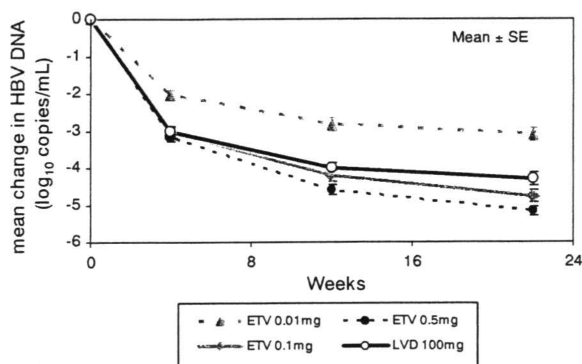

Fig. 1 Mean change from baseline in serum HBV DNA level by PCR assay through 22 weeks in patients treated with entecavir (ETV) 0.01, 0.1, and 0.5 mg and lamivudine 100 mg. Mean change in serum HBV DNA level was plotted as a function of time after the initiation of the protocol therapy (weeks). Data expressed as mean ± SE

significant dose–response relationship between $\log_{10}$ entecavir dose and reduction in $\log_{10}$ serum HBV DNA level ($P < 0.0001$).

Mean change (from baseline) in serum HBV DNA level at week 22 for the lamivudine 100 mg group was −4.29 $\log_{10}$ copies/ml (Fig. 1; Table 2). Estimated mean differences (95% CI) in serum HBV DNA level (after adjustment for baseline level and HBeAg status) were −0.39 (−0.83 to 0.05) $\log_{10}$ copies/ml between the entecavir 0.1 mg and lamivudine 100 mg groups and −0.62 (−1.06 to −0.18) $\log_{10}$ copies/ml between the entecavir 0.5 mg and lamivudine 100 mg groups, indicating the noninferiority of the entecavir 0.1 and 0.5 mg groups to the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group to the lamivudine 100 mg group ($P = 0.007$) (Table 2). In contrast, the entecavir 0.01 mg group was significantly inferior to the lamivudine 100 mg group (estimated mean difference = 1.20 [0.69–1.71]; $P < 0.0001$) (Table 2).

The secondary efficacy end point of a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or HBV DNA level less than 400 copies/ml by PCR assay was achieved by 88.6% of patients in the entecavir 0.01 mg group and by 100% of patients in the entecavir 0.1 and 0.5 mg groups at week 22. Ninety-seven percent of patients in the lamivudine 100 mg group achieved this end point at week 22. HBV DNA level less than 0.7 MEq/ml by bDNA assay was achieved by 65.7%, 94.1%, and 100% of patients in the 0.01, 0.1, and 0.5 mg entecavir groups, respectively, and by 93.9% of patients in the lamivudine 100 mg treatment group.

Serologic response

Among HBeAg-positive patients, there was no significant difference between seroconversion rates at week 22 for the entecavir 0.01, 0.1, and 0.5 mg treatment groups (10.0%, 13.3%, and 3.6%, respectively) versus the lamivudine 100 mg treatment group (3.3%; Table 2). All patients who lost HBeAg also experienced HBeAg seroconversion.

Biochemical response

At baseline, elevated serum ALT levels (>1.25 × ULN) were present in more than 90% of patients in all four treatment groups. At week 22, normal serum ALT levels (World Health Organization grade 0, <1.25 × ULN) were recorded in similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg treatment groups (75.0%, 85.3%, and 80.0% of patients, respectively) and the lamivudine treatment group (78.1% of patients), with no significant intergroup difference (Table 2).

Response

Response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg loss, and serum ALT level <1.25 × ULN for HBeAg-positive patients and HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT <1.25 × ULN for HBeAg-negative patients) was achieved by 14.3%, 20.6%, and 15.6% of patients in the entecavir 0.01, 0.1, and 0.5 mg

Table 2 Differences in HBV DNA levels between entecavir dose groups by PCR at week 22 in evaluable subjects

	0.1 mg ETV–0.01 mg ETV ($n = 34$, $n = 35$)	0.5 mg ETV–0.01 mg ETV ($n = 32$, $n = 35$)	0.5 mg ETV–0.1 mg ETV ($n = 32$, $n = 34$)
Estimated difference[a] ($\log_{10}$ copies/ml)	−1.61	−1.95	−0.23
Standard error	0.24	0.24	0.19
95% Confidence interval[b]	−2.20, −1.02	−2.53, −1.37	−0.69, 0.23
P-value	<0.0001	<0.0001	0.227

[a] Estimated differences are regression-adjusted for baseline serum HBV DNA and HBeAg status

[b] 95% Confidence interval is adjusted by modified Bonferroni procedures

ETV entecavir

Table 3 Virology and biochemical responses at week 22 and comparison of entecavir treatment groups with lamivudine in evaluable subjects

Response	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 32$)	LVD 100 mg ($n = 33$)
HBV DNA by PCR assay				
Reduction from baseline at week 22 ($\log_{10}$ copies/ml), mean ± S.E.	−3.11 ± 0.18	−4.77 ± 0.17	−5.16 ± 0.13	−4.29 ± 0.18
HBV DNA estimated difference[a] (vs. LVD) ($\log_{10}$ copies/ml)	1.20	−0.39	−0.62	-
Standard error	0.26	0.22	0.22	-
95% Confidence interval	0.69, 1.71	−0.83, 0.05	−1.06, -0.18	-
P-value	<0.0001[b]	0.081	0.007[c]	-
HBV DNA by Roche Amplicor™ PCR assay				
Change in $\log_{10}$ HBV DNA reduction >2 or HBV DNA <400 copies/ml at week 22, n (%)	31 (88.6)	34 (100)	32 (100)	32 (97.0)
P-value (vs. LVD)	0.206	NR[d]	NR[d]	-
HBV DNA by Quantiplex assay				
HBV DNA <0.7 MEq/ml (2.5 pg/ml) at week 22, n (%)	23 (65.7)	32 (94.1)	32 (100)	31 (93.9)
P-value (vs. LVD)	0.002	1.000	NR[d]	-
Normalization of ALT levels[e]				
At week 22, n/n with abnormal baseline (%)	24/32 (75.0)	29/34 (85.3)	24/30 (80.0)	25/32 (78.1)
P-value (vs. LVD)	0.842	0.439	0.880	-
Loss of HBeAg and seroconversion at week 48[f]				
HBeAg loss, n/n HBeAg positive at baseline (%)	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
HBeAg seroconversion	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
P-value (vs. LVD)	0.605	0.350	1.000	-
Response[g] at week 22, n (%)	5 (14.3)	7 (20.6)	5 (15.6)	3 (9.1)
P-value (vs. LVD)	0.735	0.190	0.480	-

[a] Estimated differences are regression-adjusted for baseline HBV DNA and HBeAg status

[b] Two-sided test indicates inferiority of the entecavir 0.01 mg dose

[c] Two-sided test indicates superiority of the entecavir dose

[d] Not reported because expected counts <5

[e] WHO grade 0, ALT <1.25 × upper limit of normal

[f] Seroconversion was defined as disappearance of HBe-antigen and appearance of HBe-antibody

[g] Response was defined as HBV DNA levels <0.7 MEq/ml, HBeAg negativity and ALT <1.25 × ULN for HBeAg-positive patients and HBV DNA levels <0.7 MEq/ml and ALT <1.25 × ULN for HBeAg-negative patients

ETV entecavir

LVD lamivudine

treatment groups, respectively, and by 9.1% of patients in the lamivudine treatment group at week 22, and there were no significant differences in the rates of response between the four treatment groups (Table 2).

Resistance analysis

During the treatment period, serum HBV DNA level increased by 1 $\log_{10}$ copies/ml or more from its nadir in one patient in the entecavir 0.01 mg group and one patient in the lamivudine 100 mg group. Nucleotide sequence analysis of the DNA polymerase coding region, using viral samples collected from these two patients at day 1 and at week 22, revealed no lamivudine-resistance substitutions (rt180 and rt204 amino acid residues) [17, 18] or entecavir-resistance substitutions (rt184, rt202, and rt250 amino acid residues) [19].

Safety

During the study, adverse events were experienced by similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg groups and the lamivudine 100 mg treatment group (97.1%, 97.1%, 91.2%, and 100.0%, respectively). Most adverse events were of mild or moderate intensity (grade 1/2) and transient. The most frequently reported adverse events (affecting ≥ 10% of patients in any one treatment group) included nasopharyngitis, headache, and

Table 4 Summary of adverse events and laboratory abnormalities during the 24-week blinded treatment phase

	ETV 0.01 mg (n = 35)	ETV 0.1 mg (n = 34)	ETV 0.5 mg (n = 34)	LVD 100 mg (n = 34)
Any adverse events	34 (97)	33 (97)	31 (91)	34 (100)
Most frequent clinical adverse events,[a] n (%)				
Nasopharyngitis	9 (25.7)	10 (29.4)	11 (32.4)	10 (29.4)
Headache	6 (17.1)	7 (20.6)	2 (5.9)	7 (20.6)
Diarrhea	1 (2.9)	1 (2.9)	4 (11.8)	4 (11.8)
Grade 3/4 clinical adverse events, n (%)	0	0	1 (2.9)	1 (2.9)
Grade 3/4 laboratory adverse events, n (%)	2 (5.7)	4 (11.8)	2 (5.9)	4 (11.8)
Any serious adverse events, n (%)	0	1 (2.9)	2 (5.9)	1 (2.9)
Discontinuations due to adverse events,[b] n (%)	0	0	1 (2.9)	1 (2.9)
ALT flares,[c] n (%)	0	1 (2.9)	1 (2.9)	2 (5.9)
Death, n (%)	0	0	0	0

[a] Occurring in at least 10% of patients

[b] One patient treated with ETV 0.5 mg discontinued the study drug due to hepatic cirrhosis. One patient treated with lamivudine discontinued due to increased ALT

[c] ALT flare defined ALT >2 × baseline and 10 × ULN

ETV entecavir

LVD lamivudine

diarrhea (Table 4). Grade 3/4 clinical adverse events occurred in one patient in the entecavir 0.5 mg group (colon carcinoma) and one patient in the lamivudine group (anal ulcer); neither of these events was considered to be related to the study drug. Serious adverse events were limited to the above-mentioned case of colon carcinoma, serum ALT elevation (entecavir 0.1 mg group [n = 1], entecavir 0.5 mg group [n = 1]), and serum aspartate aminotransferase (AST)/ALT elevation (lamivudine 100 mg group [n = 1]), but these were not considered to be causally related to the study drug and did not necessitate treatment discontinuation. Transient ALT flares (serum ALT >2 × baseline level and >10 × ULN) occurred in four patients (entecavir 0.1 mg group [n = 1], entecavir 0.5 mg group [n = 1], and lamivudine 100 mg group [n = 2]) and were associated with HBV DNA level decreases of 2 $\log_{10}$ copies/ml or more. None of the ALT flares were associated with hepatic decompensation and serum ALT and AST levels recovered to less than 1.25 × baseline level on continuation of the study treatment.

Discussion

The global ETV-005 study reported that entecavir was superior to lamivudine at reducing viral load in nucleoside-naive patients with CHB infection [15]. We conducted the present study, using an identical design to the ETV-005 study, to determine whether the findings from this earlier study are applicable to Japanese patients. In keeping with the previous findings, our results indicate that entecavir produces a dose-related reduction in serum HBV DNA level ($0.01 < 0.1 \leq 0.5$ mg) in nucleoside-naive Japanese patients with CHB; the log dose–response curves for the reduction in serum HBV DNA level with entecavir in the two studies were similar, with estimated regression curve slopes of –1.24 (Japanese study) and –1.32 (global study). In addition, both studies demonstrated the noninferiority of the entecavir 0.1 mg group compared with the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group compared with the lamivudine 100 mg group. The demonstration of a dose–response relationship for entecavir and the superiority of the entecavir 0.5 mg dose over lamivudine confirm that the antiviral activity of entecavir in Japanese patients is similar to that observed in study ETV-005. In a previous study, Ono et al. [14] demonstrated that the in vitro potency of entecavir was up to 2,200 times greater than that of lamivudine. The results presented here substantiate these earlier in vitro data and confirm the greater potency of entecavir over lamivudine in patients with CHB.

Serum ALT normalization rates with entecavir 0.5 mg and lamivudine 100 mg (~80%) were higher in the present study than those reported in the ETV-005 study (entecavir 0.5 mg, 69.0%; lamivudine 100 mg, 59.1%) [15]. In keeping with previous findings [20, 21], the incidence of entecavir-associated serum ALT flares in Japanese patients was low. The serum ALT flares occurred against a background of 2 $\log_{10}$ copies/ml or more reductions in serum

HBV DNA level, and serum ALT levels subsequently normalized without discontinuation of entecavir. Therefore, the serum ALT flare noted here may indicate recovery of the host's immune response arising from the reduction in HBV viral titer [22, 23]. ALT flares have been reported after the discontinuation of entecavir therapy [15, 16], thus necessitating long-term follow-up to identify possible posttreatment viral rebound.

In conclusion, the results of this dose-ranging study demonstrate a clear dose–response relationship for entecavir in terms of mean HBV DNA level reduction at week 22. Entecavir 0.5 mg was significantly more effective than lamivudine 100 mg in reducing HBV DNA levels in nucleoside-naive Japanese adult patients with CHB. At this dose level, entecavir treatment resulted in serum HBV DNA levels of less than 400 copies/ml in 100% of patients and normalization of serum ALT levels in 80% of patients after 22 weeks. Moreover, entecavir 0.5 mg once daily was well tolerated and showed a comparable safety profile to lamivudine.

References

1. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362–366. doi:10.1002/1096-9071(200007)61:3<362::AID-JMV14>3.0.CO;2-I
2. Kao JH, Chen DS. The natural history of hepatitis B virus infection. In Lai CL, Locarnini S, editors. Hepatitis B Virus. London: International Medical Press; 2002. 161–172
3. Lok AS, McMahon BJ. American Association for the Study of Liver Diseases (AASLD) practice guidelines. Chronic Hepatitis B. Hepatology 2007;45:507–539. doi:10.1002/hep.21513
4. Rivkin A. A review of entecavir in the treatment of chronic hepatitis B infection. Curr Med Res Opin 2005;21:1845–1856. doi:10.1185/030079905X65268
5. Wong DKH, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med 1993;119:312–323
6. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670–1677. doi:10.1002/hep.510270628
7. Chang TT, Lai CL, Chien RN, Guan R, Lim SG, Lee CM, et al. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J Gastroenterol Hepatol 2004;19(11):1276–1282. doi:10.1111/j.1440-1746.2004.03428.x
8. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351(15):1521–1531. doi:10.1056/NEJMoa033364
9. Omata M. Treatment of chronic hepatitis B infection. N Engl J Med 1998;339:114. doi:10.1056/NEJM199807093390209
10. Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. Hepatol Int 2008;2:263–283. doi:10.1007/s12072-008-9080-3
11. Genovesi EV, Lamb L, Medina I, Taylor D, Seifer M, Innaimo S, et al. Efficacy of the carbocyclic 2′-deoxyguanosine nucleoside BMS-200475 in the woodchuck model of hepatitis B virus infection. Antimicrob Agents Chemother 1998;42:3209–3217
12. Marion PL, Salazar FH, Winters MA, Colonno RJ. Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication. Antimicrob Agents Chemother 2002;46:82–88. doi:10.1128/AAC.46.1.82-88.2002
13. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. Hepatology 2006;44:1656–1665. doi:10.1002/hep.21422
14. Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, et al. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 2001;107:449–455. doi:10.1172/JCI11100
15. Lai CL, Rosmawati M, Lao J, Van Vlierberghe H, Anderson FH, Thomas N, et al. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. Gastroenterology 2002;123:1831–1838. doi:10.1053/gast.2002.37058
16. Chang TT, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, et al. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. Gastroenterology 2005;129:1198–1209. doi:10.1053/j.gastro.2005.06.055
17. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996;24:711–713. doi:10.1002/hep.510240339
18. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DLJ. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714–717
19. Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, et al. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to lamivudine. Antimicrob Agents Chemother 2004;48:3498–3507. doi:10.1128/AAC.48.9.3498-3507.2004
20. Chang TT, Gish R, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med 2006;354:1074–1076. doi:10.1056/NEJMoa051285
21. Sherman M, Yurdaydin C, Sollano J, Silva M, Liaw Y-F, Cianciara J, et al. Entecavir is superior to continued lamivudine for the treatment of lamivudine-refractory. HBeAg-positive chronic hepatitis B. Gastroenterology 2006;130:2039–2049
22. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968–975. doi:10.1172/JCI3731
23. Nair S, Perrillo RP. Serum alanine aminotransferase flares during interferon treatment of chronic hepatitis B: is sustained clearance of HBV DNA dependent on levels of pretreatment viremia? Hepatology 2001;34:1021–1026. doi:10.1053/jhep.2001.28459

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Abstract

Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.

Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.

Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.

Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/\text{mm}^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load (≥100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for ≤60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for > 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years ≥ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%≤ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	

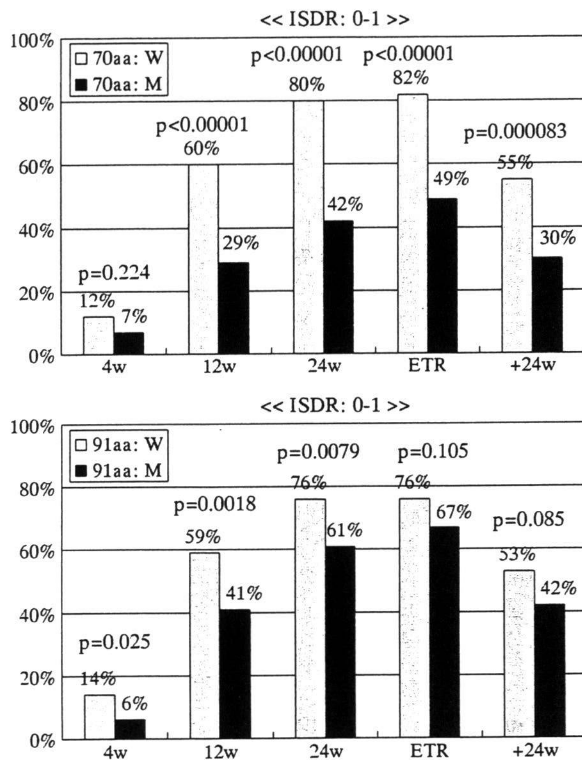

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/mm^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	*p* value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4$/mm^3)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4$/mm^3)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, *M* Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4$/mm^3 of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4$/mm^3. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

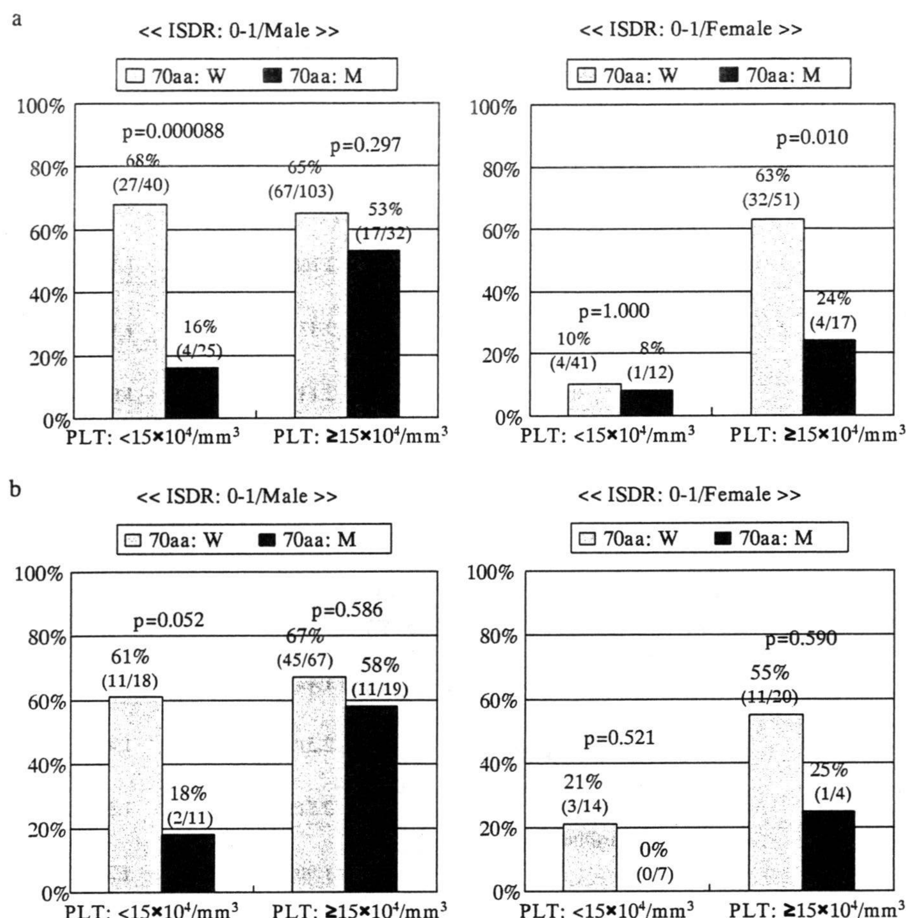

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/mm^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT count of less than $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**a**). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/mm^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/mm^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (**b**)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4/mm^3$)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, p = 0.0037) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

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References

1. Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet. 2001;358:958–65.
2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonzales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
3. Hadziyannis S, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alfa-2a plus ribavirin combination therapy in chronic hepatitis C. a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;40:346–55.
4. Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, et al. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. Hepatol Res. 2008;38:52–9.
5. Honda T, Katano Y, Urano F, Murayama M, Hayashi K, Ishigami M, et al. Efficacy of ribavirin plus interferon-α in patients aged 60 years with chronic hepatitis C. J Gastroenterol Hepatol. 2007;22:989–95.
6. Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, et al. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral load. Dig Dis Sci 2009;54:1317–24
7. Puoti C, Castellacci R, Montagness F, Zaltron S, Stornaiuolo G, Bergami N, et al. Histological and virological features and follow-up of HCV carriers with normal aminotransferase levels: the Italian Study of the Asymptomatic C Carriers (ISACC). J Hepatol. 2002;37:117–23.
8. Hui CK, Belaye T, Montegrande K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. J Hepatol. 2003;38:511–7.
9. Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, et al. A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. J Hepatol. 2005;43:599–605.
10. Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index in an independent risk factor for non response to antiviral treatment in chronic hepatitis C. Hepatology. 2003;38:639–44.
11. Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdi DM, Clouston AD, et al. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signaling 3 in patients with chronic hepatitis C, viral genotype 1. Gut. 2006;55:604–9.
12. Patton HM, Patel K, Behling C, Bylund C, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
13. Asselah T, Rubbia-Brandt L, Marcellin M, Negro F. Steatosis in chronic hepatitis C: why does it really matter? Gut. 2006; 55:123–30.
14. Romero-Gomez M, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. Gastroenterology. 2005;128: 636–41.
15. Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Cammozzi M, et al. Peginterferon alfa-2b plus ribavirin for native patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol. 2004;41:474–81.
16. Everson GT, Hoefs JC, Seeff LB, Bonkovsky HL, Naishadham D, Shiffman ML, et al. Impact of disease severity on outcome of antiviral therapy for chronic hepatitis C: lessons from the HALT-C trial. Hepatology. 2006;44:1675–84.
17. McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology. 2002;123:1061–9.
18. Jeffers LJ, Cassidy W, Howell CD, Hu S, Reddy R. Peginterferon alfa-2a (40kd) and ribavirin for black American patients with chronic HCV genotype 1. Hepatology. 2004;39:1702–6.
19. Muir AJ, Bornstein JD, Killenberg PG, Atlantic Coast Hepatitis Treatment Group. Peginterferon alfa 2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med. 2004;350:2265–71.
20. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomized trial of interferon alpha 2b plus ribavirin for 48 weeks or 24 weeks versus interferon alpha 2b plus placebo for 48 weeks for treatment for chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. 1998;352:1426–32.
21. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5 A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
22. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response in interferon-ribavirin combination therapy. Intervirology. 2005;48:372–80.
23. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the

full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol. 2007;81:8211–24.

24. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
25. Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales FL, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a ribavirin. J Hepatol. 2005;43:425–33.
26. Moucari R, Ripault M-P, Oules V, Martinot-Peignoux M, Asselah T, Boyer N, et al. High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. J Hepatol. 2007;46:596–604.
27. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1a: amino acid substitutions in the core region and low-density lipoprotein cholesterol level. J Hepatol. 2007;46:403–10.
28. Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: grading and staging. Hepatology. 1994;19:1513–20.
29. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA. 1990;87:9524–8.
30. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocelluarlr carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. J Hepatol. 1999;30:653–9.
31. Okanoue T, Itoh Y, Minami M, Hashimoto H, Yasui K, Yotsuyanagi H, et al. Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet count. Hepatol Res. 2008;38:27–36.
32. Iwasaki Y, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis. Hepatology. 2006;43:54–63.
33. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
34. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
35. Lagging M, Romero A, Westin J, Norkrans G, Dhillon AP, Palwlosky JM, et al. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. Hepatology. 2006;44:1617–25.
36. Pilli M, Zerbini A, Penna A, Orlandini A, Lukasiewicz E, Pawlotsky JM, et al. HCV-specific T-cell response in relation to viral kinetics and treatment outcome (DITTO-HCV Project). Gastroenterology. 2007;133:1132–43.
37. Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribabirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008; 48:1753–60.

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ORIGINAL ARTICLE

Successful hepatitis B vaccination in liver transplant recipients with donor-specific hyporesponsiveness

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Summary

Currently, patients are prescribed lifelong treatment with hepatitis B immunoglobulin (HBIg) after liver transplantation (LT) for hepatitis B virus (HBV)-related diseases in order to prevent reinfection with HBV. Active immunization with an HBV vaccine would be a preferable alternative; however, the immunosuppressive environment in LT recipients is believed to elicit a poor response to vaccination. Minimizing the exposure of the HBV-infected LT recipients to immunosuppressants would be beneficial in inducing adaptive immunity against HBV by vaccination. In this study, in addition to efforts to minimize immunosuppression, prophylaxis with HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation attributable to HBV-related diseases. During the observation period, the overall response rate to HBV vaccination was 64.7%. The immune status of the recipients was evaluated by a mixed lymphocyte reaction assay in response to allostimulation. Patients showing a donor-specific hyporesponse with a well-maintained response to the third-party stimulus always achieved a sustained immune response to the vaccine, whereas patients showing a hyporesponse to both the donor and the third-party stimulus were unable to do so. Thus, inducing an anti-donor-specific immunosuppressive status by minimizing immunosuppression should enable post-transplant HBV vaccination to be a promising prophylactic strategy.

Introduction

Patients face a high risk of endogenous hepatitis B virus (HBV) reinfection in the absence of postoperative prophylaxis after liver transplantation (LT) caused by HBV-related disease. Combined treatment with either a nucleoside or nucleotide analog and hepatitis B immunoglobulins (HBIg) has been the gold standard for prophylaxis of HBV reinfection after LT [1–3]. According to current recommendations, HBIg should be administered indefinitely after LT [4–6]. However, indefinite prophylaxis with HBIg has substantial drawbacks, such as increasing costs [7] and the risk of emergence of HBV envelope protein mutations [8,9]. Therefore, induction of an active immune response against the hepatitis B surface antigen (HBsAg), leading to the continuous production of specific antibodies would be

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an enormous advantage, and it would eliminate the need for lifelong replacement with HBIg [10,11].

Several groups have attempted vaccination of LT recipients against HBV [11–20]. In most of these studies, relatively low seroconversion rates as well as serum anti-HBs concentrations were observed among chronic HBV-infected LT recipients; only a minority of vaccinees developed stable antibody levels >100 IU/l, the maintenance of which is required for prevention of HBV reinfection [21]. The poor response to vaccination was probably because of the immunosuppressive environment in LT recipients. Minimizing the exposure of HBV-infected LT recipients to immunosuppressants appears to be beneficial in inducing adaptive immunity against HBV by vaccination; however, the relevance of the immune status of LT recipients to the outcome of HBV vaccination remains to be elucidated.

In this study, prophylactic HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation because of an HBV-related disease and had not experienced signs of recurrence for at least 12 months after treatment with HBIg. The immune status of these patients was evaluated by a mixed lymphocyte reaction (MLR) assay in response to anti-donor and third-party allostimulation using an intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique.

Patients and methods

Patients

In this study, we included 17 living donor LT recipients at the Hiroshima University Hospital. All patients had normal liver function without any virologic and biochemical evidence of HBV recurrence. The following were the inclusion criteria: (i) at least 3 months of HBIg plus lamivudine (100 mg/day) with/without adefovir (10 mg/day) administration and (ii) no findings of recurrent infection and negativity for HBsAg and hepatitis B viral deoxyribonucleic acid (HBV DNA) (by PCR) at the time of vaccination. For prophylaxis against reinfection, all transplanted patients were on a stable schedule of 1000–2000 IU of intravenous HBIg every 4 weeks in order to maintain an anti-HBs titer of >100 IU/l. We attempted to minimize immunosuppression in all patients with good liver function by adopting the policy of tapering off the immunosuppressants. The study protocol was approved by the Ethics Committee of Hiroshima University, and all patients provided informed consent before entering into the trial. None of the vaccinees showed clinical evidence of recurrence of HBV graft infection and the episode of rejection throughout the follow-up period, and all of them were persistently negative for both HBsAg and HBV DNA, except for one vaccinee (Patient #3) who showed temporarily positive for HBV DNA.

Vaccination protocol

All participants received a yeast-derived recombinant, adsorbed HBV vaccine (Bimmugen®; Chemotherapy and Serotherapy Laboratories Inc., Kumamoto, Japan) subcutaneously every 4 weeks at a dose of 10–20 μg (0.5–1.0 ml) in combination with HBIg and lamivudine/adefovir. HBIg immunoprophylaxis was continued during primary immunization (dose, 1000–2000 IU every 4 weeks). The response to vaccination was defined as (i) a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and (ii) sustained anti-HBs titer to >100 IU/l after discontinuation of combined administration of the vaccine and HBIg. If the anti-HBs titer exceeded the responsive increasing level, HBIg substitution and vaccine administration were discontinued. Lamivudine/adefovir prophylaxis was additionally discontinued, if the anti-HBs titer was maintained effectively without HBIg administration. The vaccine was continuously and indefinitely administered till acquired immunity was elicited.

Serologic markers and virologic assays

Serum HBsAg, hepatitis Be antigen (HBeAg), hepatitis B core antibody (HBcAb), and anti-HBsAb were measured monthly using an enzyme-linked immunoassay (Abbott Diagnostics, Chicago, IL, USA). HBV DNA was detected by the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). These quantitative assays of HBV DNA were performed at the Special Reference Laboratory, Tokyo, Japan. Positive levels of HBV DNA were defined as levels >2.6 log copies/ml. HBV recurrence was diagnosed on the basis of appearance of HBsAg or HBV DNA.

Immune monitoring by *in vitro* CFSE-MLR assay

For patients who showed completely normal liver function, CFSE-MLR was performed to determine whether immunosuppression could be further minimized. In patients with hyporesponse of anti-donor T cells, immunosuppression was successfully reduced.

For CFSE-MLR, the peripheral blood mononuclear cells prepared from the blood of the LT recipients (autologous control), donors, and healthy volunteers with same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy and those obtained from the recipients for use as the responder cells

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were labeled with 5 μM CFSE (Molecular Probes Inc., Eugene, OR, USA), as described previously [22]. The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates (BD Labware, Franklin Lakes, NJ, USA) in a total volume of 2 ml of culture medium at 37 °C under 5% CO_2 for 5 days. After culture for MLR, the harvested cells were stained with either phycoerythrin (PE)-conjugated anti-human CD4 or PE-conjugated anti-human CD8 monoclonal antibodies (mAbs; BD Pharmingen, San Diego, CA, USA) and subjected to analysis by flow cytometry (FCM). All analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from the analysis by forward scatter or propidium iodide gating. T-cell proliferation was visualized by serial-halving of the fluorescence intensity of CFSE. $CD4^+$ and $CD8^+$ T-cell proliferation and stimulation index (SI) were quantified using a previously described method [23,24]. Briefly, the number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each $CD4^+$ and $CD8^+$ T-cell subset was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of self-control.

Statistical analysis

The values are presented as the median and the range. The Mann–Whitney *U*-test was performed to analyze whether the age of the vaccinees at the time of vaccination, the time elapsed since LT, the anti-HBsAb titers at the start of the vaccination, the median tacrolimus trough levels, and the SI in anti-donor and anti-third-party MLR differed significantly between the good and poor responders and also between the moderate and poor responders. A Fisher's exact test was performed to determine whether there were differences between both the above groups with regard to gender, indication for LT, ratio of HBV DNA and HBeAg negative before LT, ratio of donor HBc and HBsAb positive before LT, and immunosuppressive monotherapy at the time of vaccine administration. *P*-values below 0.05 were considered statistically significant.

Results

Demographics

A total of 17 HBV vaccinees (four female- and 13 male subjects; age range, 20–65 years; median age, 49 years) participated in this study. The demographic and clinical data of the participants are shown in Table 1. Of them, 14 patients underwent LT for HBV-related cirrhosis and three underwent transplantation for HBV-related fulminant hepatic failure. Among the 17 vaccinees, five (29.4%) had been HBV DNA positive before LT with levels >2.6/ml, and five (29.4%) had been HBeAg positive before LT. Immunosuppressive treatment comprised either cyclosporine or tacrolimus monotherapy in 11 patients (64.7%) and additional steroid therapy (methylprednisolone, 2–4 mg/day) in six patients. Steroids were withdrawn at after a median duration of 13 months (range, 1–50 months) after LT. At the time of vaccination, a median duration of 21 months (range, 3–41 months) had elapsed since LT. The median follow-up time after commencement of vaccination was 26 months (range, 8–72 months). At the start of vaccination, a median anti-HBsAb titer was 161.4 (range, 37.7–328.4) IU/l.

Response to vaccination

During the observation period, 11 of the 17 HBV vaccinees (64.7%) achieved a sustained immune response to the HBV vaccine, which was defined as a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and no decrease in the anti-HBs titer to <100 IU/l even after discontinuation of combined administration of the vaccine and HBIg (Table 1). Within 1 year, 5/11 responders responded to the vaccine, and other six responded after 1 year from the commencement of vaccination (Fig. 1a and b). The other six HBV vaccinees did not respond to the vaccine during the study period (Fig. 1c). When the subjects were divided into three distinct groups, i.e., patients who responded to the vaccine within 1 year after commencement of vaccination (good responders), patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders), and patients who did not respond to the vaccine within 1 year and still remain receiving the vaccine (poor responders), the following factors did not exhibit statistically significant differences between the good and poor responders and also between the moderate and poor responders: age, gender, indication for LT, HBV viremia, donor HBcAb and HBsAb before LT, immunosuppressive regimen and tacrolimus trough levels and anti-HBsAb titers at the time of vaccination, duration between vaccination and transplantation and also duration between steroid withdrawal and transplantation. (Table 2) (Fig. 2).

Estimation of immunosuppressive status during vaccination by CFSE-MLR assay

Eleven patients (#1, 2, 4, 5, 7, 9, 11, 12, 13, 14 and 17) and their donors consented to be subjected to an

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Table 1. The demographic and clinical characteristics of patients.

Patient	Age*	Gender	Underlying disease	HBV DNA before LT	Recipient HBeAg before LT	Donor HBcAb before LT	Donor HBsAb before LT	Time of vaccination†	Time of steroid withdrawal†	Duration of follow-up‡	Immuno-suppressive drugs*	Tac/CsA trough level (ng/ml)*	Anti-HBsAb titer (IU/l)*
Patients who responded to the vaccine within 1 year after commencement of vaccination (good responders)													
1	62	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	41	3	34	CsA 50 mg	39.3 (CsA)	152.6
2	54	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	26	2	35	CsA 50 mg	15.0 (CsA)	189.1
3	58	M	Cirrhosis	6.4	Positive	Negative	Negative	9	2	43	Tac 2 mg	4.6	161.0
4	43	M	Cirrhosis/HCC	3.4	Negative	Negative	NA	35	45	20	Tac 3 mg + mPSL 2 mg	1.5	220.6
5	57	M	Fulminant	<2.6	Negative	Negative	Negative	9	1	15	Tac 2 mg	3.4	37.7
Patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders)													
6	34	M	Fulminant	<2.6	Negative	NA	NA	3	7	72	Tac 6 mg + mPSL 4 mg	4.2	152.1
7	38	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	35	7	49	Tac 1 mg	4.4	146.6
8	57	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	40	50	31	Tac 2 mg + mPSL 4 mg	4.4	68.3
9	46	F	Cirrhosis/HCC	4.6	Positive	Negative	Positive	17	2	33	Tac 3 mg	4.7	93.4
10	46	F	Cirrhosis	<2.6	Negative	Positive	Positive	20	1	22	Tac 1 mg	4.2	214.9
11	53	M	Cirrhosis/HCC	<2.6	Negative	Positive	Positive	13	4	15	Tac 2 mg	4.2	160.5
Patients who did not respond to the vaccine during the study period (poor responders)													
12	20	M	Fulminant	>7.6	Positive	Negative	Positive	18	29	20	Tac 3 mg + mPSL 2 mg	5.0	222.7
13	46	M	Cirrhosis	<2.6	Negative	Negative	Negative	16	1	20	Tac 1 mg	6.6	188.9
14	58	F	Cirrhosis/HCC	<2.6	Negative	NA	NA	18	20	11	Tac 2 mg + mPSL 2 mg	1.5	92.3
15	65	M	Cirrhosis/HCC	4.5	Positive	NA	NA	12	21	13	Tac 4 mg + mPSL 4 mg	8.0	328.4
16	45	F	Cirrhosis/HCC	<2.6	Negative	NA	Negative	25	23	8	Tac 0.5 mg	3.7	193.3
17	54	M	Cirrhosis/HCC	<2.6	Positive	Positive	Positive	13	1	9	Tac 2 mg	2.9	122.2

LT, liver transplantation; Tac, tacrolimus; CsA, cyclosporine; mPSL, methylprednisolone; NA, not available.
*At the time of vaccination.
†Months after liver transplantation.
‡Months after commencement of vaccination.

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