 μM EPA for 48 hr. As a precondition, the proliferative activity and viability of pitavastatin- and/or EPA-treated cells were comparable with those of control cells (data not shown). As a result, luciferase activity was significantly suppressed in EPA- and/or pitavastatin-treated cells compared with the control cells (Fig. 4A). At these concentrations, the suppressive effect was more marked in pitavastatin-treated than EPA-treated cells.

TABLE III. Univariate Analysis Between Non-Sustained Virological Response and Sustained Virological Response Groups

Factors	Non-SVR	SVR	P
Gender (M/F)	82/86	55/54	NS
Age (years)	60.5 ± 10.6	55.7 ± 12.0	0.0038
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	136/24/8	88/15/6	NS
HCV RNA (log IU/ml)	6.03 ± 0.16	5.91 ± 0.55	NS
IL-28B (rs8099917) TT/TG + GG/ND	35/44/89	12/64/33	<0.0001
ITPA (rs1127354) CC/CA + AA/ND	59/20/89	54/21/34	NS
Staging (F_{0-1}/F_{2-3}/ND)	11/59/98	31/41/37	0.0012
Treatment add-on/standard	45/123	56/53	<0.0001
ALT (IU/l)	72.3 ± 57.7	63.9 ± 45.2	NS
GGT (IU/l)	65.3 ± 56.0	41.1 ± 27.1	0.0009
WBC (/μl)	4,935 ± 1,392	4,791 ± 1,254	NS
Hemoglobin (g/dl)	13.8 ± 1.4	13.8 ± 1.4	NS
Platelet (/μl)	16.7 ± 5.7	19.1 ± 6.6	0.0132
% of patients treated with enough total doses of Peg-IFN[a]	60.9	74.4	NS
% of patients treated with enough total doses of ribavirin[b]	71.9	80.8	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.
[a]Enough total dose: >80% of planned doses.
[b]Enough total doses: >60% of planned doses.

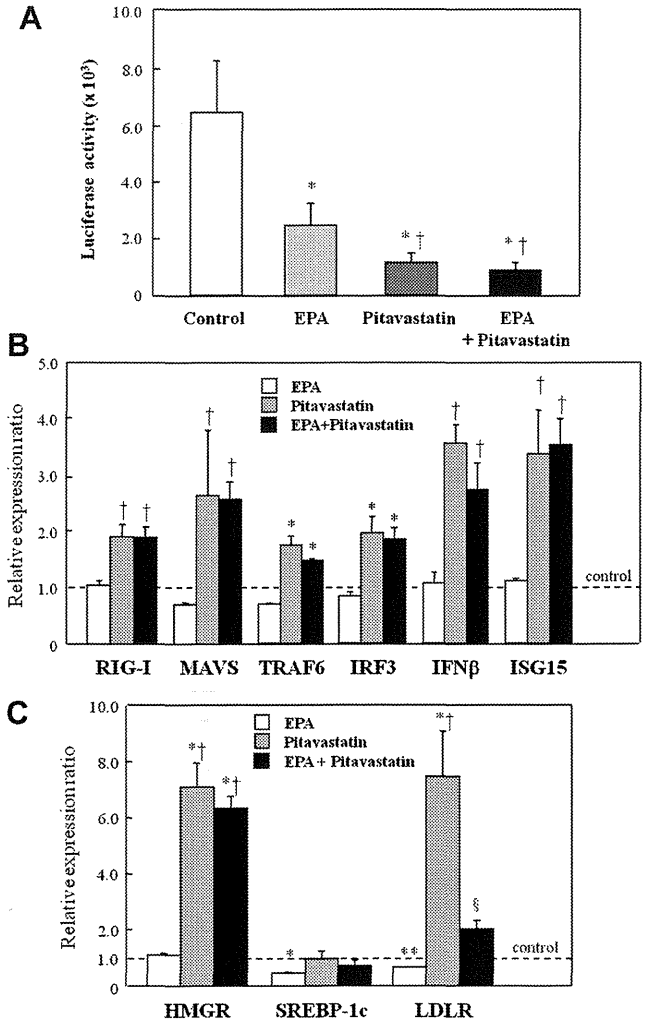

Fig. 4. Treatment of pitavastatin and/or EPA in HCV replicon cells. **A**: HCV replication was estimated by luciferase assay. Huh7/Rep-Feo-1b cells were treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{\dagger}P < 0.01$ versus EPA. **B**: Expression levels of RIG-I, MAVS, TRAF6, IRF3, IFNβ, and ISG15 genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control and EPA, $^{\dagger}P < 0.05$ versus control and EPA. **C**: Expression levels of HMGR, SREBP-1c and LDLR genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{**}P < 0.05$ versus control, $^{\dagger}P < 0.01$ versus EPA, $^{\S}P < 0.01$ versus pitavastatin.

In Huh7/Rep-Feo-1b cells, the expression levels of innate-immunity-associated genes were examined after 48 hr treatment with 10 μM pitavastatin and/or 20 μM EPA. As shown in Figure 4B, retinoic acid inducible gene I (RIG-I), mitochondrial antiviral signaling (MAVS), TNF receptor associated factor 6 (TRAF6), IFN regulatory factor 3 (IRF3), IFNβ and IFN-stimulated gene 15 (ISG15) showed similar trend in expression. Accordingly, their expression was significantly increased by pitavastatin but not by EPA, and EPA did not show an additive effect with pitavastatin. With the same treatments, expression of lipid-metabolism-associated genes was analyzed (Fig. 4C). HMG-CoA reductase (HMGR) expression was significantly enhanced by pitavastatin but not by EPA. The sterol regulatory element binding protein 1c (SREBP-1c) expression was significantly suppressed by EPA but not by pitavastatin. Low-density lipoprotein receptor (LDLR) expression was significantly suppressed by EPA, whereas the expression was activated by pitavastatin, but the activation was lost in the presence of EPA.

DISCUSSION

For ethical reasons, standard therapy could not be selected after 2008; therefore, the present study was unable to eliminate some methodological issues that limit the interpretation and drawing of firm conclusions. For example, the percentage of patients receiving sufficient total dose of peg-IFN was lower in the historical standard group although the difference was not significant and, in order to prevent dose reduction, additional means might have been performed on the add-on group after 2008. However, in univariate and multivariate analyses, total dose of peg-IFN was not detected as a significant factor for sustained virological response. Nevertheless under these limitations, the presented clinical and in vitro studies indicate some sufficient trends in treatment response.

Previous studies on hepatic lipid metabolism have shown that, in the liver of patients with HCV infection, synthesis of cholesterol and fatty acids is still activated, regardless of overaccumulation of lipids [Kohjima et al., 2009; Nakamuta et al., 2009, 2011; Fujino et al., 2010]. This means that addition of pitavastatin and EPA to standard therapy is pathophysiologically reasonable for patients with chronic hepatitis C. Sustained virological response rates in patients infected with HCV-2 were sufficiently high and comparable between the standard and add-on therapy groups (Fig. 1A). Therefore, this study was focused on patients infected with HCV-1b with high virus load. This investigation of sustained virological response in patients treated with add-on or standard therapy had two clinically important findings.

First, add-on therapy led to significantly higher sustained virological response rates than did standard therapy (Fig. 1A). Although overall sustained virological response rate in this study was lower compared with the results from some other institutions, it may be because intention to treat analysis was used in this study and the ratio of IL28B minor (TG + GG) patients was higher in the standard and add-on groups. When sustained virological response rates were compared only in patients with IL28B major or in those with IL28B minor, the sustained virological response rates were not lower compared with those in other reports (data not shown). The suppressive effect against HCV replication by statins and EPA, and their synergistic action with IFN, has already been demonstrated in some HCV replicon systems [Ye

et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007; Ikeda and Kato, 2007]. In our investigation using the luciferase assay in Huh7/Rep-Feo-1b cells, a similar suppressive effect was seen with both pitavastatin and EPA treatments (Fig. 4A). It has been reported that the statins impede HCV replication through inhibition of host protein geranylgeranylation and FBL2 has been identified as a geranylgeranylated cellular protein required for HCV RNA replication [Wang et al., 2005; Nakamuta et al., 2011]. PUFAs, including EPA, inhibit HCV replication, although the precise mechanism is still unclear but may be independent of the route regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005]. The synergistic and additive effect of EPA with pitavastatin was not significant in our luciferase assay; therefore, statins and EPA may act against cognate targets.

Second, the add-on therapy improved sustained virological response rates especially in patients with the minor type variation (TG + GG) of the IL28B gene (rs8099917), in whom sustained virological response is expected to be poor after standard therapy (Fig. 1B). Recent studies have revealed that SNPs within or adjacent to IL28B region provide a strong predictive value for the outcome of IFN-based therapy in patients infected with HCV-1b [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. With add-on therapy, sustained virological response rates were significantly higher in patients with major type (TT) than minor type (TG + GG) variations, meaning that genome variation of IL28B (rs8099917) still governs the outcome even in add-on therapy. However, in the patients with minor type variation, the sustained virological response rate (37.9%) in the add-on group was markedly higher compared with that in the standard therapy group (Fig. 1B). This sustained virological response rate may be sufficiently high for clinical use of add-on therapy in patients infected with HCV-1b with minor type variation. From this point of view, the add-on therapy is a clinically valuable strategy for chronic hepatitis C. In the analysis of viral dynamics in patients with minor type variation of IL28B, no significant difference was found in viral decline within 84 days between the standard and add-on groups (Fig. 3C). Although there is still no evidence, in the add-on therapy, late phase viral decline (3 months after treatment initiation) may be more important for the achievement of sustained virological response in patients with minor type variation.

It has been emphasized that mutation of amino acids 70 and 91 in the core region of HCV-1b as a virus-related factor, as well as genome variation of IL28B gene as a host-related factor, greatly influences the outcome of IFN-based antiviral treatments. According to recent clinical studies in patients infected with HCV-1b, substitution of core 70 is assessed as a more influential factor affecting the outcome of peg-IFN plus ribavirin combination therapy, rather than that of core 91 [Akuta et al., 2007; Hayes et al., 2011; El-Shamy et al., 2012]. Even in the latest triple therapy with peg-IFN, ribavirin and a NS3/4A protease inhibitor, telaprevir, patients infected with HCV-1b with core 70 mutation were reported to be severely resistant to the therapy [Akuta et al., 2010]. In our assessment of patients with minor type IL28B variation at rs8099917 (TG + GG), mutation at core 70 was likely not to diminish the outcome of add-on therapy, although the number of patients examined was small (Fig. 4). Therefore, the lipid modulators, pitavastatin and EPA, may be expected to be more effective for patients infected with HCV-1b with core 70 mutation, compared with an NS3/4A protease inhibitor. However, for reliable assessment, further clinical data are needed from patients treated with add-on therapy.

As part of its pathogenic strategy, HCV interferes with the innate immune response of its host; mainly in the RIG-I/MAVS pathway [Breiman et al., 2005; Tasaka et al., 2007; Baril et al., 2009; Jouan et al., 2010; Lemon, 2010; Liu and Gale, 2010; Ekisioglu et al., 2011]. RIG-I undergoes a conformational change upon HCV RNA binding and interacts with MAVS, resulting in phosphorylation and nuclear translocation of IRF3, which leads to transcriptional activation and synthesis of IFNβ. IFNβ activates the Jak–STAT (Janus kinase–signal transducer and activator of transcription) signaling pathway and acts through the expression of ISGs. TRAF6 is recruited to the MAVS complex and is required for activation of nuclear factor-κB, which forms an enhanceosome on the IFNβ promoter in coordination with IRF3. In HCV-infected cells, NS3/4A protease cleaves MAVS, and the RIG-I/MAVS pathway is impeded. In the present study, the HCV replicon system was used to examine how pitavastatin and EPA influence the RIG-I/MAVS pathway, which plays an important role in the innate antiviral host response to HCV infection. The expression profile of innate-immunity-associated genes in pitavastatin- and/or EPA-treated Huh7/Rep-Feo-1b cells showed that only pitavastatin activated expression of the tested genes, RIG-I, MAVS, IRF3, TRAF6, IFNβ, and ISG15, similarly (Fig. 4B). EPA treatment did not increase expression levels of these genes. It is unclear whether the activation of these innate-immunity-associated factors directly contributes to elimination of HCV or whether inhibition of HCV replication by pitavastatin treatment directly leads to the activation of innate immunity through lowering NS3/4A protease expression.

Cholesterol, fatty acids, and lipid rafts have been demonstrated to be critical for efficient replication, infection and secretion of HCV [Simons and Ehehalt, 2002; Kushner et al., 2003]. For example, HCV replication was suppressed by inhibition of the liver X receptor α–SREBP-1c pathway [Kapadia and Chisari, 2005]. Therefore, negative modulation of lipid synthesis may be an antiviral step of statins and EPA. In pitavastatin treatment of HCV replicon cells, HMGR

and LDLR expression was enhanced in response to inhibition of cholesterol synthesis, whereas EPA decreased the expression of SREBP-1c and LDLR, by which fatty acid synthesis and cholesterol uptake might be lowered (Fig. 4C). Of note, although pitavastatin alone enhanced the expression of LDLR, the enhancement was abolished by addition of EPA (Fig. 4C). This effect of EPA indicates the clinical significance of the add-on therapy because LDLR is known to be an important cellular factor that is required for cell entry/infection of HCV. EPA addition is expected to accelerate the antiviral effect of peg-IFN, ribavirin and pitavastatin through repression of HCV entry/infection as well as HCV replication. It has recently been reported that HCV particles are enriched in cholesterol and virion cholesterol is involved in HCV cell entry, depending on Niemann-Pick C1-like 1 (NPC1L1), which is an HCV cell entry factor as well as a cellular cholesterol uptake receptor [Yamamoto et al., 2011; Sainz et al., 2012]. The NPC1L1 may be amenable to therapeutic intervention.

The analysis of viral dynamics during add-on therapy indicated that early phase viral decline within the first 2 weeks influenced the achievement of sustained virological response in patients with major type variation (TT) but not in those with minor type variation (TG + GG; Fig. 3A,B). It cannot be explained clearly why high sustained virological response rates were obtained in patients infected with HCV-1b with minor type variation, regardless of poorer viral decline with add-on therapy. Although there is still no evidence, in patients with minor type variation, statins and EPA may show their effect in a later phase, and the EPA effect of impeding HCV entry/infection through suppression of LDLR expression may contribute partly to the achievement of sustained virological response.

In univariate analysis, addition of pitavastatin and EPA, as well as genotype TT of IL28B at rs8099917, was positively associated with sustained virological response in peg-IFN plus ribavirin combination therapy (Table III). However, this association disappeared in a multivariate analysis, and IL28B variation remained as an independent factor. One of the major reasons may be that, compared with the addition of pitavastatin and EPA, TT variation of IL28B in the profile of individuals has overwhelming weight for governing the effect of peg-IFN plus ribavirin combination therapy. In our study, SNP variation of ITPA (rs1127354) did not influence treatment outcome but the planned dose of ribavirin was maintained well in patients with minor type variation (non-CC), as reported in previous studies (Fig. 2).

In conclusion, the lipid modulators, pitavastatin and EPA, could enhance the efficacy of peg-IFN plus ribavirin combination therapy through their synergistic antiviral effect, particularly in patients infected with HCV-1b with an intractable IL-28B allele. Although the research is still in the preliminary stages, there is a possibility that addition of pitavastatin and EPA may be effective for HCV-1b with core 70 mutation, and may increase sustained virological response rates in patients treated with triple therapy of peg-IFN, ribavirin, and telaprevir.

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?
- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?
- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?
- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

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Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

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Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			
				Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

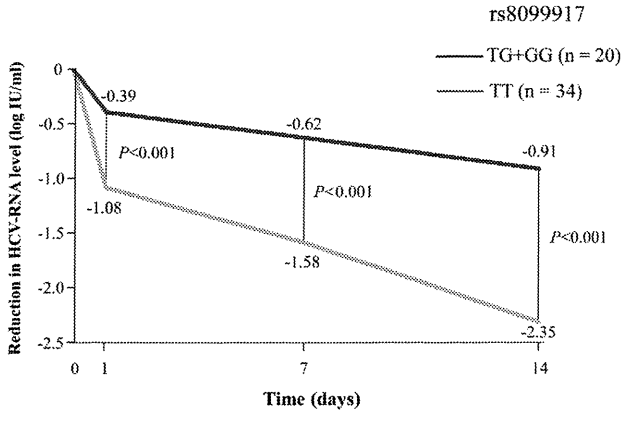

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

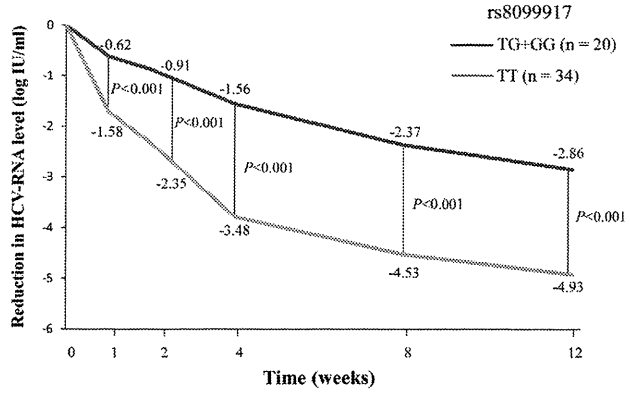

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

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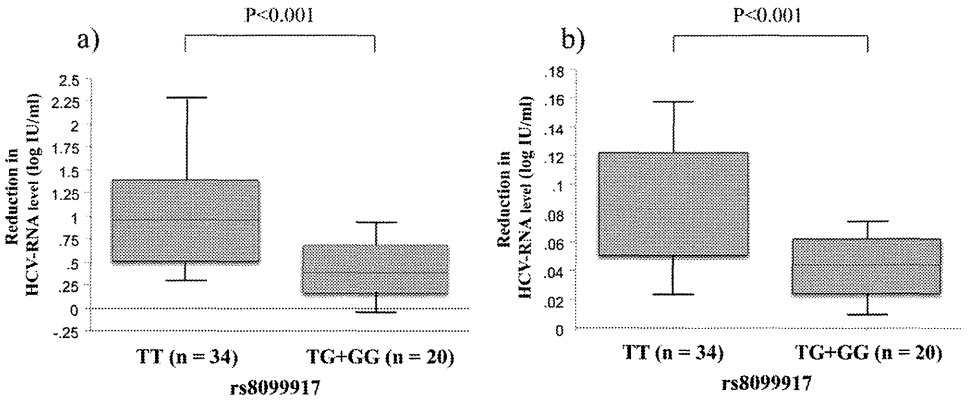

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

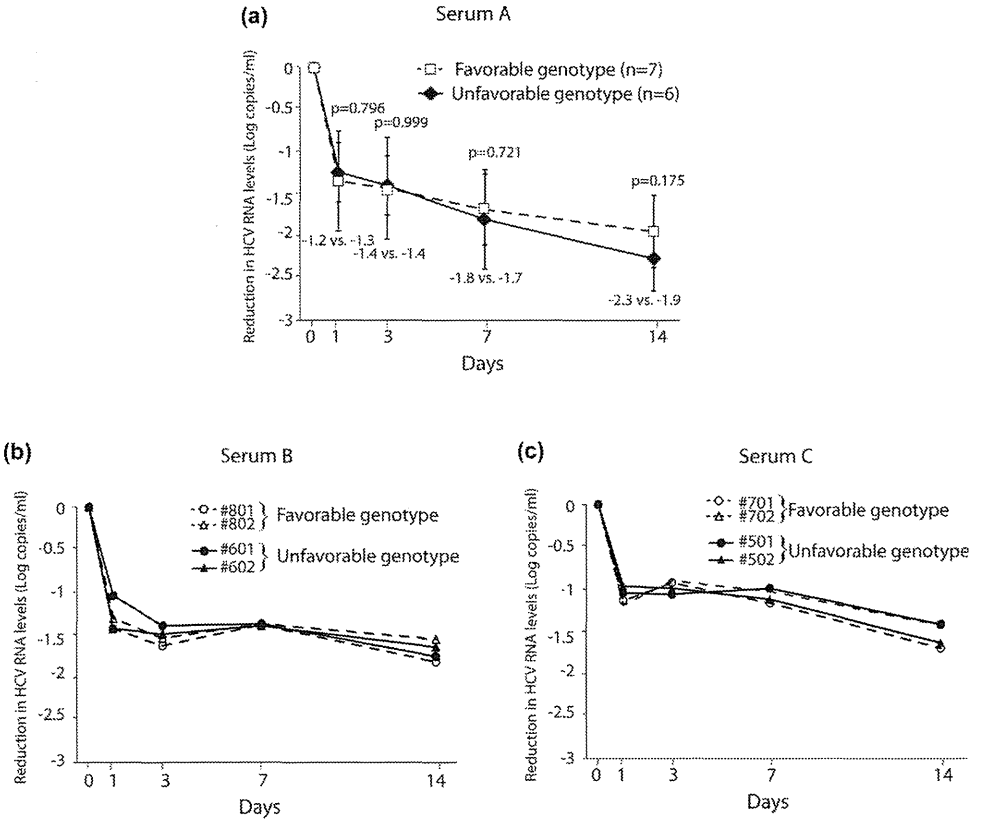

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

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(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

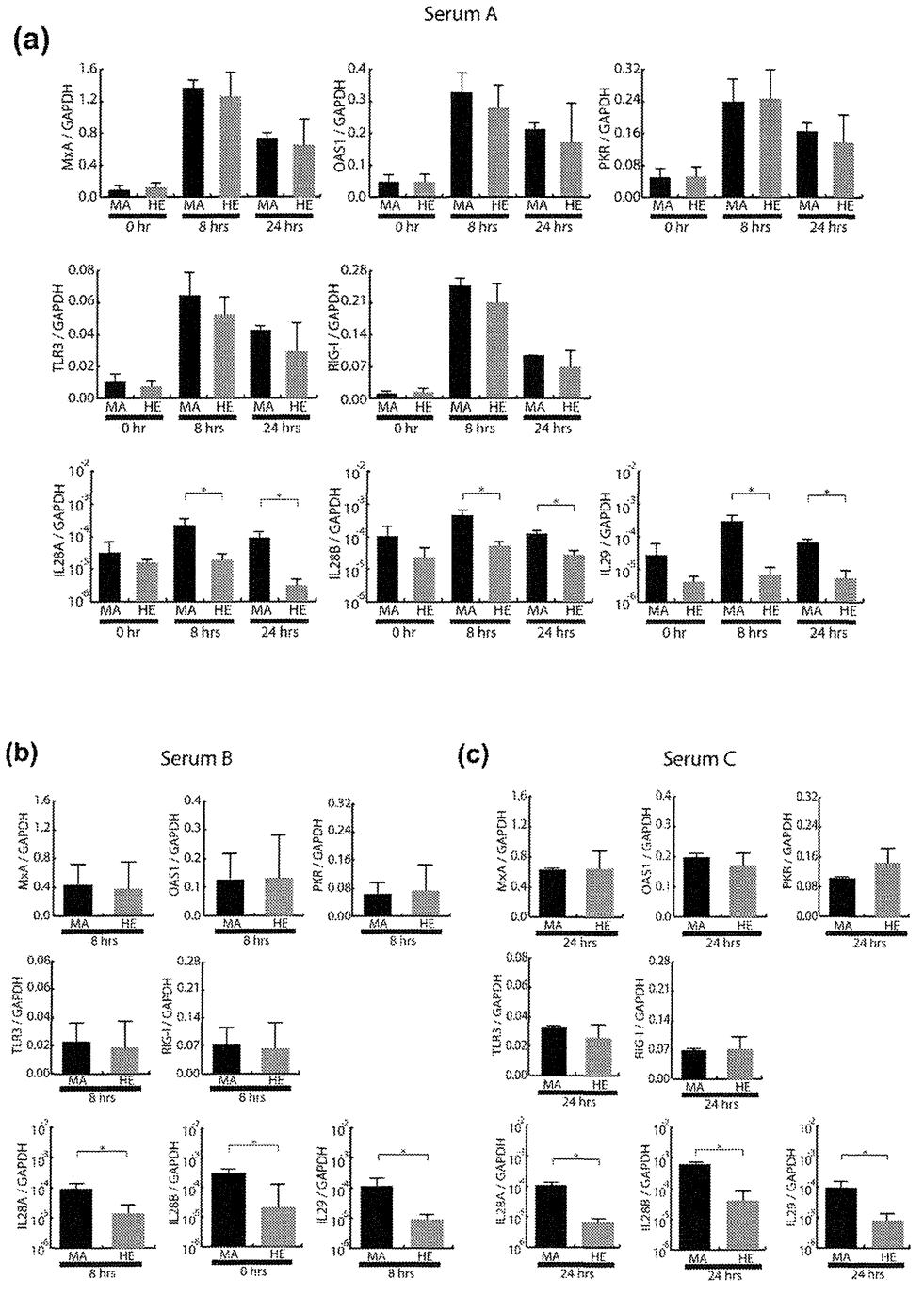

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

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expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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Evolutionary Analysis of Classical *HLA* Class I and II Genes Suggests That Recent Positive Selection Acted on *DPB1*04:01* in Japanese Population

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Abstract

The human leukocyte antigen (*HLA*) genes exhibit the highest degree of polymorphism in the human genome. This high degree of variation at classical *HLA* class I and class II loci has been maintained by balancing selection for a long evolutionary time. However, little is known about recent positive selection acting on specific *HLA* alleles in a local population. To detect the signature of recent positive selection, we genotyped six *HLA* loci, *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DQB1*, and *HLA-DPB1* in 418 Japanese subjects, and then assessed the haplotype homozygosity (*HH*) of each *HLA* allele. There were 120 *HLA* alleles across the six loci. Among the 80 *HLA* alleles with frequencies of more than 1%, *DPB1*04:01*, which had a frequency of 6.1%, showed exceptionally high *HH* (0.53). This finding raises the possibility that recent positive selection has acted on *DPB1*04:01*. The *DPB1*04:01* allele, which was present in the most common 6-locus *HLA* haplotype (4.4%), *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01*, seems to have flowed from the Korean peninsula to the Japanese archipelago in the Yayoi period. A stochastic simulation approach indicated that the strong linkage disequilibrium between *DQB1*06:04* and *DPB1*04:01* observed in Japanese cannot be explained without positive selection favoring *DPB1*04:01*. The selection coefficient of *DPB1*04:01* was estimated as 0.041 (95% credible interval 0.021–0.077). Our results suggest that *DPB1*04:01* has recently undergone strong positive selection in Japanese population.

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Introduction

The crucial immunological function of human leukocyte antigen (HLA) molecules is to present pathogen-derived antigenic peptides to T lymphocytes [1]. The HLA proteins are encoded by genes in the major histocompatibility complex region, which spans approximately 4 megabases (Mb) on the short arm of chromosome 6 (6p21.3) and includes the most polymorphic loci in the human genome [2]. A remarkable feature of the classical *HLA* class I and class II genes is the high degree of polymorphism. More than 1,750 *HLA-A*, 2,330 *HLA-B*, 1,300 *HLA-C*, 1,060 *HLA-DRB1*, 160 *HLA-DQB1*, and 150 *HLA-DPB1* alleles have been reported (IMGT/HLA database; http://www.ebi.ac.uk/imgt/hla/).

Positive selection has been shown as a driving force for the high degree of polymorphism at *HLA* loci [3,4]. The *HLA* genes show three remarkable signatures of positive selection: (1) the rate of nonsynonymous (amino acid altering) nucleotide substitution is substantially higher than that of synonymous substitution at antigen-recognition sites [5,6], (2) there are trans-species polymorphisms (i.e., similar alleles are present in multiple species) [7], and (3) there is a significant excess of heterozygosity [8,9]. Balancing selection, including overdominant selection and frequency-dependent selection, can easily account for these observations [3,4].

A number of studies have reported common long-range *HLA* haplotypes [10–16]. The extended length of common haplotype is a key feature of recent positive selection [17,18]. The *HLA* alleles on long-range haplotypes may have been subject to recent positive selection. In this study, to identify the signature of recent positive selection that has acted on specific *HLA* alleles in a local (i.e., geographically restricted) population, we investigated the allele frequencies and haplotype frequencies at *HLA-A*, *HLA-C*, *HLA-B*, *HLA-DRB1*, *HLA-DQB1*, and *HLA-DPB1* in 418 Japanese individuals. Our theoretical and computer simulation analyses suggested that *DPB1*04:01* has recently undergone strong positive selection in Japanese population.

Results

HLA Class I and Class II Alleles in Japanese

The genotypes of six *HLA* genes (three class I and three class II genes) were determined for each of 418 Japanese individuals. The frequencies of the 67 alleles found at the three *HLA* class I genes are listed in Table 1. Of the 17 *HLA-A* alleles, two–*A*02:01* and *A*24:02*–had frequencies higher than 10% (10.2 and 37.7 percent,

respectively). Of the 17 *HLA-C* alleles, four–*C*01:02*, *C*03:03*, *C*03:04*, and *C*07:02*–had frequencies higher than 10%: 16.5, 13.5, 12.6, and 14.5 percent, respectively. There were 33 *HLA-B* alleles, and not one had an allele frequency greater than 10%. The allele with the highest frequency (9.6%) was *B*52:01*; this allele was followed by *B*15:01* (8.5%), *B*51:01* (8.5%), *B*4403* (8.1%), and *B*35:01* (8.0%).

The frequencies of 53 alleles at three *HLA* class II genes are listed in Table 2. Of the 27 alleles at the *HLA-DRB1* locus, two–*DRB1*09:01* and *DRB1*04:05*–had frequencies of more than 10% (15.2% and 14.6%, respectively), and five–*DRB1*15:02* (8.4%), *DRB1*15:01* (8.0%), *DRB1*13:02* (7.8%), *DRB1*08:03* (7.5%), and *DRB1*01:01* (6.8%)–were also common. Of the 14 alleles at *HLA-DQB1*, four–*DQB1*03:03*, *DQB1*06:01*, *DQB1*04:01*, and *DQB1* 03:01*–were observed at frequencies of greater than 10% (15.9%, 15.9%, 14.6%, and 11.8%, respectively). There were four other common alleles at *HLA-DQB1–DQB1*03:02* (9.2%), *DQB1*06:02* (7.8%), *DQB1*05:01*, and *DQB1*06:04* (7.5%). Of the six *HLA* loci genotyped, *HLA-DPB1* had the fewest alleles with just 12. The *DPB1*05:01* (38.5%) and *DPB1*02:01* (25.1%) alleles were the most frequent alleles at this locus.

Of the six *HLA* loci examined, the *HLA-B* locus showed the highest heterozygosity (0.937), and *HLA-DPB1* showed the lowest (0.765) (Tables 1 and 2). None of the *HLA* class I or II loci

Table 1. Frequencies of *HLA* class I alleles.

HLA-A						HLA-C						HLA-B					
Allele	Count	Freq.	H[a]	HWE[b]	EW[c]	Allele	Count	Freq.	H[a]	HWE[b]	EW[c]	Allele	Count	Freq.	H[a]	HWE[b]	EW[c]
				P-val	P-val					P-val	P-val					P-val	P-val
*A*01:01*	10	0.012	0.810	0.667	0.294	*C*01:02*	138	0.165	0.891	0.919	0.003	*B*07:02*	57	0.068	0.937	0.286	0.002
*A*02:01*	85	0.102				*C*01:03*	4	0.005				*B*13:01*	13	0.016			
*A*02:06*	61	0.073				*C*03:02*	3	0.004				*B*15:01*	71	0.085			
*A*02:07*	23	0.028				*C*03:03*	113	0.135				*B*15:07*	5	0.006			
*A*02:10*	2	0.002				*C*03:04*	105	0.126				*B*15:11*	5	0.006			
*A*03:01*	4	0.005				*C*04:01*	42	0.050				*B*15:18*	13	0.016			
*A*03:02*	1	0.001				*C*05:01*	5	0.006				*B*15:27*	1	0.001			
*A*11:01*	80	0.096				*C*06:02*	7	0.008				*B*15:28*	1	0.001			
*A*24:02*	315	0.377				*C*07:02*	121	0.145				*B*27:04*	2	0.002			
*A*24:08*	1	0.001				*C*07:04*	7	0.008				*B*35:01*	67	0.080			
*A*24:20*	10	0.012				*C*08:01*	47	0.056				*B*37:01*	7	0.008			
*A*26:01*	67	0.080				*C*08:03*	12	0.014				*B*39:01*	34	0.041			
*A*26:02*	12	0.014				*C*12:02*	81	0.097				*B*39:04*	5	0.006			
*A*26:03*	22	0.026				*C*12:03*	1	0.001				*B*40:01*	46	0.055			
*A*26:05*	1	0.001				*C*14:02*	50	0.060				*B*40:02*	57	0.068			
*A*31:01*	66	0.079				*C*14:03*	69	0.083				*B*40:03*	7	0.008			
*A*33:03*	76	0.091				*C*15:02*	31	0.037				*B*40:06*	34	0.041			
												*B*40:52*	1	0.001			
												*B*44:02*	5	0.006			
												*B*44:03*	68	0.081			
												*B*46:01*	38	0.045			
												*B*48:01*	22	0.026			
												*B*51:01*	71	0.085			
												*B*51:02*	4	0.005			
												*B*52:01*	80	0.096			
												*B*54:01*	64	0.077			
												*B*55:02*	20	0.024			
												*B*55:04*	1	0.001			
												*B*56:01*	5	0.006			
												*B*56:03*	2	0.002			
												*B*58:01*	3	0.004			
												*B*59:01*	16	0.019			
												*B*67:01*	11	0.013			

[a]Heterozygosity.
[b]Hardy-Weinberg equilibrium test.
[c]Ewens-Watterson test.
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Table 2. Frequencies of *HLA* class II alleles.

HLA-DRB1						*HLA-DQB1*						*HLA-DPB1*					
Allele	**Count**	**Freq.**	**H[a]**	**HWE[b]**	**EW[c]**	**Allele**	**Count**	**Freq.**	**H[a]**	**HWE[b]**	**EW[c]**	**Allele**	**Count**	**Freq.**	**H[a]**	**HWE[b]**	**EW[c]**
				P-val	P-val					P-val	P-val					P-val	P-val
*DRB1*01:01*	57	0.068	0.918	0.247	0.013	*DQB1*02:01*	1	0.001	0.885	0.222	0.001	*DPB1*02:01*	210	0.251	0.765	0.398	0.225
*DRB1*03:01*	1	0.001				*DQB1*03:01*	99	0.118				*DPB1*02:02*	35	0.042			
*DRB1*04:01*	10	0.012				*DQB1*03:02*	77	0.092				*DPB1*03:01*	36	0.043			
*DRB1*04:03*	24	0.029				*DQB1*03:03*	133	0.159				*DPB1*04:01*	51	0.061			
*DRB1*04:04*	2	0.002				*DQB1*04:01*	122	0.146				*DPB1*04:02*	83	0.099			
*DRB1*04:05*	122	0.146				*DQB1*04:02*	26	0.031				*DPB1*05:01*	322	0.385			
*DRB1*04:06*	28	0.033				*DQB1*05:01*	63	0.075				*DPB1*06:01*	5	0.006			
*DRB1*04:07*	1	0.001				*DQB1*05:02*	17	0.020				*DPB1*09:01*	65	0.078			
*DRB1*04:10*	12	0.014				*DQB1*05:03*	30	0.036				*DPB1*13:01*	12	0.014			
*DRB1*08:02*	32	0.038				*DQB1*06:01*	133	0.159				*DPB1*14:01*	10	0.012			
*DRB1*08:03*	63	0.075				*DQB1*06:02*	65	0.078				*DPB1*19:01*	5	0.006			
*DRB1*09:01*	127	0.152				*DQB1*06:03*	5	0.006				*DPB1*41:01*	2	0.002			
*DRB1*10:01*	6	0.007				*DQB1*06:04*	63	0.075									
*DRB1*11:01*	23	0.028				*DQB1*06:09*	2	0.002									
*DRB1*12:01*	30	0.036															
*DRB1*12:02*	18	0.022															
*DRB1*13:01*	5	0.006															
*DRB1*13:02*	65	0.078															
*DRB1*14:02*	1	0.001															
*DRB1*14:03*	11	0.013															
*DRB1*14:05*	17	0.020															
*DRB1*14:06*	13	0.016															
*DRB1*14:07*	3	0.004															
*DRB1*14:54*	26	0.031															
*DRB1*15:01*	67	0.080															
*DRB1*15:02*	70	0.084															
*DRB1*16:02*	2	0.002															

[a]Heterozygosity.
[b]Hardy-Weinberg equilibrium test.
[c]Ewens-Watterson test.
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exhibited significant deviation from HWE. Results of a Ewens-Watterson neutrality test [19,20] of *HLA* allele frequencies in this study population revealed that the observed distributions of allele frequencies at *HLA-C* ($P = 0.003$), *HLA-B* ($P = 0.002$), *HLA-DRB1* ($P = 0.013$), and *HLA-DQB1* ($P = 0.001$) differed significantly (i.e., there was excess heterozygosity) from the distributions expected based on the assumption of neutrality, whereas there was no significant difference between the expected and observed distributions of allele frequencies at *HLA-A* or *HLA-DPB1* (Tables 1 and 2).

Pairwise LD between *HLA* Alleles

The pairwise linkage disequilibrium (LD) parameters, r^2 and $|D'|$ [21], for each possible pair of two *HLA* alleles were estimated (Figure 1 and Data S1). Most alleles at *HLA-A* were not in strong LD with any of the alleles at the other loci because the physical distance from *HLA-A* to each of the other loci is large. To evaluate the relative strength of LD between two *HLA* loci, 2-locus r^2 and 2-locus $|D'|$ (see Materials and Methods for details), were calculated based on the pairwise LD parameters for all the allelic pairs (Table S1). The values of 2-locus $|D'|$ for *HLA-C* and *HLA-B* ($|D'| = 0.91$) and for *HLA-DRB1* and *HLA-DQB1* ($|D'| = 0.80$) were high, whereas the lowest 2-locus $|D'|$ value was observed for *HLA-A* and *HLA-DPB1* ($|D'| = 0.25$). These values reflected the physical distances between the respective loci. The values of 2-locus $|D'|$ for *HLA-DRB1* and *HLA-DPB1* and for *HLA-DQB1* and *HLA-DPB1* were relatively low compared to the values for the other pairs (Figure 2). These low values probably result from the recombination hotspot in the *HLA* class II region [22–24].

Major 6-locus *HLA* Haplotypes in Japanese

Frequencies of multi-locus haplotypes were estimated using the PHASE program [25,26] (Table 3 and Tables S2, S3, S4, S5). In 418 Japanese subjects (i.e., 839 chromosomes), 489 different 6-locus *HLA* haplotypes were inferred. Based on the frequencies of 6-locus *HLA* haplotypes, the probability of selecting two identical 6-locus *HLA* haplotypes at random from the Japanese population was estimated as 0.0075. Six 6-locus *HLA* haplotypes had

frequencies higher than 1% (Table 3). Of these, *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* was the most common (4.4%).

The intensity of recombination in the *HLA* region has been estimated at 0.67 cM/Mb [27], which corresponds to a recombination fraction of approximately 2% between *HLA-A* and *HLA-DPB1*. Thus, association between the six *HLA* alleles in any 6-locus *HLA* haplotype is not generally strong due to the frequent recombination in the *HLA* region. The expected frequency of the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype is 2.5×10^{-7} under the assumption of linkage equilibrium, which is much smaller than the observed frequency of 0.044. The strong LD among *HLA* alleles on the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype may result from recent positive selection acting on one of *HLA* alleles on the haplotype, although other mechanisms such as neutral random genetic drift, recent admixture, recent migration, recent bottlenecks, and suppression of recombination can also cause the strong LD [10,12,13,15,16].

Haplotype Omozygosity

Strong positive selection leads to a rapid increase in the frequency of a selected (target) allele in a population. The number of recombination events between the target allele and the surrounding polymorphic sites is limited while the advantageous allele increases in frequency; therefore, the diversity of haplotypes carrying the advantageous allele becomes low. Accordingly, strong LD is expected in the genomic region bearing the selected allele. In this study, the degree of LD for each HLA allele was measured by haplotype homozygosity (*HH*); this term is defined as the probability that any two randomly chosen samples of haplotype bearing a focal *HLA* allele have the same 6-locus *HLA* haplotype. Like *EHH* [17], a high *HH* value can be regarded as a signature of recent positive selection acting on a focal *HLA* allele.

To detect *HLA* alleles that have been subject to recent positive selection, *HH* was calculated for each allele based on the estimated number of 6-locus haplotypes in 418 Japanese subjects. Of the 80

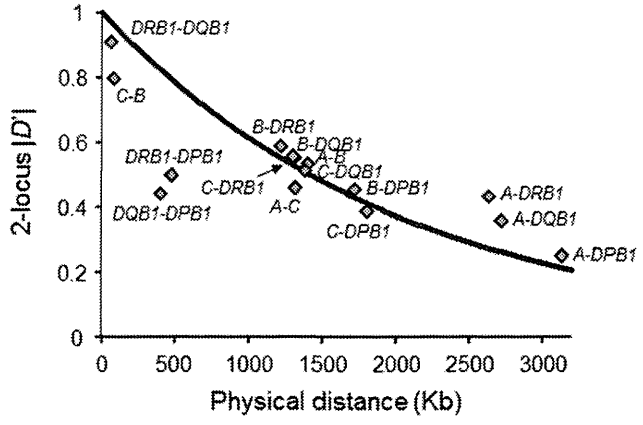

Figure 2. Relationship between two-locus |*D'*| and physical distance (Kb). A solid-line curve, $2-locus|D'|=(1-0.67\times10^{-5}\times x)^{75,13}$, was obtained using the least-squares method, where *x* represents the physical distance (Kb). The recombination rate in the *HLA* region was assumed to be 0.67 cM/Mb [27]. Spearman's rank correlation coefficient between 2-locus |*D'*| and the physical distance was −0.8607 ($P<0.0001$).
doi:10.1371/journal.pone.0046806.g002

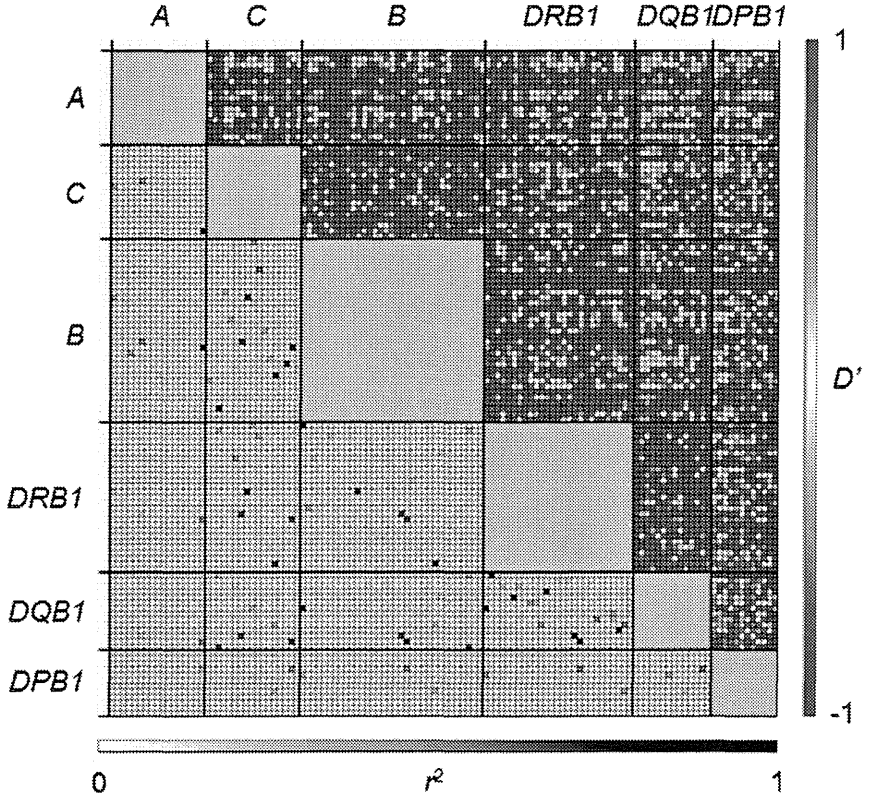

Figure 1. Pairwise estimates of LD parameters, |*D'*| (upper diagonal) and r^2 (lower diagonal) for every pair of *HLA* alleles. The name of each allele is presented in Data S1.
doi:10.1371/journal.pone.0046806.g001

Table 3. Estimated frequencies of 6-locus *HLA* haplotypes.

Association						# of haplotypes[a]	HF[b]
*A*33:03*	*C*14:03*	*B*44:03*	*DRB1*13:02*	*DQB1*06:04*	*DPB1*04:01*	37	0.044
*A*24:02*	*C*12:02*	*B*52:01*	*DRB1*15:02*	*DQB1*06:01*	*DPB1*09:01*	33	0.039
*A*24:02*	*C*07:02*	*B*07:02*	*DRB1*01:01*	*DQB1*05:01*	*DPB1*04:02*	29	0.035
*A*24:02*	*C*01:02*	*B*54:01*	*DRB1*04:05*	*DQB1*04:01*	*DPB1*05:01*	13	0.016
*A*24:02*	*C*12:02*	*B*52:01*	*DRB1*15:02*	*DQB1*06:01*	*DPB1*02:01*	12	0.014
*A*11:01*	*C*04:01*	*B*15:01*	*DRB1*04:06*	*DQB1*03:02*	*DPB1*02:01*	11	0.013

[a]Estimated by the PHASE program version 2.1.
[b]Haplotype frequency.
doi:10.1371/journal.pone.0046806.t003

HLA alleles that had frequencies of more than 1%, one allele at each class I locus (*A*33:03*, *C*14:03*, and *B*44:03*) had the highest *HH* for that locus; similarly, one allele at each class II locus (*DRB1*13:02*, *DQB1*06:04*, and *DPB1*04:01*) had the highest *HH* for that locus (Figure 3). These six *HLA* alleles made up the 6-locus haplotype, *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01*, with the highest frequency in this Japanese population (Table 3).

The *HH* values are generally reduced by loci with high heterozygosity. Therefore, it was relatively difficult for an allele at *HLA-DPB1* to show high *HH*, because heterozygosities at the other loci are high. Nevertheless, the *DPB1*04:01* allele, which had a population frequency of 6.1%, showed the highest *HH* value (0.53) of the 80 *HLA* alleles with frequencies higher than 1% (Figure 3). The values of *HH* of the remaining 79 *HLA* alleles were less than 0.33. This finding suggests that *DPB1*04:01* had undergone recent positive selection in Japan. The large *HH* values of the five other alleles (*A*33:03*, *C*14:03*, *B*44:03*, *DRB1*13:02*, and *DQB1*06:04*) in this 6-locus *HLA* haplotype (i.e., *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01*) appear to be due to the hitchhiking effect of *DPB1*04:01*.

To investigate the effect of recombination on the decay of the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype, the value of extended haplotype homozygosity (*EHH*) was calculated for *DPB1*04:01* (Figure 4). Although the *EHH* of *DPB1*04:01* was reduced at *HLA-DQB1*, the decrease in *EHH* was almost negligible at *HLA-DRB1*, *HLA-B*, and *HLA-C* loci; these findings indicate that, in this haplotype, recombination mainly has occurred between *DQB1*06:04* and *DPB1*04:01*.

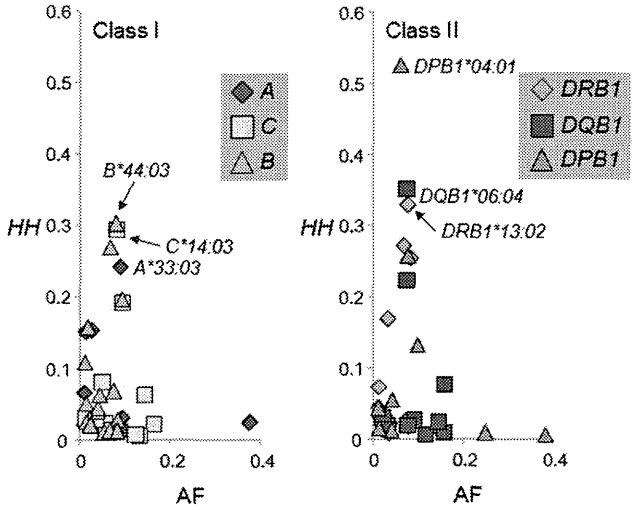

Figure 3. Haplotype homozygosity (*HH*) × allele frequency (AF) of each *HLA* allele. The left and right panels show *HH* values of *HLA* class I alleles and *HLA* class II alleles, respectively. The class I alleles were designated as follows: *HLA-A* (red diamond), *HLA-C* (yellow square), and *HLA-B* (green triangle); the class II alleles were designated as follows: *HLA-DRB1* (blue diamond), *HLA-DQB1* (purple square), and *HLA-DPB1* (pink triangle). In both panels, only *HH* values of alleles with frequencies of more than 0.01 are shown.
doi:10.1371/journal.pone.0046806.g003

Origin of *DPB1*04:01* in Japanese

*DPB1*04:01* is common (>30%) in European populations [9,28], whereas the frequency of *DPB1*04:01* is 6.1% in Japanese (Table 2). Given the worldwide distribution of *DPB1*04:01*, it is unlikely that *DPB1*04:01* originated in Japan. *DPB1*04:01* seems to have entered Japan. Archaeological studies of Japanese history have suggested that the Yayoi people came from the Korean peninsula circa 300 B.C., and mixed with the indigenous Jomon people. A recent large-scale survey of single nucleotide polymorphisms (SNPs) on autosomal chromosomes [29] revealed that most people presently inhabiting mainland Japan are genetically closer to Koreans than to Ryukuans. Ryukuans are considered to be more pure descendants of the Jomon people than are mainland Japanese. These observations indicate that a large population of Yayoi people migrated from the Korean peninsula. Although the frequency of the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype in Koreans has not been reported, *DPB1*04:01*, which was carried by *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01*, appears to have derived from the Korean population because the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04* and *DRB1*13:02-DQA1*01:02-DQB1*06:04-DPB1*04:01* haplotypes are observed at the frequencies of 4.2% and 4.7% in Korean populations [28,30,31]. These and similar haplotypes have not been reported in other Asian populations (http://www.allelefrequencies.net) [28].

If the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype has a single origin, the current genetic diversity of this haplotype must be low. To assess the genetic diversity of this haplotype, we performed a sliding window analysis of individual heterozygosity, defined as a proportion of heterozygous SNPs to all SNPs in the window (Figure 5). Reduced individual heterozygosity was only found in the *HLA* region on the short arm of chromosome 6 in all the three subjects that were

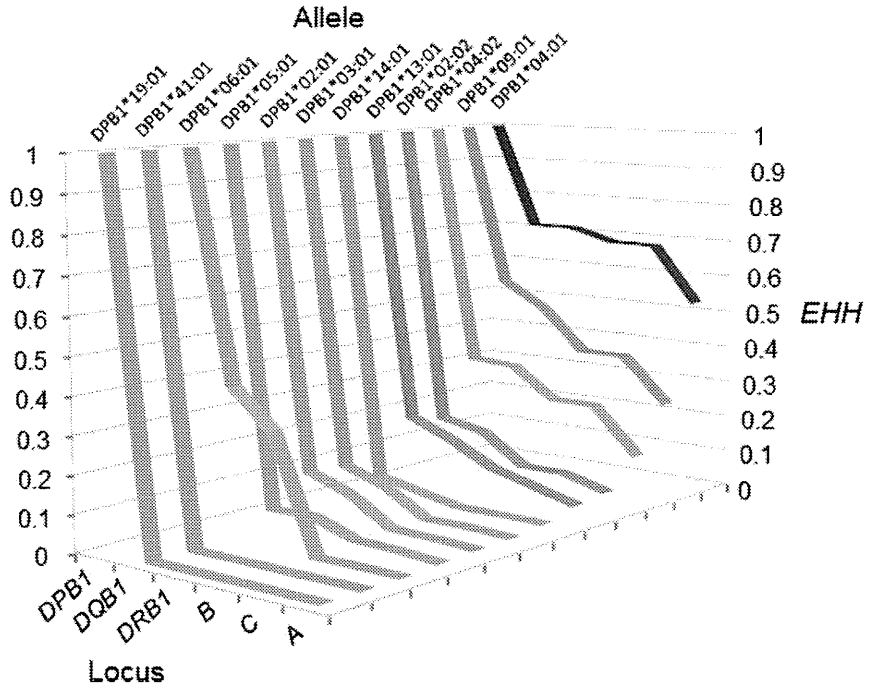

Figure 4. Extended *HH* (*EHH*) × relative locus position for12 *HLA-DPB1* alleles.
doi:10.1371/journal.pone.0046806.g004

homozygous for the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype (Figure 5A); in contrast, such a reduction was not observed in two subjects that were heterozygous for this haplotype (Figure 5B). Furthermore, three subjects that were homozygous for the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype shared the same SNP haplotype that spanned more than 4 Mb in the *HLA* region (Figure 5A). These observations suggest that the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype in Japanese has a single origin, and has not been generated repeatedly by recombination.

Computer Simulation

The analysis of *EHH* revealed that the reduction in *EHH* for *DPB1*04:01* resulted from recombination between *DQB1*06:04* and *DPB1*04:01* that inhabited the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype (Figure 4). Therefore, the relationship between *DQB1*06:04* and *DPB1*04:01* was focused in the following analyses. The high *HH* and *EHH* values of *DPB1*04:01* (Figures 3 and 4) may merely reflect that a neutral random genetic drift, rather than a recent positive selection, occurred after the Yayoi people reached the Japanese archipelago (300 B.C. or 2300 years ago). To assess this possibility, we conducted a computer simulation assuming a two-locus two-allele model in which changes in the frequency of four haplotypes carrying *DPB1*04:01* or non-*DPB1*04:01* alleles at the *HLA-DPB1* locus and *DQB1*06:04* or non-*DQB1:06:04* alleles at the *HLA-DQB1* locus were evaluated. In the simulation, the values of three parameters: selection intensity, s, recombination rate, c, and frequency of *DQB1*06:04-DPB1*04:01* haplotype, $f_1(0)$, in the beginning of the Yayoi period were drawn by a random number generator in every run. Haplotype frequencies were subject to change based on a stochastic model of positive selection, recombination, and random genetic drift. Dominant selection was assumed for *DPB1*04:01*, and, for the sake of simplicity, no selection (i.e., selectively neutral) was assumed for all alleles at the *DQB1* locus. The rejection method [18,32,33] was applied to accept only simulation runs that gave results similar to the observed values (see Materials and Methods for details). The uniform distribution was used for each parameter as a prior distribution (see Materials and Methods for detail). Figure 6A shows 2,500 parameter sets (i.e., posterior distributions) that were accepted in these simulations. The posterior distribution of the initial frequency of *DQB1*06:04-DPB1*04:01* haplotype was similar to the prior one, whereas the posterior distributions of selection intensity and recombination rate were different from the prior ones. In the posterior distribution, s ranged from 0.009 to 0.098, and the mean and 95% credible interval of s were 0.041 and 0.021−0.077, respectively (Figure 6B). It should be noted that neutral random genetic drift (i.e., $s \approx 0$) did not yield the results similar to the observed values. The findings from the simulations indicated that *DPB1*04:01* has been subject to relatively strong positive selection in Japanese since the Yayoi period.

Discussion

A number of *HLA* alleles have been shown to be associated with variations in immune responses to infectious diseases (e.g., human immunodeficiency virus [HIV]/AIDS, malaria, tuberculosis, hepatitis, leprosy, leishmaniasis, and schistosomiasis) caused by pathogenic microorganisms (see review by Blackwell et al. [34]). The most plausible explanation for positive selection favoring *DPB1*04:01* would be its function in resistance to infections. A recent genome-wide association study showed that the *DPA1*01:03-DPB1*04:01* haplotype confers protection against hepatitis B virus (HBV) infection (OR = 0.57, 95% CI = 0.33–0.96) [35]. Hepatitis B is a deadly infectious disease. Acute hepatitis B, which can cause fatal complications such as fulminant hepatitis, occurs in a percentage of the people infected with HBV. Although the estimated selection coefficient of s (0.0254–0.0550) for

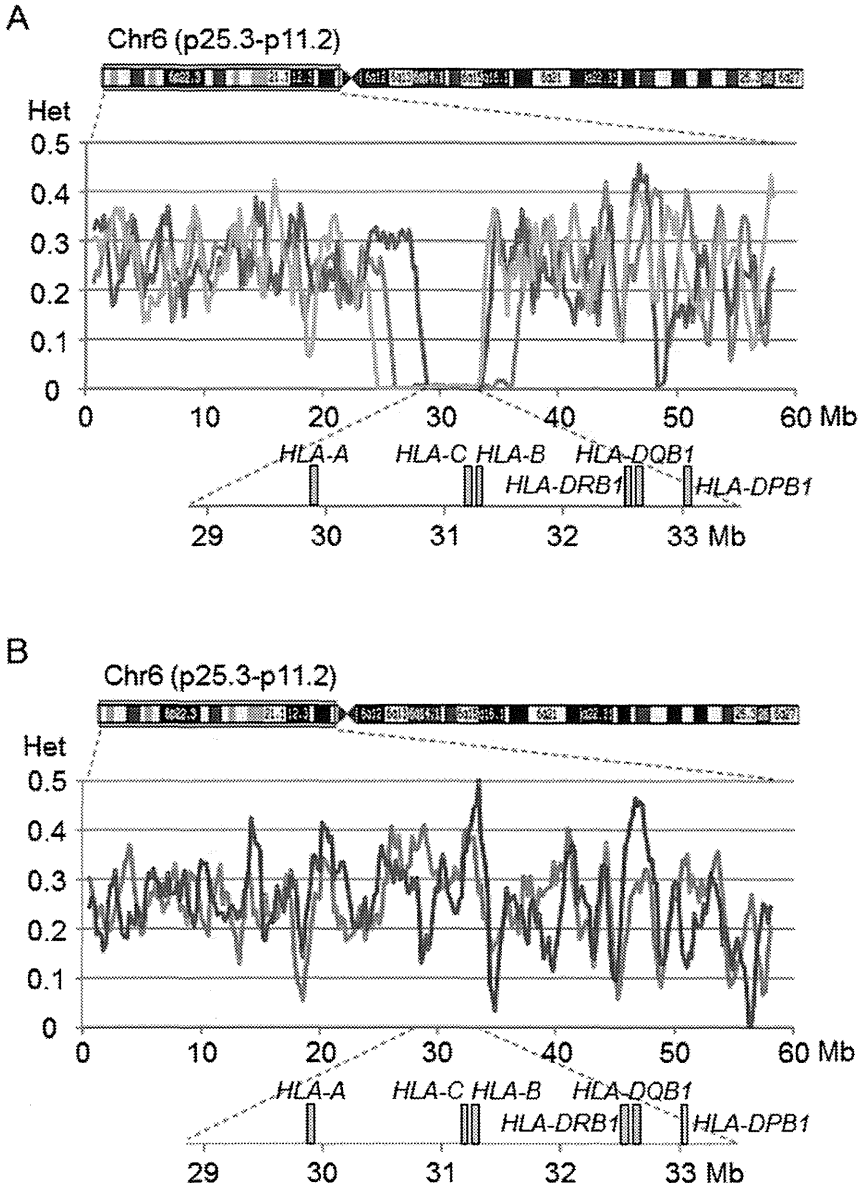

Figure 5. Individual heterozygosity of each subject with the most common 6-locus *HLA* haplotype. The individual heterozygosity in the genomic region on the short arm of chromosome 6 was assessed using the sliding window analysis; in this analysis, the window and step sizes were set to be 1 Mb and 200 kb, respectively. The individual heterozygosity was defined as a proportion of heterozygous SNPs to SNPs genotyped in a single subject. This analysis was performed for five Japanese subjects with the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype: (A) three of these five subjects were homozygous for this haplotype (blue, red, and green) and (B) two subjects had the heterozygous genotypes of the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype and the *A*24:02-C*07:02-B*07:02-DRB1*01:01–DQB1*05:01-DPB1*04:02* haplotype (orange) and of the *A*33:03-C*14:03-B*44:03-DRB1*13:02-DQB1*06:04-DPB1*04:01* haplotype and the *A*24:02-C*12:02-B*52:01-DRB1*15:02-DQB1*06:01-DPB1*09:01* haplotype (purple).
doi:10.1371/journal.pone.0046806.g005

*DPB1*04:01* does not seem to result solely from protection against infection with HBV, HBV infection may have been one of the key driving forces for the rapid increase in frequency of *DPB1*04:01* in the Japanese population.

Here, the analysis of *HH* was used to detect a signature of recent positive selection. The advantage of using *HH* in the analysis of *HLA* genes is that alleles with similar frequencies not only at the same *HLA* locus, but also at different loci, can be compared. This feature of analyses based on *HH* allows us to compare *HLA* alleles even within the same long-range haplotype. Since the same polymorphic markers are used for all *HLA* alleles in the calculation of *HH*, the effect of recombination on the value of *HH* can be well controlled. However, the *HH* analysis has a disadvantage in that the empirical distribution of *HH* value has to be obtained from only those alleles that are in the targeted region. Therefore, unlike conventional long-range haplotype tests based on *EHH* values [17,36], the statistical test based on *HH* values cannot be performed using genome-wide data. Nevertheless, *HH*-based test is thought to be suitable for analysis of *HLA* genes because each locus has a number of alleles to be examined and strong LD exists between alleles even at distant loci. The use of *HH* in the analysis of various human populations would help us to detect other *HLA*