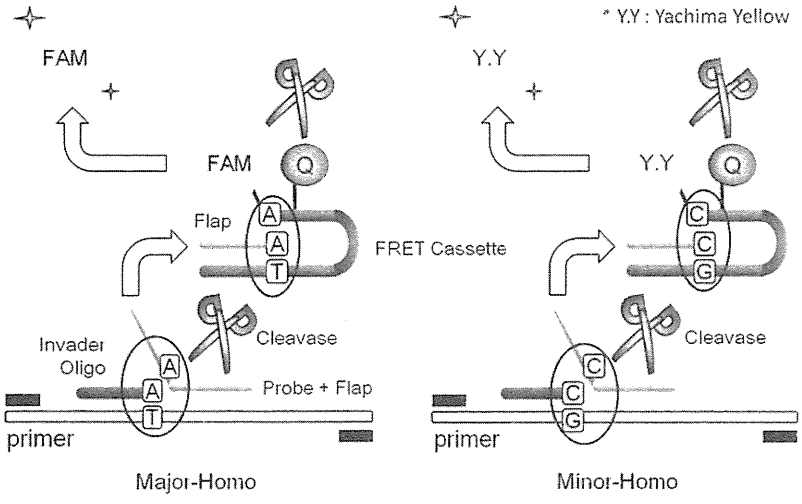

Fig. 1. Principle of the Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (for rs8099917). First, an Invader Oligo and a probe are annealed to amplified target DNA, overlapping at the SNP position, which clipped out the 5′flap fragment by the cleavase enzyme. Second, the released 5′flapanneals to the fluorescence resonance energy transfer (FRET) cassette and initiates a secondary cleavage reaction that releases the fluorescent dye. The signal is only released when the invasive structure is formed on the target DNA. 'Major allele' is left FAM signal, 'Minor allele' is right Yackima-Yellow (Y.Y) signal, and 'Hetero-type' observed both merged signals, respectively. The fluorescent dye is between major and minor alleles at rs12979860.

incubated at 99°C for 10 min to inactivate the Taq polymerase. Next, the reaction temperature is lowered to 63°C for 15 min to permit hybridization of the probe oligonucleotide and formation of the overlap flap structure (Fig. 1). Data were analyzed by endpoint genotyping software (Roche Diagnostics).

TaqMan PCR assay

The rs8099917 polymorphism was determined using TaqMan Pre-Designed SNP Genotyping Assays, as recommended by the manufacturer (Life Technologies, Carlsbad, CA, USA). The Custom TaqMan SNP Genotyping Assay Service MGB probe was used to determine the genotype of rs12979860 (Life Technologies). Each genome DNA sample (10 ng) was amplified using the master mix reagent of LightCycler480 Probe Master (Roche Diagnostics). The assays were carried out using the LC480II under the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles: 15 s at 95°C, and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

Direct sequencing assay

Before proceeding to *IL28B* SNP genotyping by the two methods, 105 DNA samples were genotyped using direct sequencing. To determine the SNP genotype of rs8099917 and rs12979860, the specific primer sets, rs8099917F (5′-AAGTAACACTTGTTCCTTRTAAAAGATTCC-3′) and rs8099917R (5′-CGCTATAATTAAAGATGTGGGAGAA-TGCAA-3′), rs12979860F (5′-CACGGTCGTGCCTGTC-GTGT-3′) and rs12979860R (5′-TGTGCTGTGCCTTCA CGCTCCGAGCA-3′) were used, respectively (Life Technologies). The amplification products were sequenced directly in both forward and reverse directions with Prism Big Dye (Life Technologies) on an ABI3100 DNA automated sequencer (Life Technologies).

RESULTS

Assay validation

In this study, the performance characteristics of the assay were determined before experimental testing. To assess the accuracy of *IL28B* SNP genotyping based on both rs8099917 and rs12979860, the results from the Invader Plus genotyping assay were compared with the results from direct sequencing. Of the 105 DNA samples, 70 samples had the major homozygous allele, 30 samples the minor heterozygous and five samples the minor homozygous allele for both SNPs by direct sequencing. This gave 100% concordant results for rs12979860 between the two assays, although one sample showed different results for rs8099917 with direct sequencing. As a result of direct sequencing, this sample had the major homozygous allele, but the results of the Invader assay and TaqMan assay both showed the minor heterozygous allele. Precision was determined by analyzing six samples (including positive and negative controls) in triplicate during three runs (three different days) with 100% concordant results. Intra-assay coefficient of variation (CV%) for rs8099917 was 0.1–1.6 (%), and for rs12979860 was 0.5–2.5 (%). Then, inter-assay CV% calculated from the angle of the heterozygous allele marker was 1.9 and 2.0 (%), respectively (Fig. 2 and Table 2). To evaluate the limit of detection, 10 samples, including three genotypes, were run in triplicate using three different DNA concentrations with no visible confusion in *IL28B* SNP genotyping or signal intensity, except in the 0.3 ng/assay (Fig. 3).

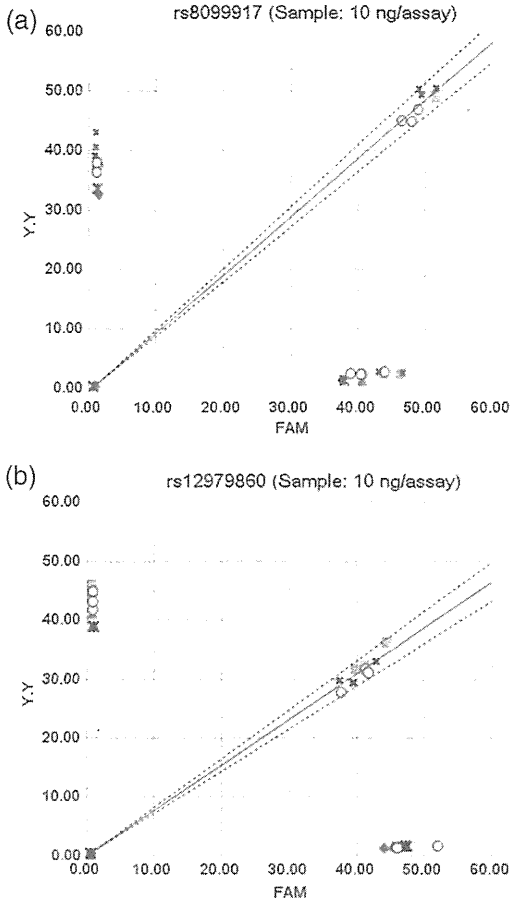

Fig. 2. Simultaneous and daily repeatability of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (three times per day and 3 days of measurement). Scatter plots of fluorescence data from intra-assay for rs8099917 (a) and for rs12979860 (b). Raw fluorescence data are plotted for each sample and control. The x-axis of rs8099917 is the FAM, corresponding to the major allele, while y-axis is Yachima-Yellow (Y.Y), corresponding to the minor allele. In contrast, the x-axis FAM and y-axis Y.Y of rs12979860 signify minor and major alleles, respectively. The line designates the mean of the angle at the heterozygous allele marker, and the broken line indicates standard deviation +2SD and −2SD. Day 1, control (•); day 1, sample (•); day 2, control (▲); day 2, sample (▲); day 3, control (×); day 3, sample (○); mean (——); +2SD (- - -); −2SD (- - -).

IL28B typing in PEG-IFN-α/RBV-treated patients

Of the 512 cases that were analyzed for the rs8099917 genotype, 361 (71%) were shown to have a major homozygous allele, 144 (28%) had a minor heterozygous, and seven (1%) had a minor homozygous allele, which gave concordant results with the TaqMan assay (Table 3). In addition, of the 512 cases that were analyzed for the rs12979860 genotype, 356 (70%) were shown to have a major homozygous allele, 149 (29%) had a minor heterozygous, and seven (1%) had a minor homozygous allele, with 100% concordant results with the TaqMan assay (Table 3). Five of the 512 cases (1%) had haplotype differences, but none of the 512 cases showed differences between the two genotyping methods.

Table 2. Simultaneous and daily repeatability of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (three times per day and 3 days of measurement)

		Coefficient of variation (%)	
		rs8099917	rs12979860
Hetero (10 ng/assay)	Day1	0.1	1.4
	Day2	1.6	2.5
	Day3	1.3	0.5
	Total	1.9	2.0

Prediction of a response to PEG-IFN-α/RBV therapy

Of the 272 patients with genotype 1 who were evaluated for the treatment response, 169 of 203 patients with the major homozygous allele in rs8099917 exhibited a higher prevalence of a virological response (83.3%), consisting of a SVR and TVR (responder), compared with those with a minor heterozygous (18/66, 27.3%) or homozygous allele (1/3, 33.3%) (Table 4). Therefore, the prevalence of responders with the major homozygous allele was 83.3%, and that of non-responders with the minor heterozygous or homozygous allele was 72.5%. Of the 36 patients with genotype 2a or 2b, patients with a major homozygous allele had 96.7% incidence of virological response and those with a heterozygous allele had 100%. For all cases, 85.2% of the patients with a major homozygous allele were responders to PEG-IFN-α/RBV therapy, and 66.7% of the patients with a minor heterozygous or homozygous allele were non-responders to that therapy.

DISCUSSION

The rate of a NVR after 48 weeks of PEG-IFN-α/RBV therapy among patients infected with HCV genotype 1 is around 20–30%. Previously, there have been no reliable predictors of a NVR or SVR. Some recent studies have focused on the *IL28B* polymorphism as one of the most critical factors with a bearing on the prediction of the treatment response (8–10). In particular, rs8099917 and rs12979860 were significantly associated with the treatment regimen; therefore, it would be useful to examine such genetic markers before IFN-based treatments in clinical practice because it would avoid the unpleasant side effects that commonly accompany the treatment. In addition, such an advanced diagnosis would be economically beneficial, as treatment costs would be reduced.

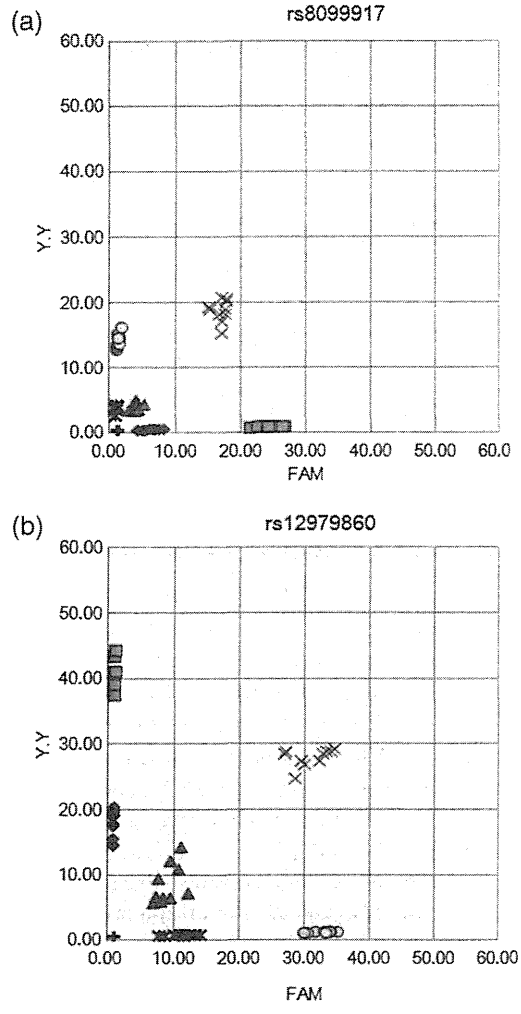

Fig. 3. Sensitivity of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs). Scatter plots of fluorescence data from intra-assay for rs8099917 (a) and for rs12979860 (b). It was defined that the major allele is "Major", hetero type is "Hetero", the minor allele is "Minor", and Negative control is "Neg", respectively. Major (0.3 ng) (◆); major (1.0 ng) (■); hetero (0.3 ng) (▲); hetero (1.0 ng) (×); minor (0.3 ng) (✱); minor (0.3 ng) (○); + Neg (+).

Table 3. Comparisons of the Invader Plus genotyping assay with TaqMan assay for rs8099917 and rs12979860

		Invader Plus			
rs8099917		Major	Hetero	Minor	total
TaqMan probe	Major	361	0	0	361
	Hetero	0	144	0	144
	Minor	0	0	7	7
	total	361	144	7	512
rs12979860		Invader Plus			
TaqMan probe	Major	356	0	0	356
	Hetero	0	149	0	149
	Minor	0	0	7	7
	total	356	149	7	512

Table 4. Effect of the rs8099917 single nucleotide polymorphisms (SNPs)on response to pegylated interferon/ ribavirin (PEG-IFN/RBV) therapy in Japanese patients with hepatitis C virus (HCV) genotype 1

	Major ($n=203$) 74.6%	Hetero ($n=66$) 24.3%	Minor ($n=3$) 1.1%	Total ($n=272$)
Mean age (SD)	57.2 (10.1)	54.9 (10.8)	65.7 (6.5)	56.7 (10.3)
Gender (%)				
Females	102 (50.2%)	35 (53.0%)	2 (66.7%)	139 (51.1%)
Males	101 (49.8%)	31 (47.0%)	1 (33.3%)	133 (48.9%)
Responder	169 (83.3%)	18 (27.3%)	1 (33.3%)	188 (69.1%)
Non-responder	34 (16.7%)	48 (72.7%)	2 (66.7%)	84 (30.9%)

Some SNPs, such as UGT1A1 polymorphism associated with irinotecan therapy, have already been exploited in clinical practice to avoid severe adverse effects (12, 13). These tailor-made therapies are expected to become more common in clinical practice (14). Similarly, *IL28B* polymorphism detection can be used in tailor-made therapies; thus, it is important to develop a genotyping assay that is convenient, swift, accurate, and inexpensive.

The Invader Plus genotyping assay *IL28B* SNP test kit to genotype *IL28B* SNPs (rs8099917 and rs12979860) from DNA samples was developed and evaluated in this study. This assay contains reagents for DNA amplification, including each SNP. Accuracy was determined by comparing the Invader assay results with the direct sequencing results. Only one sample (0.95%) showed a discrepant result for rs8099917 by comparing the Invader Plus genotyping assay and direct sequencing, because another rare SNPs existed in the forward primer binding region used for amplification and direct sequencing (15). Intra-assay (0.1–1.6% for rs8099917 and 0.5–2.5% for rs12979860) and inter-assay (1.9 and 2.0% respectively) precision were sufficient for clinical practice and tailor-made therapies. Interestingly, Invader Plus assay is more convenient, swift and inexpensive than direct sequencing. Invader Plus assay is better than TaqMan assay at specificity because no results come up without tagging both probe and Invader oligonucleotide on SNP directly.

For each or both genotypes, influences on pretreatment prediction with the *IL28B* SNP genotype were evaluated. As a result, especially for patients with genotype 1b, the positive predictive value (PPV) for a NVR was 72.5% and the negative predictive value (NPV) was 83.3%, suggesting that a convenient method using the Invader Plus assay could be useful to predict the treatment outcome in clinical practice.

In conclusion, using the Invader Plus *IL28B* SNP genotyping assay, swift and accurate selection of the optimum treatment strategy for individual patients can be improved by combining with other factors.

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DISCLOSURE

The authors have no conflict of interest.

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CLINICAL STUDIES

Hepatocellular carcinoma and survival in patients with autoimmune hepatitis (Japanese National Hospital Organization-autoimmune hepatitis prospective study)

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Keywords
autoimmune hepatitis – cirrhosis – hepatocellular carcinoma – multicentre cohort study – outcome

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Abstract

Background/Aims: Although the outcome of autoimmune hepatitis (AIH) is generally good, the natural course and likelihood of progression to cirrhosis or hepatocellular carcinoma (HCC) remain undefined, and may vary by region and population structure. Our aims were to evaluate risk factors that contribute to poor outcome and particularly development of HCC in a prospective multicentric cohort study of AIH. *Methods:* The study group comprised 193 Japanese patients with AIH who were prospectively followed up at annual intervals between 1995 and 2008. The mean follow-up period was 8.0 ± 4.5 years. *Results:* Twenty-one (10.9%) patients had cirrhosis at presentation and a further 15 (7.8%) developed cirrhosis during the follow-up period. Survival rates were 94.2% at 10 years and 89.3% at 15 years. HCC was diagnosed in seven of the 193 patients. The presence of cirrhosis at presentation was a risk factor for HCC according to a Cox proportional hazard model, and the HCC-free survival rate was significantly lower in those with cirrhosis compared to those without cirrhosis according to Kaplan–Meier analysis. *Conclusions:* Although the outcome of AIH is as good if not better among Japanese than for other populations, there was an increased risk of HCC in these patients. Cirrhosis at presentation was predictive of development of HCC in AIH in Japan.

Autoimmune hepatitis (AIH) is a chronic inflammatory disorder, dependent in part on autoimmune reactivity, that can cause cirrhosis and end-stage liver disease (1, 2). Current descriptions of features of AIH, derived mostly from Caucasian patients, cite a generally good outcome with 10- and 20-year survivals more than 80% (3). Similarly, the outcome among Japanese patients with AIH is generally good with a 10-year survival rate reported as 90% (4). In general, the natural history and course of AIH are largely defined by the degree of inflammatory activity at the onset of disease and the presence or development of cirrhosis (5). Hepatocellular carcinoma (HCC) complicating AIH is reported (6) but occurs rarely among Caucasian populations secondary to AIH (7): the true incidence remains uncertain and factors contributing to development of HCC in AIH are not fully elucidated. To better understand the natural history and outcome of AIH, and to establish comparisons of AIH among Japanese and Caucasian patients, a nation-wide multicentre cohort study was developed, and herein, we describe the clinical presentation, course and efficacy of treatment of 193 consecutive AIH patients enrolled in the Japanese National Hospital Organization (NHO)-AIH register. We particularly assessed risk factors for a fatal outcome and development of HCC.

Patients and methods

Study population

There were 212 patients initially enrolled in the register of the Japanese National Hospital Organization (NHO) liver-network study, contributed to medical facilities in Japan. Of these 212 patients, 193 were retained and prospectively followed between 1995 and 2008 as a multicentre cohort population. All patients satisfied the 1999 revised criteria of International Autoimmune Hepatitis Group (IAIHG) for a diagnosis of definite (114 cases) or probable (79 cases) AIH (8). Patients were excluded

from study if there was histological evidence of cholangitis or non-alcoholic steatohepatitis. Also, patients who were positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA were excluded and other causes of liver disease, such as excess alcohol, or drugs had been excluded by appropriate history and investigations. The study protocol was approved by the Ethics Committees of all institutes.

Clinical and histological assessments

Follow-up assessments were made at annual intervals. Standard laboratory tests of liver inflammation and function were measured at each assessment. Liver tissue from percutaneous biopsy performed at the referring facility was available for the majority of the patients at the time of entry (143/193, 74.1%) and at subsequent follow-up examination for some (39/193, 20.2%). The histological variables examined included degree of fibrosis (0; absent, 1; expansion of fibrosis to parenchyma, 2; portal-central or portal-portal bridging fibrosis, 3; presence of numerous fibrous septa, 4; multi-nodular cirrhosis). The histological diagnosis of cirrhosis required loss of normal lobular architecture, reconstruction of hepatic nodules and presence of regenerative nodules (9). Biopsy samples from AIH patients developing to HCC were examined in a blinded fashion by a dedicated pathologist (MI). Anti-nuclear antibodies (ANA) and smooth muscle antibodies (SMA) were measured by indirect immunofluorescence on HEp-2 cell respectively and cut-off titres for positivity were 1:40. Clinical relapse was defined as an increase of serum ALT levels to beyond three-fold of the upper limit of normal range (ULN) (10). Asymptomatic patients or patients with lower serum aminotransferase, total bilirubin or IgG were managed with ursodeoxycholic acid (UDCA) therapy alone, which was demonstrated to be effective in Japanese patients with type I autoimmune hepatitis (11).

Variables at study entry

Demographic and other characteristics of the 193 retained patients were recorded as a data-base at the initial assessment. Data included gender, age at diagnosis, time of onset of symptoms or other evidence of liver disease, markers of infection with hepatitis viruses HBV and HCV, alcohol intake, coexisting autoimmune diseases, serum levels of ALT, AST, alkaline phosphatase and bilirubin, platelet count and prothrombin time.

Occurrence of hepatocellular carcinoma

Abdominal ultrasound and serum alpha-fetoprotein determinations were performed annually. Viral hepatitis was excluded by testing for HBsAg and HCV-RNA by polymerase chain reaction (PCR). Subjects with antibodies to HCV were subsequently screened for HCV-RNA using nested PCR. A diagnosis of HCC was made based on the typical patterns on imaging studies, such as early-phase hyperattenuation and late-phase hypoattenuation by dynamic computerized tomography, magnetic resonance imaging and finally, by ultrasonography-guided tumour biopsy.

Statistical analysis

For quantitative data, analysis was performed using a Mann–Whitney test for comparison of two independent groups. Differences in proportions were analysed by the Fisher's exact test when the number of subjects was <5, and the chi-squared test for 2×2 tables when the number of subjects was >5. Prognostic factors for HCC were analysed using the univariate and multivariate Cox proportional hazard model with SPSS software (Chicago, Illinois, USA). The *P*-values of entering variables for multivariate Cox proportional hazard model were <0.1. Survival, related to follow-up time, was analysed using the Kaplan–Meier method and compared using the log-rank test. A value of $P \leq 0.05$ was considered statistically significant.

Results

Baseline data at entry

Of the original 212 recruited patients registered as AIH, 19 were excluded from analysis owing to loss of follow-up (Fig. 1). The retained 193 were considered eligible for the study. Table 1 presents the demographic data for the cohort at entry. The age at diagnosis ranged from 16 to 84 years (mean, 56.6 ± 13.9 years), greater than that in earlier studies on Caucasian patients, and female patients predominated (91.7%). In 51 patients (26.4%) there was concurrent symptomatic autoimmune disease, notably Hashimoto thyroiditis in 11, rheumatoid arthritis in 20, systemic lupus erythematosus in 6, Sjögren's syndrome in 14 and systemic sclerosis in 2. Regarding tests for autoantibodies, data for SMA were lacking in 105 and for ANA in 5. Of those tested, 158 (84.0%) gave positive tests (titre >1:40) for ANA and 36 (40.9%) for SMA. Regarding treatment, 144 patients (74.6%) had been treated with prednisolone, and 43 (22.3%) with ursodeoxycholic acid alone. Relapse occurred in 47 (24.4%) during the follow-up period.

Patient outcome and survival

Seven patients (3.6%) died as a result of complications of AIH. Liver-related death (HCC 2, ruptured oesophageal varices 1, hepatic failure 1) was confirmed in four AIH patients with cirrhosis at presentation (19.0%), one patient (hepatic failure) who subsequently developed cirrhosis during follow-up (6.7%) and one patient (fulminant hepatitis) without cirrhosis during the follow-up period (Fig. 1, Table 2). The overall survival in the AIH patients is shown in Figure 2. The calculated

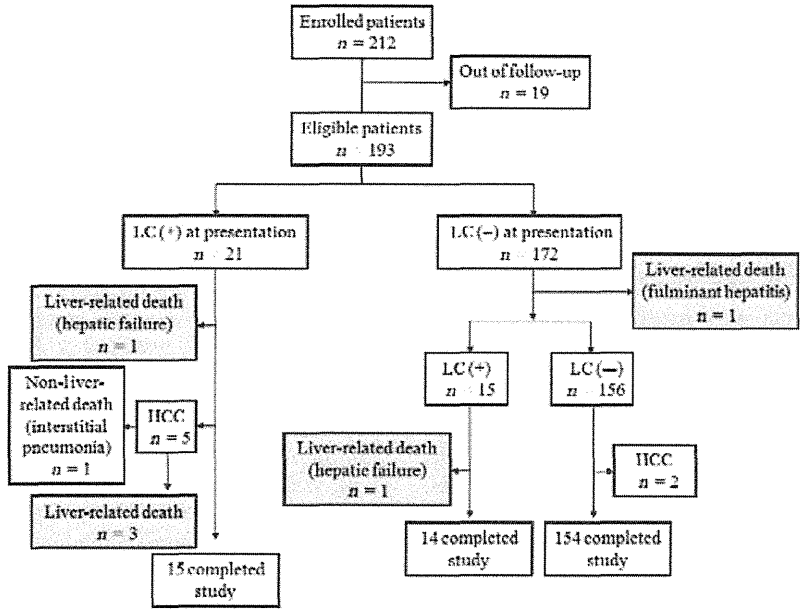

Fig. 1. Flow diagram of patient selection and clinical outcome of autoimmune hepatitis (AIH) patients in the present cohort study. C; cirrhosis, HCC; hepatocellular carcinoma.

Table 1. Baseline characteristics of AIH patients

	n = 193
Gender (male/female)	16/177
Mean age at presentation (years)	56.6 ± 13.9 (16–84)
Mean age	
Age ≥ 60year	98 (50.8%)
Age<60year	95 (49.2%)
Other autoimmune diseases	51 (26.4%)
Mean follow-up (years)	8.0 ± 4.5 (0.1–21)
Baseline Laboratory Values	
AST (<40IU/L)	392.00 ± 450.65 (29–2718)
ALT (<40IU/L)	408.55 ± 421.21 (18–2020)
ALP (<112U/L)	453.18 ± 270.04 (112–2135)
Bilirubin (mg/dl)	3.95 ± 5.66 (0.27–31.8)
Albumin (3.5–5.0g/L)	3.76 ± 0.61 (2.00–5.10)
IgG (500–1300mg/dl)	2517.49 ± 913.43 (210.2–5221)
Platelets (15–40×10^4μl)	19.25 ± 8.02 × 10^4 (2.00–57.00 × 10^4)
ANA+ (≥ 1:40)	158/188 (84.0%)
SMA+ (≥ 1:40)	36/88 (40.9%)
Cirrhosis at presentation	21 (10.9%)
Received treatment	
Mean PSL (mg/day)	28.71 ± 72.98 (0–1000)
PSL ≥ 20mg	126 (65.3%)
PSL alone	100 (51.8%)
PSL+UDCA	42 (21.8%)
PSL+Aza	2 (1.0%)
UDCA alone	43 (22.3%)
Relapse	47 (24.4%)

ALP, alkaline phosphate; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; Aza, azathioprine; PSL, prednisolone; SMA, anti-smooth muscle antibody; UDCA, ursodeoxy cholic acid.

Table 2. Fatal outcome of patients with type 1 autoimmune hepatitis

	Cirrhosis at entry (*n* = 21)	Developed cirrhosis (*n* = 15)	No cirrhosis (*n* = 157)
Deaths (*n*)	5	2	5
Liver-related	4	1	1
HCC	2	0	0
Ruptured oesophageal varices	1	0	0
Fulminant hepatitis	0	0	1
Liver failure	1	1	0
Non-Liver-related	1	1	4
CVA	0	0	3
Lymphoma	0	1	0
Lung cancer	0	0	1
Interstitial pneumonia	1	0	0
Follow-up after cirrhosis (year)	10.0 ± 4.8	4.3 ± 3.9	NA
Total follow-up (year)	10.0 ± 4.8	9.5 ± 4.6	7.6 ± 4.4

Note: Numbers in parentheses represent percentages. Data are expressed as mean ± SD.
HCC, hepatocellular carcinoma; CVA, c erebrovascular accident.

survival for the entire cohort was 94.2% at 10 years and 89.3% at 15 years.

Development of HCC

Hepatocellular carcinoma was diagnosed in as many as seven of the 193 (3.6%) patients with AIH, in five

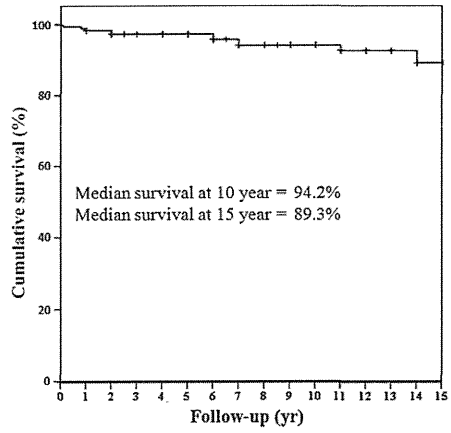

Fig. 2. Survival curve of Japanese patients with AIH. The 10-year survival was 94.2% and 15-year survival was 89.3%.

female patients (2.6%) and in two male patients (1.0%). The mean age at diagnosis was 62.0 ± 10.5 years (range, 43–73 years; median 65 years). Of the seven patients who developed HCC, five had antecedent cirrhosis (mean duration of cirrhosis, 9.0 ± 3.5 years; range, 4–12 years; median 11 years); two who developed HCC did not have antecedent cirrhosis (Fig. 1). Two patients with cirrhosis and HCC died within 2 years after the diagnosis of HCC and three were surviving at the last follow-up. HCC developed in seven patients. Table 3 summarizes the clinical features, and outcome of these HCC patients. At diagnosis of HCC, five patients had cirrhosis and the remaining two patients did not progress to cirrhosis. Overall, four patients died, among which two patients died of HCC, one of ruptured oesophageal varies and one of interstitial pneumonia. The mean survival time from HCC diagnosis was 3.2 ± 2.2 years in these four patients. The remaining three patients still alive and the mean duration from HCC diagnosis to the final surveillance was 5.0 ± 1.3 years.

Initial clinical features and laboratory data at diagnosis of AIH were compared between patients with and without HCC (Table 4). In patients who developed HCC, the frequencies of the association with cirrhosis were significantly higher. Also lower platelet counts, total bilirubin and ALT were observed in the group with HCC. Using time-dependent univariate analysis (Cox proportional hazard model), the following variables at

Table 3. Clinical features of 7 patients who developed HCC

Patient no	Case1	Case2	Case3	Case4	Case5	Case6	Case7
Age at diagnosis (years)	72	60	73	43	55	65	66
Gender	Female	Female	Female	Female	Male	Male	Female
Labo data at diagnosis							
Platelet (/μl)	126000	196000	172000	94000	129000	135000	44000
AST/ALT (IU/ml)	244/86	102/55	140/107	57/44	95/185	100/159	80/66
Cirrhosis at presentation	(+)	(−)	(+)	(+)	(+)	(−)	(+)
Liver histology at presentation							
Grading A score	NT	NT	A2	A2	A2	A2	A2
Staging F score	NT	NT	F3	F4	F4	FI	F3
Other autoimmune diseases	SLE	RA	(+)	Chronic thyroiditis	(−)	(−)	(−)
Initial treatments	PSL30 mg/day	PSL2.5 mg/day	PSL30 mg/day	PSL40 mg/day	UDCA600 mg/day	PSL30 mg/day UDCA60 0 mg/day	PSL30 mg/day UDCA300 mg/day
Age at HCC diagnosis (years)	84	72	79	54	67	71	70
Duration from AIH diagnosis (years)	11.5	11.1	5.7	11.5	11.5	5.9	4.0
Cirrhosis at HCC diagnosis	(+)	(−)	(+)	(−)	(+)	(−)	(+)
Labo data at HCC diagnosis							
AST/ALT (IU/ml)	59/30	81/49	27/40	46/25	74/56	25/36	187/90
Treatments	TACE	Surgery TACE	TACE	TACE	TACE/RFA	TACE	TACE/PEIT
Survival (survival' death)	Death	Survival	Death	Death	Survival	Survival	Death
Cause of death	HCC		HCC	Interstitial pneumonia			Ruptured oesophageal varices
Survial time from HCC diagnosis (years)	2.0 year (death)	6.1 year (alive)	1.4 year (death)	6.4 year (death)	4.5 year (alive)	40 year (alive)	3.0 year (death)

HCC, hepatocellular carcinoma; PEIT, precutaneous ethanol injection therapy; RA, rheumatoid arthritis; RFA, radio frequency ablation; SLE, systemic lupus erythematosus; TACE, transcatheter arterial chemoembolization; UDCA, ursodeoxycholic acid.

Table 4. Baseline characteristics of AIH patients with or without HCC

	HCC (+) $n = 7$	HCC (−) $n = 186$	P
Mean age			
Age ≥ 60year	5	93	0.235
Age<60year	2	93	
Gender (male female)	215	14/172	0.106
Mean age at presentation (years)	62.0 ± 10.5 (43–73)	56.4 ± 14.0 (16–84)	0.321
Other autoimmune diseases	3 (42.9%)	48 (25.8%)	0.271
Mean follow-up (years)	12.9 ± 4.8 (7–18)	7.9 ± 4.4 (0.1–21)	0.011
Baseline laboratory values			
AST(<40IUL)	114.00 ± 54.59 (57–224)	402.44 ± 455.67 (29–2718)	0.050
ALT (<40IU/L)	100.29 = 53.65 (44–185)	420.15 ± 424.62 (18–2020)	0.014*
ALP (<112IU/L)	383.43 ± 197.06 (135–679)	455.89 ± 272.53 (112–2135)	0.611
Bilirubin (mg/dl)	0.74 ± 0.38 (0.3–1.5)	4.07 ± 5.73 (0.27–31.8)	0.015*
Albumin (3.5–5.0g/L)	3.66 ± 0.60 (2.7–4.4)	3.77 ± 0.61 (2.00–5.10)	0.633
IgG (500–1300mg/dl)	2842.43 ± 1008.55 (1480–4280)	2504.64 ± 910.22 (210.2–5211)	0.322
Platelets (15–40×10/μl)	12.80 ± 5.00 × 10^4 (4.40–19.60 × 10^4)	19.51 ± 8.02 × 10^4 (2.00–57.05 × 10^4)	0.015*
ANA+(≥ 1:40)	6/7 (85.7%)	152/181 (84.0%)	0.690
SMA+(≥ 1:40)	0/2	36/86 (41.9%)	0.346
HCV Ab (+)	1 (14.3%)	4 (2.2%)	0.175
Cirrhosis at presentation	5 (71.4%)	16 (8.6%)	<0.001**
Received treatment			
Mean PSL (mg/day)	23.21 ± 15.46 (0–40)	28.92 ± 74.30 (0–1000)	0.991
PSL ≥ 220mg	5 (71.4%)	117 (62.9%)	0.490
PSL aloilff	4 (57.1%)	89 (47.8%)	0.460
PSL+UDCA	2 (28.6%)	39 (21.0%)	0.459
PSL+Aza	0	2 (1.1%)	0.929
UDCA alone	1 (14.3%)	37 (19.9)	0.584
Relapse	4(57.1%)	43 (23.1)	0.061
Liver biopsy specimen available at presentation	(n = 5)	(n = 138)	
Stage of fibrosis			
F0	0	10 (7.2%)	
Fl	1 (20.0%)	34 (24.6%)	
F2	0	35 (25.4%)	
F3	2 (40.0%)	31 (22.5%)	
F4	2 (40.0%)	7 (7.1%)	
ALT	($n = 6$)	($n = 164$)	
1year after first treatment	54.0 ± 27.31 (31–96)	44.7 ± 65.2 (6–521)	0.020*

*P < 0.05; **P < 0.01.

ALP, alkaline phosphate; ALT, alanine aminotransferase; ANA, anti-nuclear antibody; AST, aspartate aminotransferase; Aza, azithioprine; PSL, prednisolone; SMA, anti-smooth muscle antibody; UDCA, ursodeoxy cholic acid.

accession were associated with risk for HCC; male gender ($P = 0.033$) and the presence of cirrhosis ($P = 0.002$) at presentation (Table 5). By multivariate Cox analysis, the presence of cirrhosis (Hazard ratio 11.47, 95% CI 2.13–64.60, $P = 0.005$) was associated independently with risk for HCC (Table 6). These data suggest that male patients and cirrhosis at the onset are at particular risk for HCC.

HCC-free survival rates

Figure 3 presents Kaplan–Meier estimates for the cumulative HCC-free survival rate, based upon the presence or absence of cirrhosis at enrolment. The 15-year survival rate without HCC was 96.6% ±2.7 in AIH patients without cirrhosis, and 62.2% ±13.9 in those with cirrhosis. A log-rank test of the two curves showed a significant difference in that the HCC-free survival rate of AIH patients with cirrhosis was significantly lower than that of those without cirrhosis ($P < 0.0001$).

Discussion

Autoimmune hepatitis is a chronic progressive liver disease caused by immune-mediated destruction of hepatic parenchymal cells. Accurate diagnosis depends on a combination of features scored as recommended by the IAIHG (8), with critical criteria including interface hepatitis histologically, hypergammaglobulinemia and characteristic serum autoantibodies (1). The precise pathogenic processes that lead to AIH are uncertain, but likely depend on a genetic predisposition of the host to

Table 5. *Variables associated with increased risk factor for HCC (Univariate Cox proportional hazard model)*

Characteristics	Subgroup	HCC		HR (95% CI)	*P* value
		Yes (*n* = 7)	No (*n* = 186)		
Gender	Male	2 (28.6%)	14 (7.5%)	6.058 (1.151–31.869)	0.033
Age	<50	1 (14.3%)	51 (27.4%)	0.325 (0.039–2.707)	0.299
	50–59	1 (14.3%)	42 (22.6%)	0.496 (0.060–4.124)	0.517
	≥60	5 (71.4%)	93 (50.0%)	3.617 (0.699–18.723)	0.125
Other autoimmune disease	(+)	3 (42.9%)	48 (25.8%)	1.730 (0.386–7.750)	0.474
Cirrhosis at presentation	(+)	5 (71.4%)	16 (8.6%)	13.878 (2.670–72.142)	0.002
PSL	(–)	1 (14.3%)	55 (29.6%)	0.330 (0.040–2.748)	0.306
	1–19 mg/day	1 (14.3%)	13 (7.0%)	1.164 (0.138–9.827)	0.889
	20–39 mg/day	4 (57.1%)	60 (32.3%)	3.352 (0.750–14.994)	0.113
	≥40 mg/day	1 (14.3%)	57 (30.6%)	0.484 (0.058–4.053)	0.503
Relapse	(+)	4 (57.1%)	43 (23.1%)	3.789 (0.848–16.936)	0.081

HCC, hepatocellular carcinoma; HR, hazard ratio; PSL, prednisolone.

Table 6. Multivariate analysis of predictive factor for HCC in AIH patients (Cox proportional hazards model)

Variables	*P*	HR (95% CI)
Male gender	0.275	2.572 (0.472–14.008)
Cirrhosis at presentation	0.005	11.741 (2.134–64.602)

CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio.

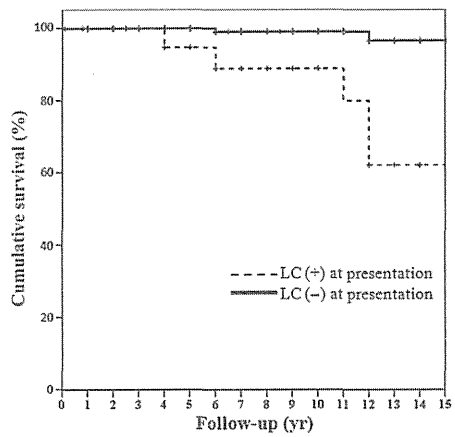

Fig. 3. Fifteen-year HCC-free survival for AIH patients with and without cirrhosis at entry. Kaplan–Meier survival curve comparing HCC-free survival among patients with or without cirrhosis at baseline. The 15-year HCC-free survival was 62.2% for patients with cirrhosis compared with 96.6% for patients without cirrhosis at baseline ($P < 0.0001$).

specific autoimmune reactivity to self-antigens with ensuing hepatic inflammation mediated by T-cell cytotoxicity mechanisms (12, 13).

Our present study has revealed some features of AIH particular to the Japanese population, as well as some differences in practice between Japanese and 'western'-trained internists. In our longitudinal multicentre study on Japanese AIH patients, 71% were treated with corticosteroid during the mean 8-year follow-up period giving an estimated 10-year survival rate as high as 94%. Thus, the survival for corticosteroid-treated type 1 AIH is generally good as in previous reports (3, 4). However, our study did not confirm previous experience that the incidence in AIH of HCC was low, since this did not pertain in patients with histological cirrhosis.

There are a number of reports that patients with chronic viral hepatitis, whether owing to HBV or HCV, are prone to develop HCC in contrast to its infrequency in AIH (7, 14). Park *et al.* (14) reported low incidence of HCC in Caucasian patients with AIH and cirrhosis, observing only one case of HCC among 88 patients with cirrhosis caused by AIH among a total of 212 patients overall, suggesting an incidence of HCC of about 0.1% per patient year (14). In our cohort of 193 patients with AIH, 21 (10.9%) patients suffered from cirrhosis at presentation and, during the follow-up period, seven developed HCC. The patients in our AIH cohort comprised a proportion with cirrhosis similar to that of Park but our follow-up time 8.0 ± 4.5 years was longer. Perhaps the true risk of HCC in AIH patients differs according to ethnicity of the population. Werner *et al.* (6) in the Swedish national-wide AIH cohort showed an overall increase in risk in AIH for all malignancies, mainly contributed to by hepatobiliary cancer. To reiterate, our data indicate that cirrhosis at presentation of AIH was indeed associated with a risk for occurrence of HCC, contrary to traditional belief (15), so that, although HCC occurrence in AIH is lower than that seen in viral-mediated liver disease (15, 16), long-standing cirrhosis may well be a significant risk factor for HCC even in AIH, as described previously (17).

Regarding gender as a risk factor for HCC, Montano-Loza *et al.* (17) reported that male gender and long-standing cirrhosis are combined risk factors for development of HCC in AIH patients. In the present

study, we found a correlation between gender and progression to HCC only in univariate Cox proportional hazard model and not in multivariate Cox proportional hazard model. Thus furthermore large-scale studies are needed in AIH to elucidate the link between gender and HCC.

Although previous concerns have been raised regarding occult viral hepatitis infection as being instrumental in the aetiology of HCC in AIH (18), all patients in our study were negative for serological markers of HBV infection and as well for persisting HCV infection. International diagnostic criteria of AIH (8) allocate negative points for positive HBV or HCV diagnostic tests. Although, seven had anti-HCV antibodies none of these had any evidence of active HCV infection (HCV-RNA negative), and all fulfilled the IAIHG criteria. Whilst seropositivity for HCV was not a statistically significant risk factor for HCC development, the unlikely possibility exists that pre-existing HCV infection was a contributing risk factor for HCC in a small subset of AIH patients. Recently, non-alcoholic steatohepatitis (NASH) has been recognized as an important cause of HCC even in Japan (19). In patients with risk factors for non-alcoholic fatty liver diseases (NAFLD), diagnosis of AIH was confirmed according to the liver histological findings (20). However, we could not rule out the possibility that NASH-related LC was included in our population completely.

In AIH, cirrhosis at presentation is reported to be an important prognostic factor (20, 21). Our study suggests that the long-term outcome for AIH patients with cirrhosis is relatively unfavourable, since the 15-year HCC-free survival rate was only 64% and further that HCC-related death partly contributed to the poorer outcome in AIH with cirrhosis. Consistent with this, Feld *et al.* (22) reported that AIH patients with cirrhosis at presentation had an inferior 10-year survival vs. those without cirrhosis [61.9% vs. 94.0%].

In conclusion, the outcome for Japanese patients with AIH is good, consistent with previous reports. However, 7 (3.6%) of 193 developed HCC and so, among Japanese AIH patients, HCC may not be an uncommon final event in patients with AIH. The presence of cirrhosis at presentation confers an increased risk for future HCC, albeit less commonly than with hepatitis virus-related liver diseases. These risk factors call for a regular screening strategy for HCC in AIH patients with cirrhosis.

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Original article

Age and total ribavirin dose are independent predictors of relapse after interferon therapy in chronic hepatitis C revealed by data mining analysis

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Background: This study aimed to define factors associated with relapse among responders to pegylated interferon (PEG-IFN) plus ribavirin (RBV) therapy in chronic hepatitis C.

Methods: A cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN plus RBV and who had an undetectable HCV RNA by week 12 (n=951) were randomly assigned to model derivation (n=636) or internal validation (n=315) groups. An independent cohort (n=598) were used for an external validation. A decision tree model for relapse was explored using data mining analysis.

Results: The data mining analysis defined five subgroups of patients with variable rates of relapse ranging from 13% to 52%. The reproducibility of the model was confirmed by internal and external validations (r^2=0.79 and 0.83, respectively). Patients with undetectable HCV RNA at week 4 had the lowest risk of relapse (13%), followed by patients <60 years with undetectable HCV RNA at week 5–12 who received ≥3.0 g/kg of body weight of RBV (16%). Older patients with a total RBV dose <3.0 g/kg had the highest risk of relapse (52%). Higher RBV dose beyond 3.0 g/kg was associated with further decrease of relapse rate among patients <60 years (up to 11%) but not among older patients whose relapse rate remained stable around 30%.

Conclusions: Data mining analysis revealed that time to HCV RNA negativity, age and total RBV dose was associated with relapse. To prevent relapse, ≥3.0 g/kg of RBV should be administered. Higher dose of RBV may be beneficial in patients <60 years.

Introduction

The currently recommended therapy for chronic hepatitis C is a combination of pegylated interferon (PEG-IFN) plus ribavirin (RBV) [1]. This therapy is effective in 50% of patients with HCV genotype 1b [2,3]. The most reliable predictor of sustained virological response (SVR) is the response during early weeks of therapy. A satisfactory response to therapy in the early weeks is associated with a high rate of SVR [4–8]. A basic concept of response-guided therapy is to modify the duration of therapy according to the time to HCV RNA negativity. Extended therapy may be given to patients with delayed virological response [9–13]. Modification of duration of therapy or drug dose may also be necessary in patients with early virological

response (EVR), because approximately 20% of these patients experience relapse after the completion of 48 weeks of therapy. Recent reports have revealed that single nucleotide polymorphisms located near the *IL28B* gene are strongly associated with SVR or a null response to PEG-IFN plus RBV therapy [14–16]. However, single nucleotide polymorphisms located near the *IL28B* gene are not associated with relapse after EVR [17]. Identification of risk factors for relapse among patients with virological response may lead to more individualized therapy and improved SVR rate.

Decision tree analysis, a core component of data mining analysis, is a method that explores data to develop predictive models [18]. This method has been originally used in business and recently in medical fields [19–25]. Decision tree analysis was successfully used to build a predictive model of EVR [26] and SVR to PEG-IFN plus RBV combination therapy in chronic hepatitis C [17,27,28]. The results of the analysis are presented as a tree structure, which is easy to understand and use in clinical practice. Patients can be allocated into subgroups by simply following the flowchart form of the decision tree [29].

In the present study, we used decision tree analysis to identify predictors of relapse among patients who achieved EVR to PEG-IFN plus RBV therapy, and to define a more individualized therapeutic strategy beyond response-guided therapy.

Table 1. Background of study population

Characteristic	Value
Age, years	54.9 (10.8)
Gender	–
Male, *n* (%)	557 (59)
Female, *n* (%)	394 (41)
Body mass index, kg/m^2	23.2 (3.3)
Albumin, g/dl	4.1 (1.8)
Creatinine, mg/dl	0.7 (0.2)
AST, IU/l	60.6 (46.2)
ALT, IU/l	80.7 (77.2)
GGT, IU/l	52.0 (60.0)
White blood cell count, cells/μl	4,993 (1,363)
Haemoglobin, g/dl	15.9 (52.6)
Platelets, 10^9/l	174.4 (6.1)
HCV RNA, KIU/ml	1,655 (1,455)
Fibrosis stage	–
F1-2, *n* (%)	626 (66)
F3-4, *n* (%)	98 (10)
NA, *n* (%)	227 (24)
Time to HCV RNA negativity 4/8/12 weeks	–
4 Weeks, *n* (%)	233 (24)
8 Weeks, *n* (%)	386 (41)
12 Weeks, *n* (%)	332 (35)
Treatment duration, weeks	42 (13)
Total RBV dose, g/kg body weight	3.1 (1.3)
Total PEG-IFN dose, μg/kg body weight	62.5 (38.6)
Outcome	–
Relapse, *n* (%)	238 (25)
SVR, *n* (%)	713 (75)

Total *n*=951. Data are expressed as mean (SD) unless otherwise indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; NA, not available; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR: sustained virological response.

Methods

Patients

This is a multicentre retrospective cohort study involving Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University, Yamanashi University, Osaka City University, and their related hospitals. The inclusion criteria were chronic hepatitis C patients treated with PEG-IFN-α2b plus RBV, genotype 1b, pretreatment HCV RNA titre >100 KIU/ml as confirmed by quantitative PCR; Cobas Amplicor HCV Monitor version 2.0; Roche Diagnostic Systems, Pleasanton, CA, USA), an undetectable HCV RNA level within week 12 after the start of therapy, no coinfection with HBV or HIV, and no other causes of liver disease. Patients were treated with PEG-IFN-α2b (1.5 μg/kg) subcutaneously every week plus a daily weight-adjusted RBV dose (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg and 1,000 mg for patients weighing >80 kg). Dose reduction or discontinuation of PEG-IFN and RBV was considered based on the recommendations of the package inserts and the discretion of physicians at each university and hospital. The standard duration of therapy was set at 48 weeks, but extension of duration was allowed and implemented at the discretion of each physician. The duration of therapy was extended beyond 48 weeks in 118 patients (mean duration was 56.3 weeks, ranging from 49 to 72 weeks). Although the exact reason for the prolonged treatment in each case was not available, one reason may be that each physician tried to achieve high adherence of RBV by extending the duration of therapy. Another reason may be the late time point of HCV RNA negativity even within early virological response. Among 118 patients, time to HCV RNA negativity was between 9 to 12 weeks in 56% of patients.

A total of 951 patients fulfilled the study criteria. The baseline characteristics and representative laboratory test results are listed in Table 1. For analysis, patients were randomly assigned to either the model derivation (636 patients) or internal validation (315 patients) groups. There were no significant differences in the clinical backgrounds between these two groups. For external validation of the model, we collaborated with another multicentre study group consisting of 29 medical centres and hospitals belonging to the National

Hospital Organization (Japan). A dataset collected from 598 patients who were treated with PEG-IFN-α2b plus RBV and had undetectable HCV RNA within week 12 were used for external validation. Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committees of all concerned hospitals.

Laboratory tests

Haematological tests, blood chemistry and HCV RNA titre were analysed before therapy and at least once every month during therapy. Rapid virological response (RVR) was defined as an undetectable HCV RNA level at week 4, and complete early virological response (cEVR) was defined as an undetectable HCV RNA level at week 5 through week 12 after the start of therapy. SVR was defined as an undetectable HCV RNA level 24 weeks after the completion of therapy. Detection of HCV RNA level was based on qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor; Roche Diagnostic Systems). A database of pretreatment variables included haematological tests (haemoglobin level, white blood cell count and platelet count), blood chemistry tests (serum levels of creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, total cholesterol, triglycerides and HCV RNA titre), stage of histological fibrosis and patient characteristics (age, sex and body mass index). Post-treatment variables included time to HCV RNA negativity, calculated total RBV dose (g/kg of body weight), and calculated total PEG-IFN dose (μg/kg of body weight).

Statistical analysis

The Student's *t*-test was used for the univariable comparison of quantitative variables and Fisher's exact test was used for the comparison of qualitative variables. Logistic regression models with backward selection procedures were used for multivariable analysis of factors associated with relapse. IBM SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. For the decision tree analysis [30], the data mining software IBM SPSS Modeler 14 (SPSS Inc.) was used, as reported previously [17,26–28]. The decision tree analysis, the core component of the data mining, belongs to a family of non-parametric regression methods based on binary recursive partitioning of data. In this analysis, the software automatically explored the database to determine optimal split variables to build a decision tree structure. A statistical search algorithm evaluate the model derivation group to determine the optimum variables and cutoff values and to yield the most significant division of patients into two subgroups that were as homogeneous as possible for the probability of relapse. Once patients were divided into 2 subgroups, the analysis was automatically repeated on each subgroup in the same way until either no additional significant variable was detected or the number of patients was <20. Finally all patients were classified into particular subgroups that are homogeneous with respect to the probabilities of relapse.

Results

The decision tree model for the prediction of relapse

The overall rate of relapse was 26% in the model derivation group. The decision tree analysis selected three variables that are associated with relapse: time to HCV RNA negativity, age and total RBV dose (Figure 1). Time to HCV RNA negativity was selected as the best predictor of relapse. The rate of relapse was 13% for patients with RVR compared to 30% for patients with cEVR. Among patients with cEVR, age was selected as the variable of second split. Patients <60 years had a lower probability of relapse (22%) compared with those ≥60 years (41%). The total RBV dose was selected as the third variable of split with an optimal cutoff of 3.0 g/kg of body weight. The rate of relapse was lower in patients who received ≥3.0 g/kg of body weight of RBV compared to patients who received <3.0 g/kg of body weight (among patients <60 years rates were 16% versus 32% and among patients ≥60 years rates were 26% versus 52%, respectively).

According to this decision tree, the patients were divided into five groups with different rates of relapse ranging from 13% to 52%. Patients with RVR had the lowest risk of relapse. Among patients with cEVR, patients <60 years who received ≥3.0 g/kg of body weight of RBV also had a low risk of relapse (16%). By contrast, patients who received <3.0 g/kg of body weight of RBV had higher than the average risk of relapse, especially in patients ≥60 years (52%).

Validation of the decision tree model

The decision tree model was validated using an internal validation group that was not included in the model derivation. The rates of relapse for each subgroup of patients were correlated closely between the model derivation and the internal validation group (r^2=0.79; Figure 2A). When validated using an external validation group, the rates of relapse for each subgroup of patients were again correlated closely between the model derivation and the external validation group. (r^2=0.83; Figure 2B).

Multivariable logistic regression analysis for factors associated with relapse

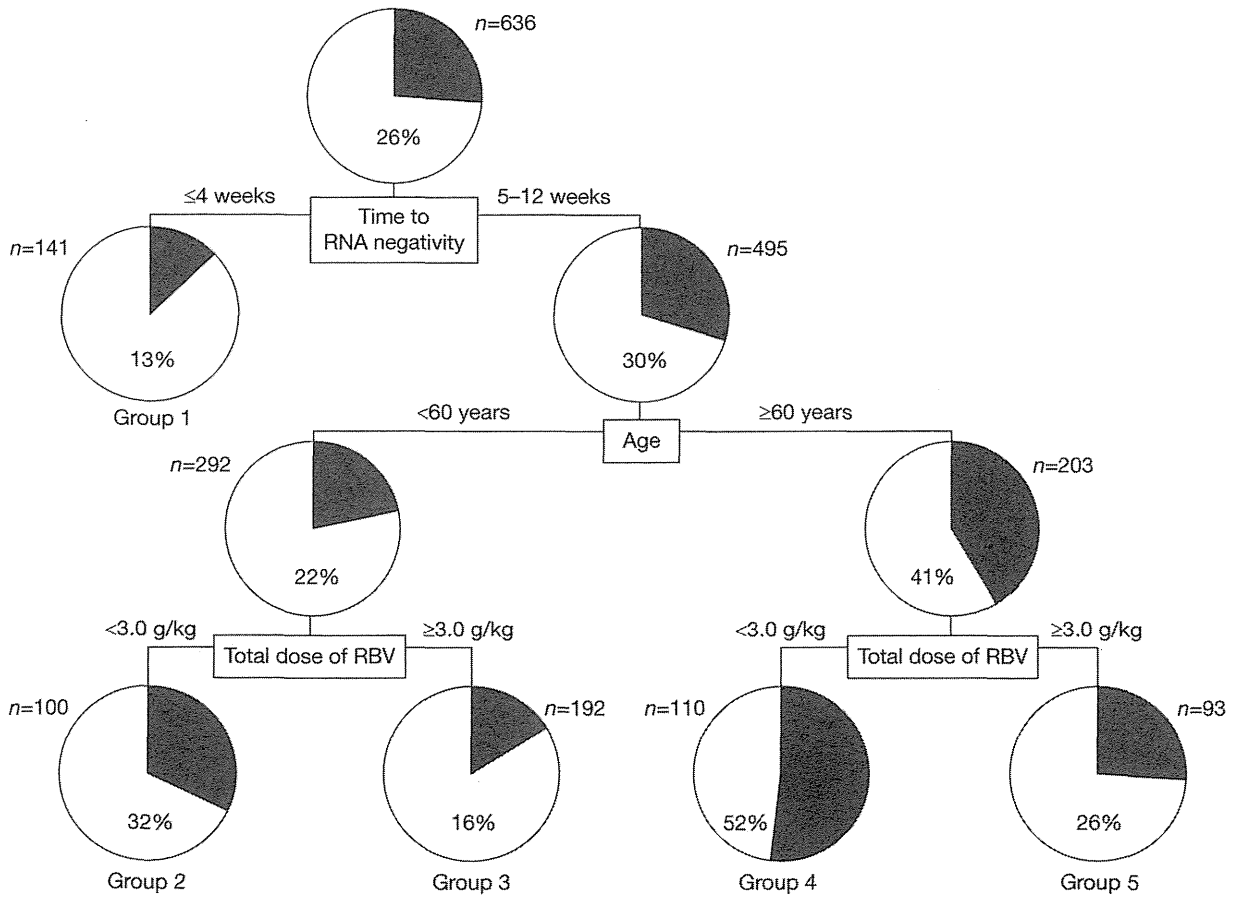
Univariable and multivariable analysis was performed using the combined population of model derivation and internal validation group. Univariable analysis found

that age, sex, serum levels of creatinine, haemoglobin, platelet count, HCV RNA titre, time to HCV RNA negativity, total PEG-IFN dose and total RBV dose were associated with relapse. Duration of therapy was not associated with reduction in relapse rate. Multivariable analysis including these factors showed that age, total RBV dose, serum level of creatinine, and time to HCV RNA negativity were independent predictors of relapse (Table 2). Creatinine was not selected as a splitting variable in data mining analysis probably due to the limitation to stop the analysis when the number of patients was <20. Using the combined population of model derivation and internal validation group, patients in each subgroup of decision tree model were further stratified by creatinine levels and the effect of creatinine level on relapse was analysed. Among patients with RVR, the rate of relapse did not differ between patients with creatinine levels of <0.7 g/dl and ≥0.7 g/dl and were 12% and 12%, respectively. Among patients with cEVR, the rate of relapse was higher in patients with creatinine levels of <0.7 g/dl compared to those with creatinine levels of ≥0.7 g/dl and were 39% versus 23%, respectively, for patients <60 years who received <3.0 g/kg of body weight of RBV, 19% versus 14% for patients <60 years who received ≥3.0 g/kg of body weight of RBV, 58% versus 41% for patients ≥60 years who received <3.0 g/kg of body weight of RBV, and 42% versus 26% for patients ≥60 years who received ≥3.0 g/kg of body weight of RBV.

Effect of age and total RBV dose on relapse among patients with cEVR

The effect of total RBV dose on relapse was analysed among patients with cEVR in a combined group of

Figure 1. The decision-tree model of relapse among patients with rapid virological response or complete early virological response

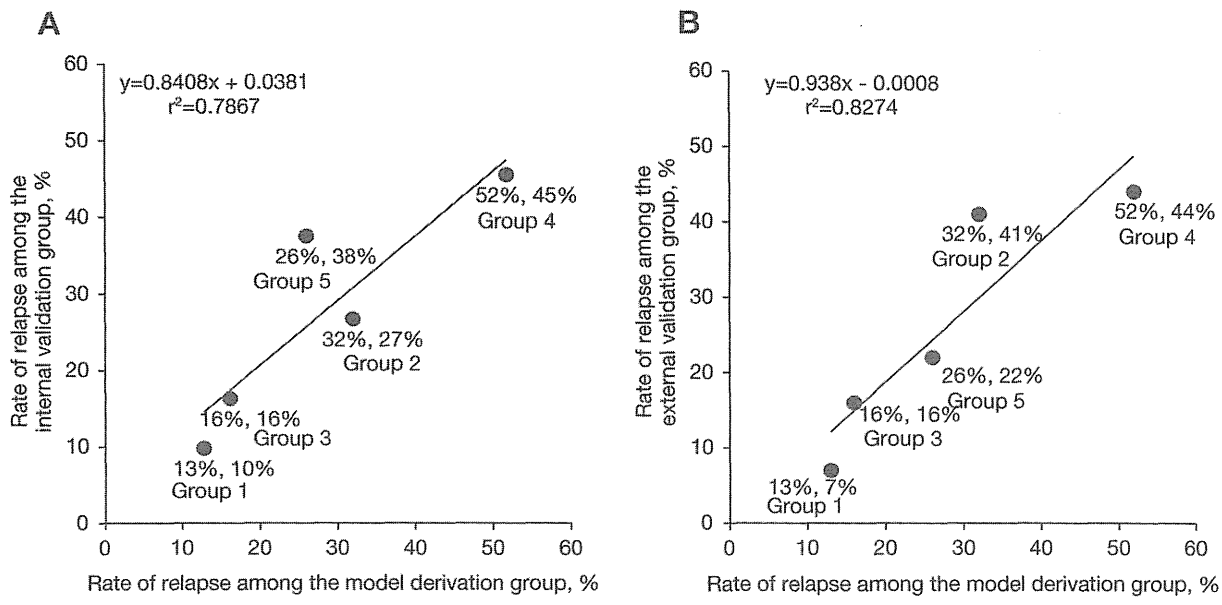


Boxes indicate the factors used for splitting and the cutoff values for the split. Pie charts indicate the rate of relapse for each group of patients after splitting. Terminal groups of patients discriminated by the analysis are numbered from 1 to 5. The rate of relapse was higher than average (>26%) in subgroups 2 and 4, where total ribavirin (RBV) dose was <3 g/kg of body weight.

model derivation and internal validation (n=718). The relapse rate decreased with an increase in RBV dose (Figure 3A). When patients were stratified into two groups according to age, the relapse rate decreased with an increase in RBV dose in patients <60 years. The relapse rate was lowest (11%) in patients <60 years who received ≥4.0 g/kg of body weight of RBV. By contrast, among patients ≥60 years, the relapse rate decreased with an increase in RBV dose up to 3.0 g/kg of body weight, but remained relatively stable despite a further increase in the RBV dose beyond 3.0 g/kg of body weight. The rate of relapse was 31% to 33% in patients who received ≥3.0 g/kg of body weight. Patients ≥60 years had higher relapse rate compared with patients <60 years after stratification by RBV dose (P=0.044 for RBV <2.5 g/kg, P=0.009 for RBV 2.5–2.9 g/kg, P=0.150 for RBV 3.0–3.4 g/kg, P=0.036 for RBV 3.5–3.9 g/kg and P=0.006 for RBV ≥4.0 g/kg).

To exclude the effect of the duration of therapy, patients who received 42–54 weeks of therapy were selected (n=544). Again, the relapse rate decreased with an increase in RBV dose in patients <60 years but remained stable despite a further increase in the RBV dose beyond 3.0 g/kg of body weight in patients ≥60 years (Figure 3B); in addition, patients ≥60 years had a higher relapse rate compared with younger patients after stratification by

Figure 2. Internal and external validation of the decision-tree model: subgroup-stratified comparison of the rate of relapse between the model derivation and validation groups



Each patient in the internal and external validation population was allocated to groups 1 to 5 following the flowchart of the decision tree. The rates of relapse were then calculated for each group and a graph was plotted. The rate of relapse in the (A) internal and (B) external validation groups are shown. The rates of relapse are shown as percentages below data points: the value on the left is from the model derivation group and on the right is from the validation group. The rates of relapse in each group of patients correlated closely between the model derivation group and the validation group (correlation coefficient: r^2=0.79 and 0.83, respectively).

Table 2. Multivariable analysis of factors associated with relapse among patients with RVR/cEVR

Factor	OR	95% CI	P-value
No-RVR	4.07	2.57–6.43	<0.0001
Total RBV dose <3.0 g/kg body weight	2.19	1.58 –3.03	<0.0001
Creatinine <0.7 g/dl	1.67	1.22–2.29	0.001
Age ≥60 years	2.37	1.73–3.24	<0.0001

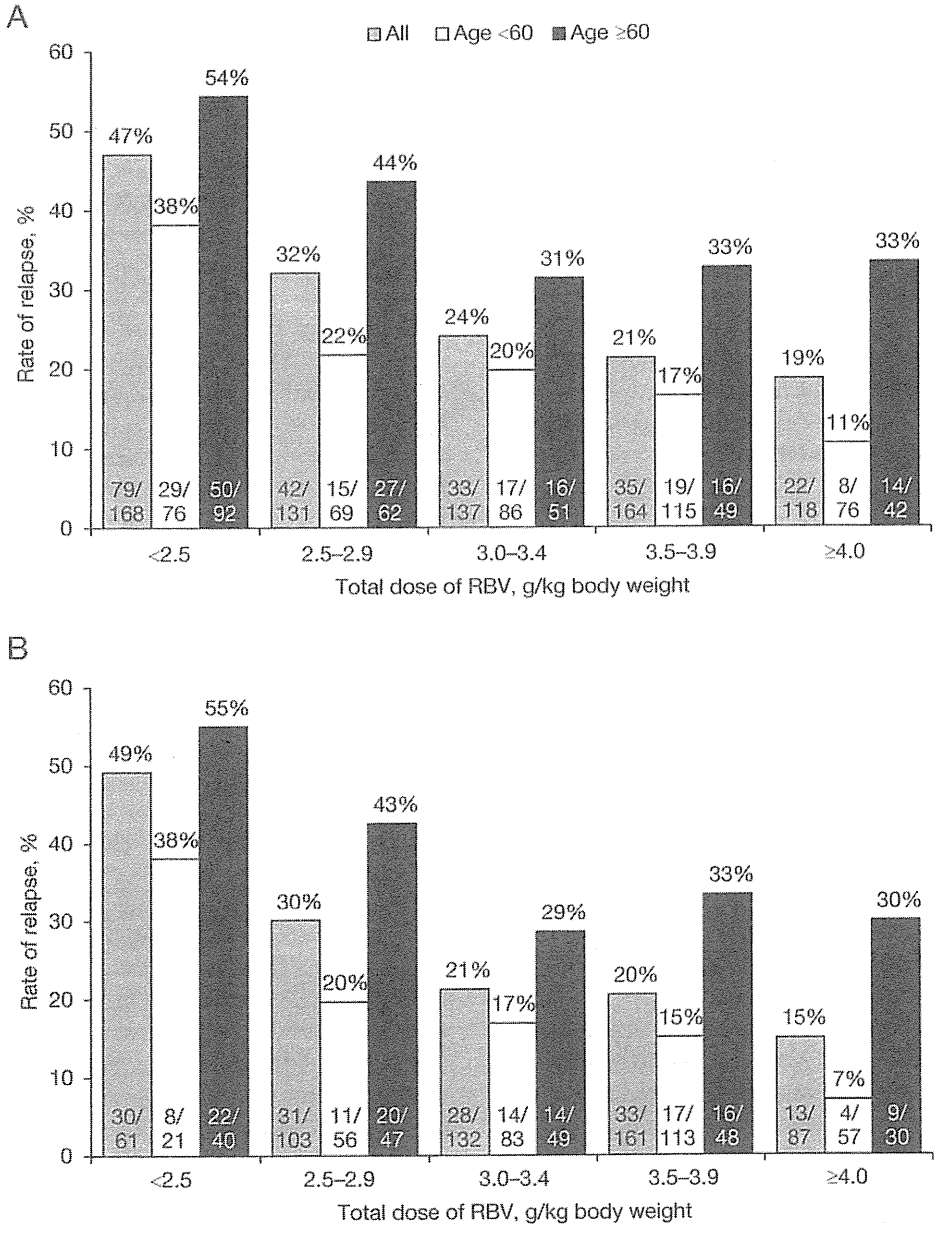
cEVR, complete early virological response (HCV-RNA-positive at week 4, but negative at week 12); RBV, ribavirin; RVR, rapid virological response (HCV-RNA-negative at week 4).

RBV dose (P=0.283 for RBV <2.5 g/kg, P=0.017 for RBV 2.5–2.9 g/kg, P=0.127 for RBV 3.0–3.4 g/kg, P=0.011 for RBV 3.5–3.9 g/kg and P=0.009 for RBV ≥4.0 g/kg).

Total dose of RBV was associated with relapse independently of PEG-IFN dose. The cutoff value of 58 zμg/kg of PEG-IFN was selected, which corresponds to the 80% of 1.5 μg/kg dose for 48 weeks. In patients who received <58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 24% and 42%, respectively. In patients who received ≥58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 21% and 38%, respectively.

The data mining analysis procedure did not select further split variables among RVR patients. However,

Figure 3. Correlation between the rate of relapse and total RBV dose among patients with cEVR after stratification by age



Association between the total ribavirin (RBV) dose and the rate of relapse among patients with complete early virological response (cEVR) is shown. (A) Higher dose of RBV was associated with reduced rate of relapse. (B) These associations were also confirmed in selected patients who received 42–54 weeks of therapy.

when analysed separately, the rate of relapse was also associated with age and total RBV dose among patients with RVR. The rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 5% and 14%, respectively. The rate of relapse for patients <60 and ≥60 years was 9% and 18%, respectively. Collectively, the rate of relapse for patients <60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 2% and 11%, respectively, whereas the rate of relapse for patients ≥60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 12% and 20%, respectively.

Discussion

The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse among patients with cEVR to PEG-IFN plus RBV. Older patients (≥60 years) who received a total RBV dose <3.0 g/kg of body weight had the highest risk of relapse (52%), whereas younger patients who received a total RBV dose ≥3.0 g/kg of body weight had the lowest risk of relapse (16%). The rate of relapse decreased depending on the total RBV dose in younger patients, but remained stable in older patients despite a further increase in the RBV dose beyond 3.0 g/kg of body weight. These findings imply that the target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR, and further increase in RBV dose up to 4.0 g/kg of body weight or greater may be recommended in patients <60 years.

The associations between the drug adherence and virological response had been reported with inconsistent results. In an earlier study, patients who received >80% of the planned dose of PEG-IFN plus RBV for >80% of the planned duration of therapy had a higher rate of SVR compared to those who received a lesser dose (51% versus 34%) [31]. Consistent results were obtained in a study reporting that patients who received >80% of the planned dose of PEG-IFN and RBV within the first 12 weeks of therapy had a higher rate of EVR compared with those who received a lesser dose of both drugs (80% versus 33%) [4]. By contrast, a large-scale multicentre study showed that reducing the PEG-IFN dose during the first 20 weeks reduced SVR; however, reducing RBV did not affect SVR as long as RBV was not prematurely discontinued [32]. The reason for these inconsistencies is unclear. One reason may be the differences in the backgrounds of patients enrolled in the study, and hence the last study was limited to patients with advanced fibrosis and prior non-responders to PEG-IFN therapy. Because the probability of SVR is affected by virological response and relapse after response, the effect of drug dosing should be analysed separately with respect to these two factors.

In the present study, we focused on factors predictive of relapse after early virological response. According to the decision tree model, relapse was less likely in patients with RVR compared with cEVR. Among patients with cEVR, older patients (≥60 years) had a higher risk of relapse compared to younger patients (41% versus 22%). In addition, our results emphasized the effect of RBV dose for the prevention of relapse. In our study, a total RBV dose of ≥3.0 g/kg of body weight was repeatedly associated with a suppressed rate of relapse in the model derivation and validation groups. The rate of relapse in patients <60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 32% versus 16%, 27% versus 16%, and 41% versus 16%, respectively. The rate of relapse in patients ≥60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 52% versus 26%, 45% versus 38%, and 44% versus 22%, respectively. It has been reported that the rate of relapse is suppressed in 48 weeks of IFN plus RBV combination therapy compared to IFN monotherapy, indicating that RBV contributes to the increase in SVR by reducing relapse [2,3]. Another study, focused on the associations between the drug dose reduction and relapse in patients with virological response, found that maintaining RBV dose ≥12 mg/kg/day during 48 weeks of treatment, which can be translated into a total dose of 4.0 g/kg of body weight, suppressed relapse [33]. Results of the present study are in accordance with this report.

The importance of drug dosing on reduction in relapse is also supported by the findings that extending therapy from 48 to 72 weeks in patients with delayed virological response improved SVR rates by reducing relapse [9–13]. Apart from these clinical studies, in the real world of clinical practice, duration of therapy is extended – even in patients with cEVR – at the physician's discretion. The relationship between duration of therapy or RBV dose, and relapse among patients with cEVR and treated with various lengths of therapy has not been examined. In the combined group of our study, extending the duration of therapy was not associated with a reduction in relapse rate. Rather, the rate of relapse decreased depending on the total RBV dose. These findings suggest that acquiring a sufficient total RBV dose, either within 48 weeks or by extending the duration of therapy, is essential to prevent relapse among patients with cEVR. The limitation of the present study was that the mean duration of therapy was only 56.3 weeks in patients whose duration of therapy was extended beyond 48 weeks. It is probable that extended duration of therapy was not long enough for the prevention of relapse. Further studies with

longer durations of therapy are necessary to confirm the effect of extended duration of therapy on reduction of relapse among patients with cEVR.

Previous reports did not consider the effects of age in setting the optimal dose of RBV. In the present study, the relapse rate decreased with an increase in RBV dose from <2.5 to 3.0–3.5 g/kg of body weight, but remained relatively stable despite a further increase in the RBV dose in older patients. Thus, a total RBV dose ≥3.0 g/kg of body weight should be the target dose for patients ≥60 years with cEVR. By contrast, ≥3.0 g/kg of body weight of RBV was associated with lower risk of relapse in patients <60 with cEVR (16% versus 32%), and a further increase in RBV dose led to a more profound reduction in relapse rates, as low as 11% in patients who received ≥4.0 g/kg of body weight. Thus, a total dose of ≥4.0 g/kg of body weight or even greater should be the target dose in patients <60 years.

In the near future, more potent therapies, such as direct antiviral agents [34,35], may become available. These drugs require RBV and PEG-IFN in combination. However, not all patients may be able to tolerate this triple combination therapy due to adverse drug reactions, such as severe anaemia or skin eruption. In particular, it may be difficult to administer a full dose of triple drugs to older patients. Thus, personalizing the PEG-IFN and RBV combination therapy based on this model may be beneficial to patients who were intolerant to triple combination therapy.

In the present study creatinine was an independent predictor of relapse by multivariable logistic regression analysis. However creatinine was not selected as a splitting variable in decision tree, which may be due to the unique property of data mining analysis. In data mining analysis, limitation is imposed to stop the analysis when the number of patients is <20. This limitation is used to avoid dividing patients into too small subgroups which lead to the generation of rules that only apply to the model derivation population and not reproduced when applied to other populations. This phenomenon is called the over-fitting of the model. Due to this limitation, the variables selected in the data mining analysis are not necessarily identical to the variables that are significant by ordinary multivariable analysis. In a separate analysis, lower level of creatinine was associated with higher rate of relapse in each subgroup of patients with cEVR. The reason for this association is not clear, but lower creatinine level may be related to more efficient clearance of RBV leading to lower serum level of RBV. Further research is needed to confirm this speculation.

A potential limitation of the present study is that data mining analysis has an intrinsic risk of showing relationships that fit to the original dataset, but are not reproducible in different groups. Although internal and external validations showed that our model had high reproducibility, we recognized that further validation on a larger external validation cohort, especially in groups other than Japanese, may be necessary to further verify the reliability of our model.

In conclusion, we built a decision tree model for the prediction of relapse among patients with EVR to PEG-IFN plus RBV. The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse. The target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR. A further increase in RBV dose up to 4.0 g/kg of body weight may be warranted in patients <60 years.

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Disclosure statement

The authors declare no competing interests.

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