Table 1
Clinical characteristics of patients ($n = 49$).

Gender (male/female)	38/11
Age (years) (mean ± SD)	58 ± 11 (29–80)
Etiology (HBV/HCV/alcohol/others)	8/30/8/3
Child-Pugh classification (A/B/C)	5/34/10
Variceal size (F1/F2/F3)[a]	10/28/11
Red color sign (RC0/RC1/RC2/RC3)[a]	6/27/15/1
HCC (presence/absence)	21/28
HCC stage (1/2/3/4)[b]	0/4/7/10
Vp (0,1,2/3,4)[b]	32/17
Total bilirubin (≤2/>2) (mg/dl)	34/15
Serum albumin (≥3.0/<3.0) (g/dl)	26/23
Cr (<1.0/≥1.0) (mg/dl)	44/5
Previous treatment for esophageal varices (yes/no)	18/31

HCC: hepatocellular carcinoma.
[a] Evaluated according to the classification system of the Japanese Society for Portal Hypertension and Esophageal Varices.
[b] Classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer [11].

tion system of the Japanese Society for Portal Hypertension and Esophageal Varices [10]. The form (F) of EV was classified as complete eradication after treatment (F0), small straight (F1), enlarged tortuous (F2), and large coiled-shaped (F3). Red color sign (RC) was defined as endoscopically detected dark red spots on the mucosa of the lower esophagus. To evaluate the risk of hemorrhage and provide a rough estimate of intravascular pressure within the EV, RC was classified into four grades: RC0: no mucosal coloring; RC1: a few localized red spots; RC2: between RC1 and RC3; and RC3: several mucosal red spots throughout the circumference of the lower esophagus. Endoscopic findings of EV before treatment were F1 in 10 patients, F2 in 28 patients, and F3 in 11 patients, as well as RC0 in 6 patients, RC1 in 27 patients, RC2 in 15 patients, and RC3 in 1 patient. HCC was present in 21 patients and absent in 28 patients. HCCs were classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer [11]. The stage of HCC before treatment was evaluated as stage II in 4 patients, stage III in 7 patients, and stage IV in 10 patients; no patients showed stage I HCC. The severity of tumor thrombus in the portal vein was Vp 0, 1, or 2 in 32 patients and Vp 3 or 4 in 17 patients.

The institutional review board approved this study, which was based on the Declaration of Helsinki as declared by the World Health Organization. All subjects gave informed consent.

2.2. EVL procedure

The endoscopic variceal device (Sumitomo Bakelite, Tokyo, Japan) consists of an outer housing cylinder, an inner banding cylinder, and an elastic O-ring. After confirming variceal bleeding and the need for banding, the endoscope was removed and the banding device assembled by securing the housing cylinder to the end of the scope. The target varices were identified and the scope advanced under direct vision until the banding cylinder was in full 360° contact with the varices. Suction was then applied by depressing the endoscopic aspiration control valve, thus drawing the varices and surrounding mucosa into the banding chamber. Once the chamber was completely filled, the elastic band over the varices was ejected. The engorged varices were thus strangulated at the mucosal junction [12].

2.3. EIS procedure

The EIS technique was designed to embolize EV feeding vessels within the portosystemic collaterals by injecting a sclerosing agent. A balloon, referred to as the oral side balloon in this study, was attached to the tip of an endoscope (model GIF-XQ 240, Olympus, Tokyo) and inflated as iopamidol contrast medium (Iopamiron; Schering, Berlin, Germany) was injected to prevent the sclerosant agent (5% ethanolamine oleate) from flowing out of the varices into the systemic circulation. The flow of sclerosant was monitored using X-ray fluoroscopy from the start of injection into the EV. The injection was terminated when the sclerosant started to fill the portosystemic collaterals.

2.4. CT examination

When the CT located the liver, the contrast medium: 100 ml of iopamidol 300 heated to 37 °C, was injected using a power injector (Auto Enhance A-250; Nemoto-Kyorindo, Tokyo), at a rate of 4.0 ml/s through a 22-gauge IV catheter inserted into an antecubital vein. Four sets of images were acquired in a craniocaudal direction at 20, 40, 65, and 180 s after injection of the contrast medium. The first and second acquisitions were used for hepatic arterial phase imaging, the third acquisition for portal venous phase imaging, and the fourth acquisition to image the hepatic venous phase. The third set of images was obtained during 20-s breath holding, while those of other acquisitions were achieved during 10-s breath holding. This protocol is routinely used in all patients with chronic liver disease at our institution, and the data of the third acquisition are used for construction of three-dimensional (3D) images of the portosystemic collaterals. All scans were performed on a LightSpeed QX/I CT scanner (Generic Electric Medical Systems, Milwaukee, WI) using the high-quality scan mode, at 1.25-mm slice thickness, and reconstruction intervals of 0.625 mm for portal venous phase imaging. MDCT was performed using a Virtual Place Advance (AZE, Tokyo) [13]. There are currently three reformatting techniques available, but MPR was used for image reconstruction in this study. CT examination was performed in every patient, within 1 month prior to endoscopic treatment and after the final session.

2.5. Evaluation of portosystemic collaterals

Portosystemic collaterals were assessed independently on MDCT-MPR imaging before and after endoscopic treatment for EV. The diameter of the main portosystemic collateral vessel such as the left gastric vein (LGV), paraesophageal vein (PEV), which drains the EV, was measured at the thickest portion of the vessel before and after endoscopic treatment. Changes in the feeding vessels after endoscopic treatment were estimated by calculating the rate of reduction, using the following formula: rate of reduction of the diameter of feeding vessel (%) = [(diameter of feeding vessel before endoscopic treatment − diameter of feeding vessel after endoscopic treatment)/diameter of feeding vessel before endoscopic treatment] × 100. Patients were divided into two groups according to the rate of reduction of the vessel diameter. Patients with a reduction rate of >20% were classified as the narrowing group, while those with a rate of ≤20% were classified into the no-change group. The cutoff rate of 20% represented the median reduction of the feeding vessel diameter in this series. Patients who showed no enhancement of the feeding vessel on MDCT were included in the narrowing group. Moreover, based on the diameter of PEV, which drains the EV, patients were divided into two groups: the large PEV group (≥3 mm) and the small PEV group (<3 mm). A cutoff diameter of 3 mm represented the median PEV diameter. Patients with PEV too narrow to be recognized on MDCT were classified as the small PEV group.

2.6. Follow-up

Radical endoscopic treatment such as EIS and EVL was repeated after one to two weeks, when needed to complete one treatment series and eliminate all varices. Additional treatment was applied until EV was classified as F0 endoscopically. Rebleeding after endo-

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scopic treatment was also assessed by endoscopy performed at 6-month intervals after treatment. We defined rebleeding of EV as the primary endpoint and survival as the secondary endpoint. The relationship between hemodynamic changes in the portosystemic collaterals and prognosis of patients with hemorrhagic EV after endoscopic treatment was analyzed by MDCT-MPR imaging and image analysis.

2.7. Statistical analysis

The cumulative rebleeding rate and cumulative survival rate between the groups classified according to rate of reduction after treatment were determined using the Kaplan–Meier method and statistical software (SPSS, Chicago, IL). Significance was tested using a generalized log-rank test and *t*-test. A *P* value < 0.05 was regarded as statistically significant.

3. Results

3.1. Portosystemic collaterals recognized by MDCT

Portosystemic collaterals were recognized on MDCT-MPR images of all patients with EV (Table 2). The LGV was the main EV feeding vessel in all patients, with a mean diameter of 5.8 mm. PEV was detected by MDCT-MPR in 80% of patients, with a mean diameter of 3.4 mm.

Twenty-four patients were classified as the narrowing group while 25 patients were considered the no-change group based on the rate of diameter change of portosystemic collaterals after endoscopic treatment. Fig. 3a and b shows typical MPR images of portosystemic collaterals in a patient of the narrowing group and another of the no-change group. Of patients who underwent EIS, 24 of the narrowing group and 13 of the no-change group, while all patients who underwent EVL were of the no-change group. However, the method of endoscopic treatment did not influence the rebleeding rate.

3.2. Cumulative rebleeding rates

The cumulative rebleeding rates of EV after endoscopic treatment were 14, 20, 28, and 42% at 1, 2, 3, and 5 years after treatment, respectively, for all patients (Fig. 4a). The median follow-up period was 26 months. Fig. 4b shows the cumulative rebleeding rates according to changes in the portosystemic collaterals after endoscopic treatment. The rates at 1, 2, 3, and 5 years after endoscopic treatment were 10, 15, 23, and 23%, respectively, for patients in the narrowing group, and 17, 24, 35, and 67%, respectively, for no-change group patients. There were no significant differences in cumulative rebleeding rates between the narrowing and no-change groups ($P = 0.068$).

3.3. Cumulative rebleeding rates with or without PEV

Patients in both groups were divided further based on PEV diameter, using a cutoff value of 3 mm. There were no significant differences in cumulative rebleeding rates between the large PEV group and small PEV group after esophageal treatment in patients of the narrowing group. However, in the no-change group, cumulative rebleeding rates at 1, 2, 3, and 5 years after endoscopic treatment were 7, 7, 20, and 60%, respectively, for the large PEV group and 32, 66, 100, and 100%, respectively, for the small PEV group (Fig. 4c). Thus, the diameter of PEV significantly influenced the cumulative rebleeding rates only in the no-change group after endoscopic treatment ($P = 0.027$). The cumulative rebleeding rates of the small PEV group in no-change patients were significantly higher than those measured in the narrowing group ($P = 0.018$). However, no significant change in cumulative rebleeding rates was apparent between the large PEV group in no-change patients and the narrowing group ($P = 0.435$).

Table 2
Portosystemic collaterals identified on multidetector-row computed tomography images.

Portosystemic collaterals	Percentage	Diameter (mm)[a]
Left gastric vein	100% ($n = 49$)	5.8 ± 2.2 (3.3–15)
Paraesophageal vein	80% ($n = 39$)	3.4 ± 1.5 (2.0–8.8)

[a] Data are mean ± SD.

3.4. Cumulative survival rates

The overall cumulative survival rates after endoscopic treatment were 67, 58, 46, and 34% at 1, 2, 3, and 5 years, respectively (Fig. 5). There were no significant differences in cumulative survival rate between the narrowing group and no-change group. The cause of death in this study was HCC in 14 patients, liver failure in 11 patients, other disease in one patient, and variceal rebleeding in 2 patients. The remaining 21 patients are still alive.

4. Discussion

This study retrospectively examined changes in portosystemic collaterals on MDCT-MPR imaging, as well as rebleeding rates and survival rates, after endoscopic treatment of 49 patients with hemorrhaged EV. The cumulative rebleeding rates in patients showing a feeding vessel diameter reduction rate of >20% (narrowing group) after endoscopic therapy tended to be lower than those in no-change group. Moreover, the PEV diameter was significant to the cumulative rebleeding rates only in the no-change group, with rates significantly lower in patients with larger PEV compared to those with smaller PEV. Despite this, there was also no difference in the cumulative rebleeding rates between narrowing group and no-change group with large PEV diameters. These results therefore suggest a close relationship between cumulative rebleeding rates after endoscopic therapy and portosystemic collaterals on MDCT-MPR imaging. No such relationship was identified between cumulative survival rates after endoscopic therapy and portosystemic collaterals.

MDCT achieves more rapid acquisition and higher longitudinal resolution than single-detector CT [14]. The accompanying use of MPR significantly improves the imaging of portosystemic collaterals and the sites of confluence [15,16]. Noninvasive MDCT with MPR before endoscopic therapy for patients with EV thus provides detailed information on the hemodynamics of the portosystemic collaterals [15]. The present study used this technique in patients with bleeding EV to evaluate the relationship between changes in portosystemic collaterals and prognosis [8].

The results of our study using MDCT-MPR imaging in patients with EV emphasized the importance of eradication of feeding vessels by endoscopic treatment in reducing the rebleeding rate. EV rebleeding rates were definitely higher in patients with inadequate eradication of the feeding vessel than in those with adequate eradication. However, even in patients of the former group, such as the no-change group in the present study, EV rebleeding rates were significantly lower in patients with a large PEV than in patients with a small PEV. It is probable that a large-diameter PEV enhances vein drainage from LGV to PEV, thus making EV rebleeding less likely even in patients without complete eradication of the feeding vessel. Thus, obliterating EV on the esophageal mucosa alone by EVL, without eradicating the feeding vessel, might favor low EV rebleeding rates [9].

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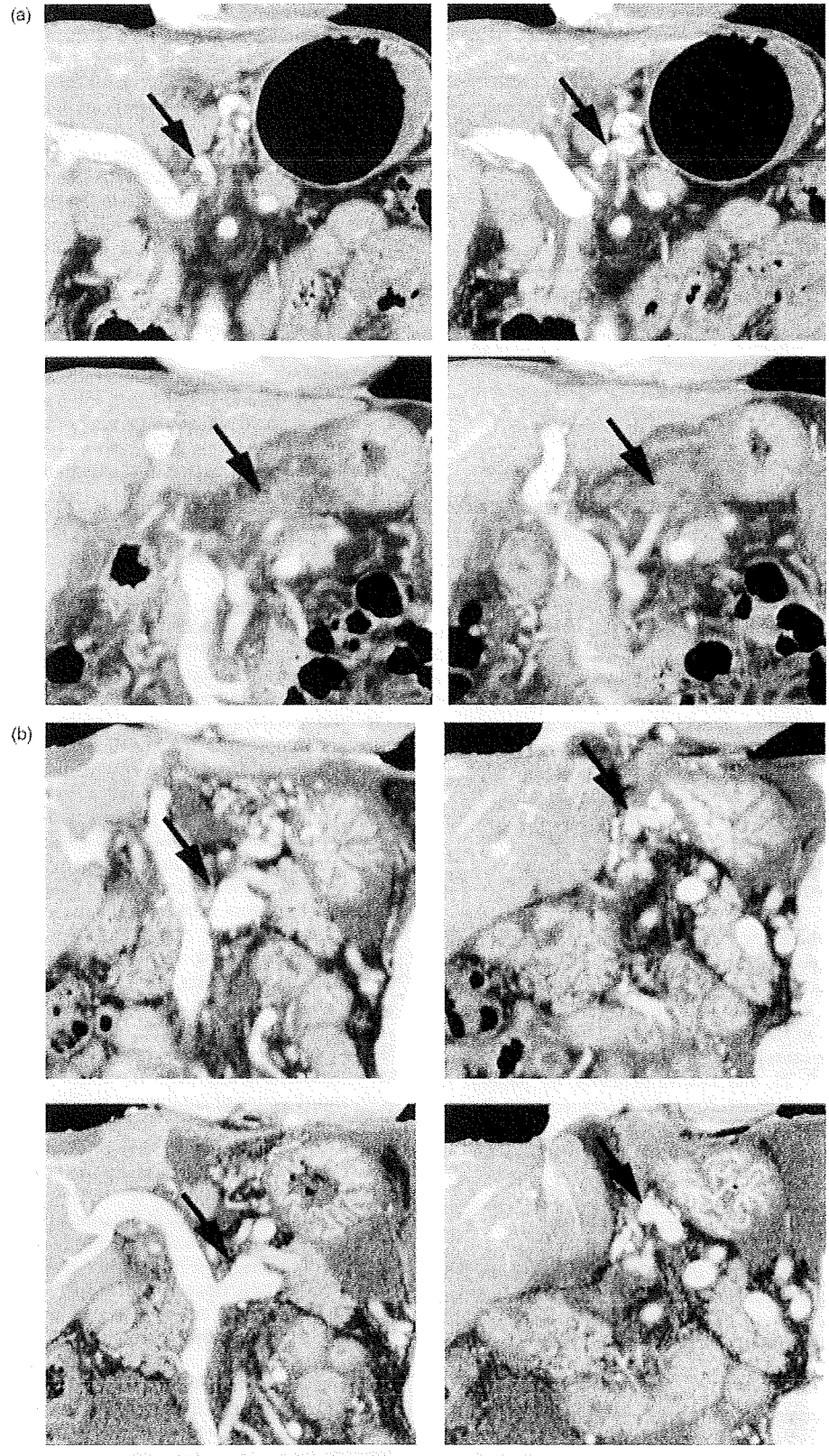

Fig. 3. Typical multiplanar reconstruction images of portosystemic collaterals in representative patients of the narrowing group (a) and no-change group (b), showing left gastric vein (arrow). *Top*: before endoscopic treatment and *Bottom*: after endoscopic treatment. See text for definition of each group.

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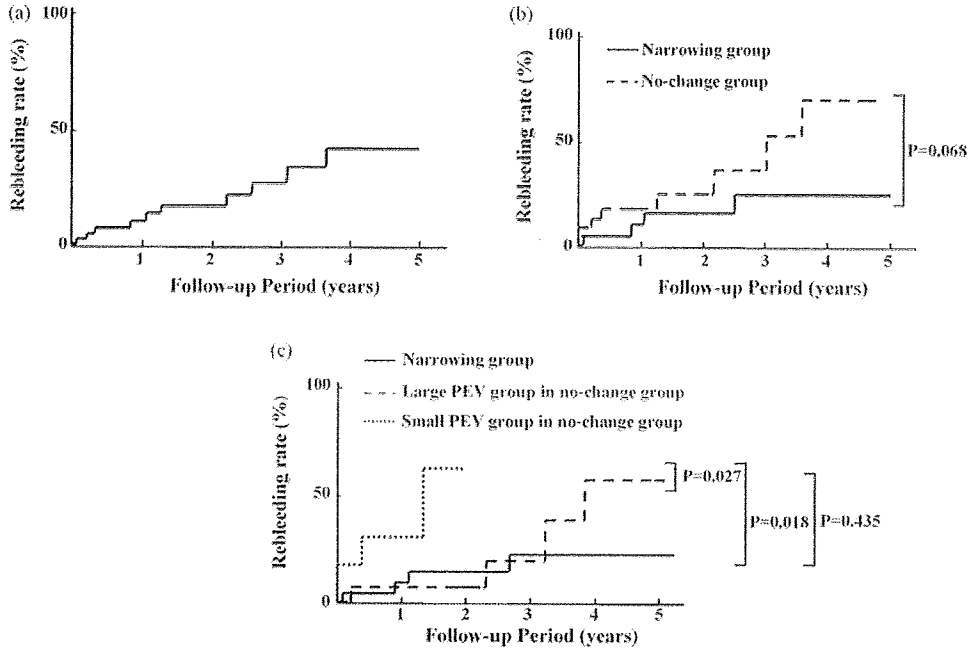

Fig. 4. (a) Cumulative rebleeding rates of hemorrhagic esophageal varices after endoscopic treatment for all patients. (b) Cumulative rebleeding rates of hemorrhagic esophageal varices according to the rate of reduction of feeding vessel after endoscopic treatment. (c) Cumulative rebleeding rates of hemorrhagic esophageal varices according to the rate of reduction of feeding vessel and development of paraesophageal vein after endoscopic treatment.

The guidelines in western countries for treatment of acute variceal bleeding recommend EVL and pharmacological treatment [2–4], as well as reduction in the hepatic venous pressure gradient (HVPG) [17], to prevent rebleeding of EV. On the other hand, our results showed a close relationship between the outcome of endoscopic treatment for variceal bleeding and variability in the portosystemic collaterals. Monitoring changes in these vessels by MDCT-MPR imaging after endoscopic treatment might therefore prove useful to predict outcome and to select the subsequent EV treatment modality, such as EIS or EVL. For example, EIS might be preferable after hemorrhagic EV for patients with a small-diameter PEV to obliterate the feeding vessel completely. On the other hand, EVL only might be necessary for patients with a large-diameter PEV to eradicate EV on esophageal mucosa alone. The results highlight the potential clinical importance of evaluating portosystemic collaterals by MDCT-MPR imaging, used in conjunction with endoscopic treatment, to manage acute variceal bleeding. Although MDCT-MPR images are of high quality, this technology has limitations. The technique can indeed measure vessel diameter, but not the equally important factors of blood pressure and direction of blood flow. Further evaluation of both HVPG and the portosystemic collaterals might therefore be beneficial in assessing the treatment effect.

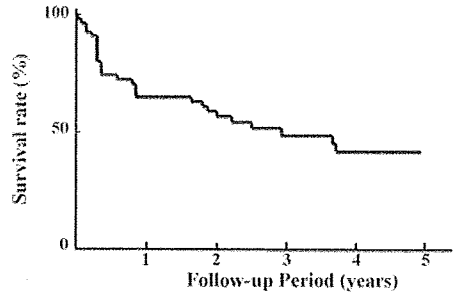

Fig. 5. Cumulative survival rates of hemorrhagic esophageal varices after endoscopic treatment for all patients.

5. Conclusion

Assessing the portosystemic collaterals using MDCT-MPR imaging could help predict EV rebleeding after endoscopic treatment and might prove valuable in the selection of subsequent endoscopic therapy.

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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 $15 \times 10^4/mm^3$ 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 $\geq$ 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%$\leq$ 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%)	

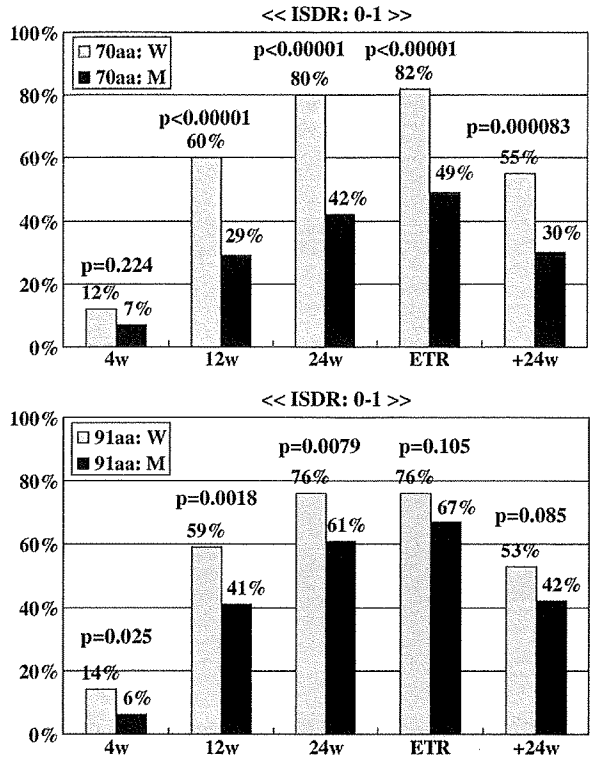

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core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

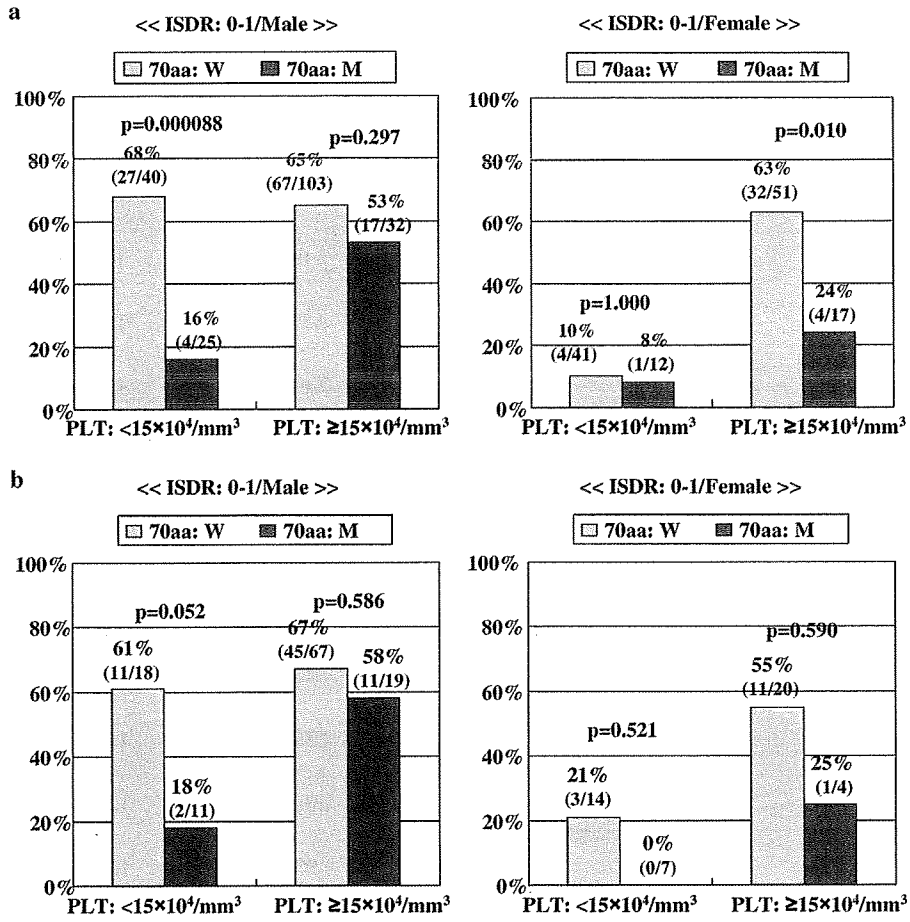

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino 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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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

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

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