Table 2 Clinical backgrounds of SVR and non-SVR cEVR patients (48 weeks of treatment, $n = 104$)

	SVR ($n = 82$)	Non-SVR ($n = 22$)	*P*-value
Male/female	57/25	8/14	0.004
Age (years)	53 (25–71)	56.5 (37–69)	0.279
BMI (kg/m^2)	22.9 (16–31.2)	21.9 (18.7–27.0)	0.192
HCV RNA (log IU/mL)	6.2 (5.2–6.7)	6.3 (5.3–6.7)	0.147
Hb (g/dL)	14.6 (10.7–17.6)	13.8 (11.9–16.7)	0.105
PLT ($\times 10^{-4}$/µL)	18.3 (9.9–39.9)	16.7 (10.0–26.5)	0.044
Neu (/µL)	2617 (1033–6222)	2638 (778–3652)	0.803
ALT (IU/L)	61 (15–740)	54.5 (19–144)	0.494
HCV RNA negativity (8w/12w)	17/65	3/19	0.340
Total PEG IFN (µg/kg)	68.4 (22.0–94.2)	65.5 (27.9–82.8)	0.431
Total RBV (mg/kg)	3422 (1100–5050)	2817 (2021–3926)	0.003

ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early virological response; Hb, hemoglobin; HCV RNA, hepatitis C virus RNA; Neu, neutrophil count; PEG IFN, peginterferon; PLT, platelet count; RBV, ribavirin; SVR, sustained virological response; w, weeks.

treated for 52–64 weeks; however, no significant differences were noted, possibly because of the small number of patients (Table 4).

Effect of treatment extension on the SVR rate of cEVR patients

We analyzed the SVR rate according to the timing of HCV RNA negativity in the serum in the 48-week-treated group (Fig. 2). While the SVR rate in females was comparable to that in males with initial HCV RNA negativity at treatment week 8 (78.8% vs 90.9%, $P = 0.413$), it was significantly lower than that in males with initial HCV RNA negativity at treatment week 12 (60.0% vs 87.0%, $P = 0.005$).

In cEVR patients who were initially serum HCV RNA negative at treatment week 12 and were treated for

Table 3 Multivariate logistic regression analysis of factors associated with SVR in cEVR patients (48 weeks of treatment, $n = 104$)

	Odds ratio	95% CI	*P*-value
Total RBV dose (mg/kg)	1.0008	1.0001–1.0015	0.017

CI, confidence interval; RBV, ribavirin.

Table 4 Clinical backgrounds of SVR and non-SVR cEVR patients (52–64 weeks of treatment, $n = 46$)

	SVR ($n = 37$)	Non-SVR ($n = 9$)	*P*-value
Male/female	24/13	5/4	0.604
Age (years)	55 (35–74)	55 (27–68)	0.533
BMI (kg/m^2)	22.5 (17.7–35.6)	25.3 (18.5–37.4)	0.136
HCV RNA (log IU/mL)	6.2 (5.2–6.7)	6.4 (5.6–6.7)	0.144
Hb (g/dL)	14.6 (11.5–18.1)	13.4 (12.3–16.4)	0.076
PLT ($\times 10^{-4}$/µL)	16.5 (8.9–45.5)	14.9 (10.9–23.7)	0.382
Neu (/µL)	2518 (1318–7772)	2070 (1579–3358)	0.089
ALT (IU/L)	74 (22–285)	53 (24–201)	0.261
HCV RNA negativity (8w/12w)	0/37	0/9	–
Total PEG IFN (µg/kg)	81.8 (35.9–96.0)	78.9 (38.7–84.0)	0.325
Total RBV (mg/kg)	4400 (1725–5016)	4004 (2619–4940)	0.160

ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early virological response; Hb, hemoglobin; HCV RNA, hepatitis C virus RNA; Neu, neutrophil count; PEG IFN, peginterferon; PLT, platelet count; RBV, ribavirin; SVR, sustained virological response; w, weeks.
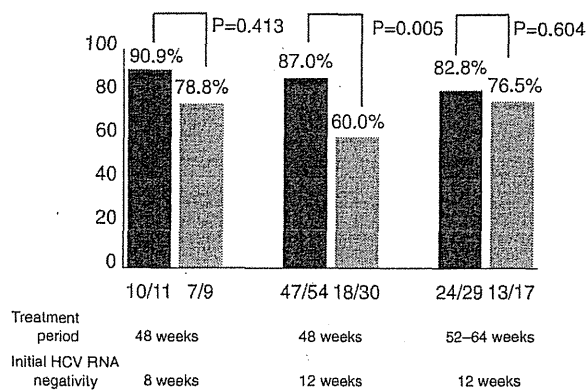
Figure 2   SVR rate of cEVR in male and female patients classified by the time of HCV RNA negativity in 48-week-treated or 52–64-week-treated groups. cEVR, complete early virological response; HCV RNA, hepatitis C virus RNA; SVR, sustained virological response. (■) Male; (▨) female.

52–64 weeks, the SVR rate in females was 76.5%, which was not significantly ($P = 0.604$) different from that in males (82.8%) (Fig. 2).

Comparison of clinical backgrounds between male and female cEVR patients with HCV RNA negativity at treatment week 12

We compared clinical backgrounds between males and females in 48-week-treated (Table 5) and 52–64-week-treated (Table 6) groups. In both groups, hemoglobin concentration was significantly higher in males. Whereas the SVR rate in females was similar to that in males in the 52–64-week-treated group (76.5% vs

82.8%, $P = 0.604$, Fig. 2), the total RBV dose beyond treatment week 48 was significantly lower in females than in males ($P = 0.027$, Table 6).

We also compared the SVR rate of female cEVR patients with initial HCV RNA negativity at treatment week 12 in 48-week-treated and 52–64-week-treated groups; however, no statistical significance was observed ($P = 0.252$, data not shown). The rate of advanced hepatic fibrosis (F2 and F3) tended to be higher in the 52–64-week-treated group compared to those in the 48-week-treated group ($P = 0.086$, Table 7).

DISCUSSION

IN THE PRESENT study, we retrospectively analyzed 150 genotype 1 CH-C patients demonstrating cEVR with PEG IFN and RBV combination therapy. In the group treated for 48 weeks, patients without SVR were more often female ($P = 0.004$), had lower platelet counts ($P = 0.044$) and received lower total RBV doses ($P = 0.003$). These findings are in accordance with a consensus report from Japan demonstrating that sex, severity of liver fibrosis and adherence to drug treatment are important predictors of the outcome with PEG IFN and RBV combination therapy.[1]

Ribavirin is an antiviral drug effective for HCV when used in combination with IFN-α/β or PEG IFN-α. Several publications have reported that short-term or cumulative RBV exposure is closely associated with EVR or SVR,[9–12] and a recent report presented evidence that RBV can promote IFN signaling in patients with CH-C in vivo.[13] In clinical practice, Hiramatsu et al. demonstrated that RBV dose reduction is associated with relapse of hepatitis in patients with cEVR.[14]

Table 5   Comparison of clinical backgrounds between male and female cEVR patients with HCV RNA negativity at week 12 (48 weeks of treatment, $n = 84$)

	Male ($n = 54$)	Female ($n = 30$)	P-value
Age (years)	56 (25–70)	54 (30–71)	0.926
BMI (kg/m^2)	22.7 (16.0–30.5)	22.7 (18.7–29.6)	0.259
HCV RNA (Log IU/mL)	6.2 (5.1–6.7)	6.2 (5.2–6.7)	0.411
Hb (g/dL)	15.1 (11.3–16.9)	13.5 (10.7–15.2)	<0.001
PLT (×10^{-4}/µL)	16.8 (10.0–36.6)	18.7 (9.9–39.9)	0.373
Neu (/µL)	2601 (1033–5017)	2435 (1033–4344)	0.173
ALT (IU/L)	64 (21–740)	53 (15–126)	0.040
Total PEG IFN (µg/kg)	66.7 (22.0–94.2)	68.6 (44.2–82.8)	0.115
Total RBV (mg/kg)	3460 (1444–5050)	3.427 (1357–4454)	0.575

ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early virological response; Hb, hemoglobin; HCV RNA, hepatitis C virus RNA; Neu, neutrophil count; PEG IFN, peginterferon; PLT, platelet count; RBV, ribavirin; SVR, sustained virological response; w, weeks.

Table 6 Comparison of clinical backgrounds between male and female cEVR patients with HCV RNA negativity at week 12 (52–64 weeks of treatment, $n = 46$)

	Male ($n = 29$)	Female ($n = 17$)	P-value
Age (years)	53 (27–74)	58 (40–71)	0.056
BMI (kg/m^2)	23.4 (17.7–31.1)	21.3 (18.5–37.4)	0.220
HCV RNA (log IU/mL)	6.3 (5.5–6.7)	6.1 (5.2–6.7)	0.317
Hb (g/dL)	15.0 (12.9–18.1)	13.0 (11.5–15.3)	<0.001
PLT ($\times 10^{-4}$/μL)	17.4 (11.6–45.5)	14.9 (8.9–23.7)	0.057
Neu (/μL)	2541 (1319–7772)	2322 (1677–3800)	0.303
ALT (IU/L)	80 (22–285)	42 (24–125)	0.033
Total PEG IFN (μg/kg)	81.8 (35.9–93.3)	77.9 (43.1–96.0)	0.432
Total RBV (mg/kg)	4400 (2620–5012)	4174 (1725–5016)	0.142
RBV 48–64w (mg/kg)	902 (148–1.167)	643 (139–988)	0.027
Total treatment period	59.1 (52.1–62.1)	60.1 (53.1–64.0)	0.227

ALT, alanine aminotransferase; BMI, body mass index; cEVR, complete early virological response; Hb, hemoglobin; HCV RNA, hepatitis C virus RNA; Neu, neutrophil count; PEG IFN, peginterferon; PLT, platelet count; RBV, ribavirin; SVR, sustained virological response; w, weeks.

Because the hemoglobin concentrations at baseline were significantly lower in females than in males (Tables 5,6), the dosage of RBV should be investigated in males and females separately. We first speculated that the inferior outcome of 48-week-treated female cEVR patients may be attributable to the lower total RBV dose they received. On the contrary, the total RBV dose was not significantly different between males and females, both in the early and late periods of the 48-week therapy (data not shown). Similarly, the total RBV dose was not significantly different between males and females in the 52–64-week-treated group (Table 6).

The rate of SVR in patients treated for 48 weeks was similar in males and females when HCV RNA was initially negative at treatment week 8 (Fig 2); however, the SVR rate was significantly lower in females than in males who were initially HCV RNA negative at treatment week

Table 7 Evaluation of hepatic fibrosis in patients with HCV RNA negativity at treatment week 12

Total treatment period	48 weeks ($n = 60$)	52–64 weeks ($n = 40$)
Fibrosis score (0,1/2,3)		
Male	24/14[a]	12/13[b]
Female	12/10[c]	5/10[d]
Total	36/24[e]	17/23[f]

Liver biopsy was performed in 60 patients treated for 48 weeks and 40 patients treated for 52–64 weeks.
[a] vs [b], $P = 0.204$; [c] vs [d], $P = 0.234$; [a] vs [c], $P = 0.512$; [b] vs [d], $P = 0.364$; [e] vs [f], $P = 0.086$.
Data evaluated by χ^2-test.

12 (60.0% vs 87.0%, $P = 0.005$, Fig. 2). In the 52–64-week-treated group who were also initially HCV RNA negative at treatment week 12, the rate of SVR in females was comparable to that in males (76.5% vs 82.8%, $P = 0.604$, Fig. 2), even though the RBV dose was significantly lower in females during the extension period ($P = 0.027$, Table 6). Therefore, we believe that treatment extension may benefit female cEVR patients who are initially HCV RNA negative at treatment week 12 even if the RBV dose is lower. A recent paper from Japan demonstrated that age and total RBV dose were independent predictors of relapse.[15] However, whether the treatment extension itself or increased total RBV dose benefited the treatment outcome in females remains to be studied.

We compared the fibrosis scores in cEVR patients with initial HCV RNA negativity at treatment week 12 and 8 (data not shown). In the 48-week-treated group ($n = 60$), the fibrosis score in SVR patients was not significantly ($P = 0.340$) different from that in non-SVR patients. Similarly, in the 52–64-week-treated group ($n = 40$), the fibrosis score in SVR patients was not significantly ($P = 0.155$) different from that in non-SVR patients. In addition, as we showed in Table 7, the fibrosis scores were not significantly different between males and females or 48-week-treated and 52–64-week-treated groups.

A preliminary study by Nomura *et al.* showed that extended therapy with IFN-α monotherapy for 24 weeks following PEG IFN and RBV combination therapy was effective for patients with cEVR.[16] In addition, short-term extension of PEG IFN and RBV combination

therapy for cEVR patients up to 60 weeks in genotype 1 CH-C patients has been reported to achieve an 80.5% SVR rate.[17] In the present study, the SVR rate of female cEVR patients with initial HCV RNA negativity at treatment week 12 and then treated for 52–64 weeks was not significantly higher than those treated for 48 weeks because of the small number of patients. We believe that further studies with a larger sample size may demonstrate a statistically significant difference between the two groups.

Response-guided therapy has been the standard approach in PEG IFN and RBV combination therapy for CH-C.[18,19] While response-guided therapy is not directly applicable to triple therapy, precise management of PEG IFN and/or RBV dosage during treatment should be closely monitored because continuous use of telaprevir is limited due to its side-effects.

Triple combination therapy with PEG IFN-α-2b, RBV and telaprevir has now become the first-choice treatment for genotype 1 CH-C in Japan[7] whereas skillful management of triple therapy for patients with lower hemoglobin levels at baseline should be intended to avoid severe anemia. Patients suffering from severe skin rashes during therapy must be reassigned to PEG IFN and RBV combination therapy; therefore, the optimal use of RBV is an important issue to consider regardless of whether double or triple therapy is prescribed.

Then, we suppose that longer RBV use in IFN-based therapy for female CH-C patients is an important point even if the RBV dose was lower than the scheduled dose.

The response to PEG IFN and RBV combination therapy is, to some extent, predicted by measuring the number of mutations in the interferon sensitivity-determining region (ISDR) in the non-structural protein 5A (NS5A) or the amino acid substitution of HCV core 70.[20,21] Because approximately 80% of cEVR patients were cured in this study, it is impossible to examine these viral factors retrospectively. A strong association between *interleukin 28B (IL28B)* gene polymorphism and a response to PEG IFN and RBV combination therapy, or *inosine triphosphate (ITPA)* gene polymorphism and RBV-induced anemia were reported for CH-C patients.[22–24] Although patients who could attain cEVR are unlikely to have "IFN-unresponsive" single nucleotide polymorphisms, *ITPA* polymorphism has a greater effect on RBV-induced anemia.

Martino *et al.* reported a result of meta-analysis of randomized controlled trials of response-guided PEG IFN and RBV combination therapy for genotype 1 showing that no significant benefit was demonstrated for 72 weeks of treatment as compared with 48 weeks of

treatment in patients with undetectable HCV RNA at week 12,[25] although their study included the report of Ferenci *et al.* which showed that the SVR rate of 72 weeks of treatment was higher than that of 48 weeks of treatment in patients with undetectable HCV RNA at week 12.[26] The discrepancy between the report by Martino *et al.* and our study may be attributed to two points.

First, we investigated the difference in SVR rate between males and females separately although Martino *et al.* did not. Because the reports from Japan concluded that the response to PEG IFN and RBV combination therapy was lower in older females,[3–5] and because the hemoglobin concentrations were significantly lower in females in our study, we considered it better to investigate whether the inferiority in females in treatment outcome could be overcome by extended treatment or not. Another point is that we studied the SVR rate in patients with initial HCV RNA negativity at treatment week 8 and at week 12 separately because the SVR rate of female patients with initial HCV RNA negativity at week 8 is high. We suppose that the benefit of treatment extension in females is restricted to the patients with HCV RNA positivity at week 8 and negativity at week 12.

The limitations of the present study are that it is a retrospective analysis and the number of cEVR patients is small. It may be best to examine the benefit of treatment extension among the groups of patients classified by each host and viral factor associated with the treatment outcome in a large multicenter study. Therefore, the results in the present study must be evaluated keeping the aforementioned information of host and viral factors in mind.

ACKNOWLEDGMENTS

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Inhibition of hepatocellular carcinoma by PegIFNα-2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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Abstract

Background We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C. *Methods* Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a

(PegIFNα-2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90 μg PegIFNα-2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFNα-2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFNα-2a (PegIFNα-2a group) and 59 patients who did not receive PegIFNα-2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

Results Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA, ≤40 IU/L alanine aminotransferase (ALT), or ≤10 ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group.

Conclusions Low-dose and long-term maintenance administration of PegIFNα-2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

Keywords Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients

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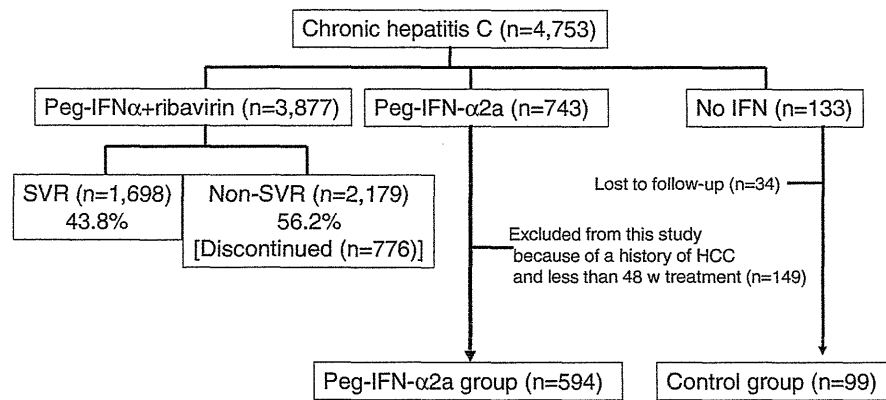Ehime University, Matsuyama, Japan

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65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90 μg peginterferon alpha-2a (PegIFNα-2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFNα-2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFNα-2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFNα-2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90 μg PegIFNα-2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

Patients and methods

Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFNα-2a

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90 μg of PegIFNα-2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180 μg) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFNα-2a for analysis. At the 21 centers involved in this

study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFNα-2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFNα-2a alone were not indicated for Peg-IFNα and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90 μg PegIFNα-2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFNα-2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pretreatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the

Table 1 Background data of patients treated with PegIFNα-2a (*n* = 594)

	n = 594
Age (years)	61.7 ± 11.7
Sex (male/female)	258/336
BMI	23.2 ± 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption (≥60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 ± 31.1
Fasting blood sugar (mg/dL)	106.3 ± 28.5
White blood cell count (/mm^3)	4,360 ± 1,470
Red blood cell count (×10^6/μL)	423.8 ± 56.4
Hemoglobin (g/dL)	13.3 ± 1.8
Platelet count (×10^3/μL)	137 ± 56
Albumin (g/dL)	4.0 ± 0.5
Total bilirubin (mg/dL)	0.8 ± 0.6
AST (IU/L)	65.8 ± 47.8
ALT (IU/L)	72.1 ± 68.0
Gamma-GTP (IU/L)	55.2 ± 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFNα-2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

PegIFN pegylated interferon, *BMI* body mass index, *ASC* asymtomatic carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *LDL* low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* guanosine triphosphate, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma

Values are means ± SD, with ranges in parentheses

Mann–Whitney *U*-test, and the χ^2 test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFNα-2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFNα-2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of ≥41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of ≥10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFNα-2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

Study 2: incidence of HCC in the PegIFNα-2a therapy and non-administration (control) groups in comparison with propensity-matched controls

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFNα and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFNα-2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

Table 2 Comparison of HCC and non-HCC patients with long-term PegIFNα-2a administration (*n* = 594)

	Patients with or without development of HCC		*p* value
	With HCC (*n* = 49)	Without HCC (*n* = 545)	
Pretreatment parameters			
Age (years)	63.8 ± 1.7	61.3 ± 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 ± 0.5	23.1 ± 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption (≥60 g/day; yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 ± 9.0	94.7 ± 2.6	n.s.
White blood cell count (/mm^3)	4,355 ± 210	4,360 ± 64	n.s.
Red blood cell count (×10^6/μL)	420.8 ± 8.1	424.1 ± 2.6	n.s.
Hemoglobin (g/dL)	13.6 ± 0.3	13.3 ± 0.1	n.s.
Platelet count (×10^3/μL)	106 ± 8	140 ± 2	<0.001
Albumin (g/dL)	3.8 ± 0.1	4.0 ± 0.1	<0.001
Total bilirubin (mg/dL)	1.2 ± 0.1	0.8 ± 0.1	<0.001
AST (IU/L)	78.1 ± 6.8	64.6 ± 2.1	n.s.
ALT (IU/L)	72.8 ± 9.7	72.0 ± 2.9	n.s.
Gamma-GTP (IU/L)	68.7 ± 7.5	53.9 ± 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 ± 5.7	44.6 ± 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher's exact test. The distributions of continuous variables were analyzed using Student's *t*-test and the Mann–Whitney *U*-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at $p < 0.05$. Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFNα-2a group were included.

Results

Study 1

We analyzed the factors involved in the development of HCC in patients who received 90 μg PegIFNα-2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFNα-2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pre-treatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFNα-2a maintenance treatment (Table 2).

Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of ≥41 IU/L and AFP levels of ≥10 ng/L 24 weeks after the start of the PegIFNα-2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of ≤40 IU/L than in those with ALT levels of ≥41 IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of <10 ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of

≥10 ng/mL (Fig. 3). The dose of PegIFNα-2a was reduced to 45 μg in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFNα-2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

Table 3 Independent risk factors for HCC development in patients treated with 90 μg PegIFNα-2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

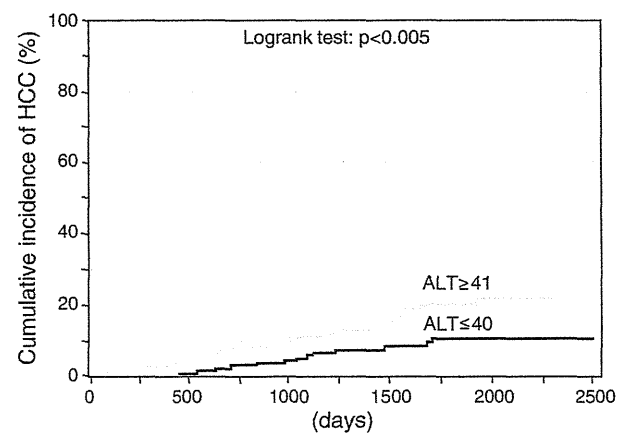|	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	p
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count (<120 × 10³/μL vs. ≥120 × 10³/μL)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) (≥41 vs. <40 IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) (≥10 vs. <10 ng/L)	3.78	1.92–11.8	<0.01

Fig. 2 Comparison of HCC rates in patients administered with PegIFNα-2a ($n = 594$) with respect to alanine aminotransferase (*ALT*) levels 24 weeks after the start of therapy. *Black line* patients with ALT ≥41 IU/L in the first 24 weeks, *gray line* patients with ALT ≤40 IU/L in the first 24 weeks
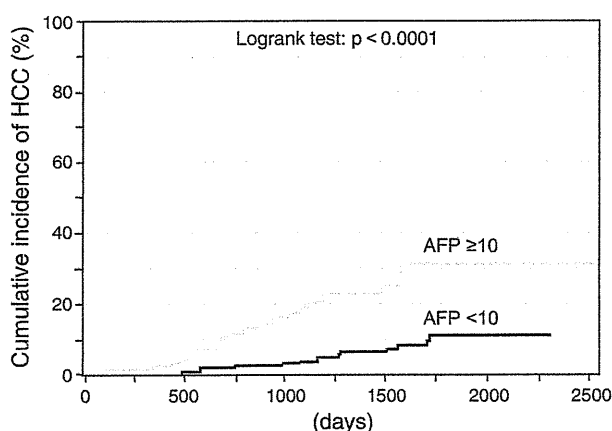
Fig. 3 Comparison of HCC rates in patients administered PegIFNα-2a ($n = 594$) with respect to alpha-fetoprotein (*AFP*) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP ≥ 10 ng/mL at 24 weeks, *gray line* patients with AFP <10 ng/mL at 24 weeks

Study 2

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFNα-2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFNα-2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFNα-2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFNα-2a group was significantly lower than that in the control group (log-rank test, $p = 0.0187$; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFNα-2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFNα-2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

Discussion

The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the

Table 4 Backgrounds of the patients in the propensity-matched control study (PegIFNα-2a group, $n = 59$; control group, $n = 59$)

	PegIFNα-2a group ($n = 59$)	Control group ($n = 59$)	p value
Age (years)	60.5 ± 13.0	63.3 ± 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 ± 3.6	22.9 ± 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	<0.05
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	<0.05
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 ± 23.8	117.0 ± 4.2	n.s.
White blood cell count (/mm^3)	4,260 ± 1,239	5,193 ± 2,078	<0.05
Red blood cell count (×10^{-4}/μL)	430 ± 57.8	441 ± 44.9	n.s.
Hemoglobin (g/dL)	13.6 ± 1.5	13.6 ± 1.9	n.s.
Platelet count (×10^{-3}/μL)	14.5 ± 5.7	15.8 ± 5.7	n.s.
Albumin (g/dL)	4.1 ± 0.5	4.1 ± 0.4	n.s.
Total bilirubin (mg/dL)	0.7 ± 0.5	0.9 ± 0.7	n.s.
AST (IU/L)	58.3 ± 47.7	49.7 ± 26.6	n.s.
ALT (IU/L)	63.6 ± 68.7	58.0 ± 39.2	n.s.
Gamma-GTP (IU/L)	78.3 ± 81.3	55.3 ± 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

incidence of HCC is reduced in patients who achieve an SVR to IFN [6–9] Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90 μg PegIFNα-2a weekly or biweekly and had AFP values of <10 ng/mL and ALT values of ≤40 IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFNα-2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between
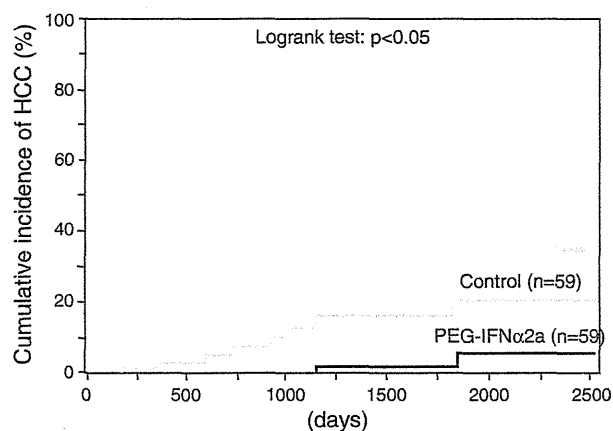
Fig. 4 Comparison of HCC rates between the long-term PegIFNα-2a administration group ($n = 59$) and non-administration group ($n = 59$) in the propensity-matched control study (Kaplan–Meier log-rank test, $p = 0.019$)

Table 5 Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	p value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every $10 \times 10^3/\mu L$)	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. ≥10 ng/L)	4.07	0.59–40.12	n.s.

the PegIFNα-2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFNα-2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to Peg-IFNα and ribavirin combination therapy [20, 21]. However, the mechanism of IL28B involvement in the response to PegIFNα and ribavirin has not been elucidated completely. A recent report has shown that IL28B is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between IL28B and inhibition of the development of HCC by PegIFNα in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25,

26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFNα-2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anti-carcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFNα-2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFNα-2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

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PLoS one

Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

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Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $P_{meta} = 1.89 \times 10^{-12}$ for rs3077 and $P_{meta} = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ($P_{meta} = 4.40 \times 10^{-19}$ for rs3077 and $P_{meta} = 1.28 \times 10^{-15}$ for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the HLA-DP molecule.

Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPB1* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–18]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

Results

Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in

order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate $\geq 95\%$; (ii) minor allele frequency (MAF) $\geq 1\%$ for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3′ UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPB1*, respectively (rs3077, $P = 1.14 \times 10^{-7}$, and rs9277542, $P = 5.32 \times 10^{-8}$, respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion $P < 8.36 \times 10^{-8}$ (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077, $P = 2.70 \times 10^{-8}$, OR = 0.48 and rs9277542, $P = 3.33 \times 10^{-6}$, OR = 0.54; replication-2 (Korean); rs3077, $P = 2.08 \times 10^{-6}$, OR = 0.47 and rs9277542, $P = 8.29 \times 10^{-5}$, OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ($P_{het} = 0.80$ for rs3077 and 0.40 for rs9277542). P_{meta} values were 4.40×10^{-19} for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and 1.28×10^{-15} for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate $\geq 95\%$ and HWE p-value ≥ 0.01. Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel P values of 2.80×10^{-7} (OR = 0.56, 95% CI = 0.45–0.70) and 1.09×10^{-7} (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ($P_{het} = 0.67$ for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed $P_{meta} = 3.94 \times 10^{-45}$, OR = 0.55 for rs3077, $P_{meta} = 1.74 \times 10^{-21}$, OR = 0.61 for rs9277535 and $P_{meta} = 1.69 \times 10^{-15}$, OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of $r^2 = 0.955$ on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with $P_{het} > 0.01$.

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).
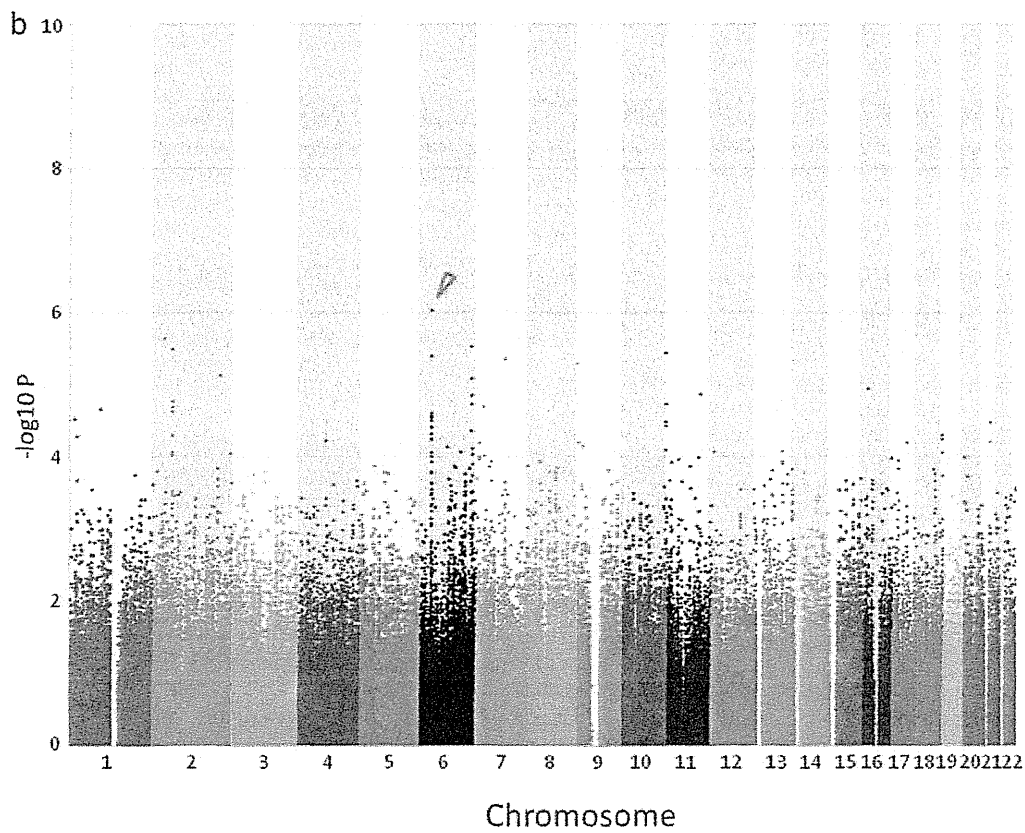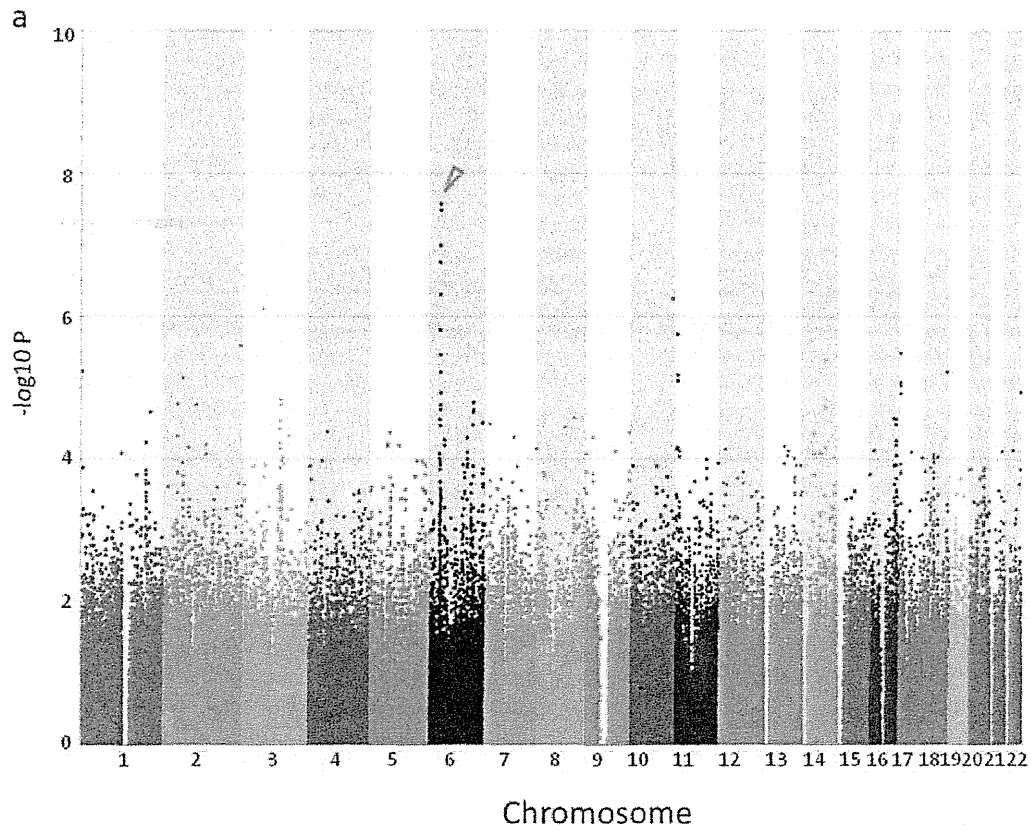
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Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. P values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance.
doi:10.1371/journal.pone.0039175.g001

Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P = 9.24 \times 10^{-7}$ and $P = 3.15 \times 10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1:256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2:344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P = 3.32 \times 10^{-2}$, OR = 0.72 and rs9277542, $P = 1.25 \times 10^{-2}$, OR = 0.68; replication-2 (Korean): rs3077, $P = 2.35 \times 10^{-7}$, OR = 0.41 and rs9277542, $P = 4.97 \times 10^{-6}$, OR = 0.46; Table 3]. Meta-analysis using random effects model showed $P_{meta} = 1.56 \times 10^{-4}$ for rs3077 (OR = 0.51, 95% CI = 0.36–0.72), and 5.91×10^{-7} for rs9277542 (OR = 0.55, 95% CI = 0.43–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het} = 0.03$) and no evidence for rs9277542 ($P_{het} = 0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta} = 3.28 \times 10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including HLA-DQ gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel P values of 2.10×10^{-5} (OR = 0.59) and 1.10×10^{-5} (OR = 0.56), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het} = 0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were

observed with $P_{meta} = 0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta} = 1.48 \times 10^{-9}$, OR = 0.60 for rs3077, $P_{meta} = 1.08 \times 10^{-17}$, OR = 0.66 for rs9277535 and $P_{meta} = 5.14 \times 10^{-5}$, OR = 0.55 for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het} = 0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het} = 9.57 \times 10^{-6}$ across 5 independent studies, including our study.

URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including HLA-DPA1 and HLA-DPB1 genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as HLA-DPA1 and HLA-DPB1 and protective effects against CHB in Korean and Japanese individuals. Mata-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of HLA-DP contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the HLA-DPA1 and HLA-DPB1 genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het} = 0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta} = 5.88 \times 10^{-24}$, OR = 0.56 (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het} = 0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P = 3.32 \times 10^{-2}$, OR = 0.72). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta} = 1.89 \times 10^{-12}$, OR = 0.43 for rs3077 with no evidence of heterogeneity ($P_{het} = 0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of HLA-DPA1/−DPB1 with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR = 0.43 for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR = 0.46 for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

Table 1. Number of study samples.

population		GWAS Japanese	Replication-1 Japanese	Replication-2 Korean
HBV carriers	Total	181	256	344
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls		184	236	151
Resolved individuals		185	150	106

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
doi:10.1371/journal.pone.0039175.t001

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Table 2. Results of replication study for protective effects against CHB.

dbSNP rsID	Chr	Position Buld 36.3	Nearest Gene	MAF[a] (allele)	Allele (1/2)	Stage (population)	\(HBV carriers\) 11	12	22	\(Healthy controls\) 11	12	22	HWEp	OR[b] 95% CI	P-value[c]	Phet[d]
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10^{-7}	
				(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
						Replication-1	26	95	134	46	125	65	0.309	0.48	2.70×10^{-8}	
						(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
						Replication-2	23	81	111	31	74	40	0.767	0.47	2.08×10^{-6}	
						(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
						Meta-analysis[e]								0.46	4.40×10^{-19}	0.80
														(0.39–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10^{-8}	
				(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
						Replication-1	30	106	118	54	114	67	0.681	0.54	3.33×10^{-6}	
						(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
						Replication-2	30	87	94	35	72	36	0.933	0.54	8.29×10^{-5}	
						(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
						Meta-analysis[e]								0.50	1.28×10^{-15}	0.40
														(0.43–0.60)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
doi:10.1371/journal.pone.0039175.t002

comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (HLA-DPA1, HLA-DPB1 and HLA-DQB2), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2<0.1$) was observed between HLA-DQB2 locus and HLA-DPA1/−DPB1 loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of HLA-DP (rs3077 and rs92775542) with use of HLA-DQ (rs9276431 and rs768538) as covariates showed that the same level of significant associations of HLA-DP with CHB and HBV clearance as shown in the single-point association analysis, while no associations of HLA-DQ with P_{log} >0.05 were detected both in Japanese and in Korean (Table S5). These results show that HLA-DP is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of HLA-DQB2 would result from linkage disequilibrium of HLA-DPA1/−DPB1.

In this study, we confirmed the significant associations between HLA-DPA1 and HLA-DPB1, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the HLA-DP locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover,

there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the HLA-DP molecule, identification of novel host genetic factors other than HLA-DP, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the a priori approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

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Table 3. Results of replication study for clearance of hepatitis B virus.

		Position		MAF^a	Allele	Stage	HBV carriers			Resolved individuals			OR^b		
dbSNP rsID	Chr	Buld 36.3	Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	95% CI	P-value^c	P_het^d
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10^{-7}	
				(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
						Replication-1	26	95	134	20	64	60	0.72	3.32×10^{-2}	
						(Japanese)	(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
						Replication-2	23	81	111	29	48	28	0.41	2.35×10^{-7}	
						(Korean)	(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
						Meta-analysis^e							0.51	1.56×10^{-4}	0.03
													(0.36–0.72)		
						Meta-analysis^e							0.43	1.89×10^{-12}	0.75
						(GWAS+replication-2)							(0.34–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15×10^{-5}	
				(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
						Replication-1	30	106	118	28	62	52	0.68	1.25×10^{-2}	
						(Japanese)	(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
						Replication-2	30	87	94	30	53	22	0.46	4.97×10^{-6}	
						(Korean)	(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
						Meta-analysis^e							0.55	5.91×10^{-7}	0.19
													(0.43–0.69)		
						Meta-analysis^e							0.49	9.69×10^{-10}	0.65
						(GWAS+replication-2)							(0.39–0.61)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
doi:10.1371/journal.pone.0039175.t003

City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

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 portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm³, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (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 agree-

ment (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained 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.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE P-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

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