

enlighten the differential signaling pathways between hepatocytes and lymphocytes.

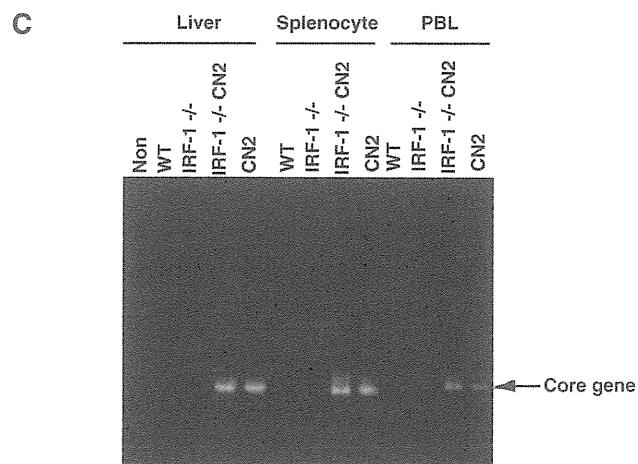
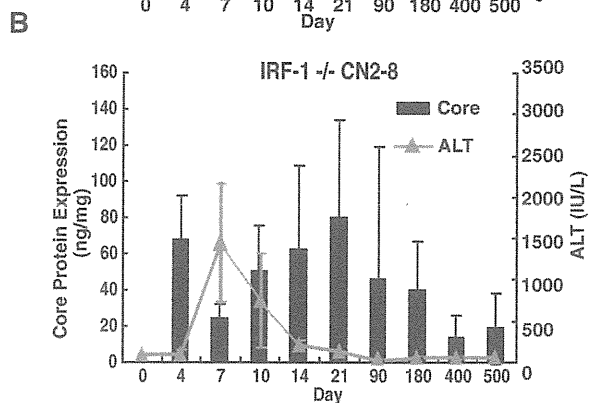
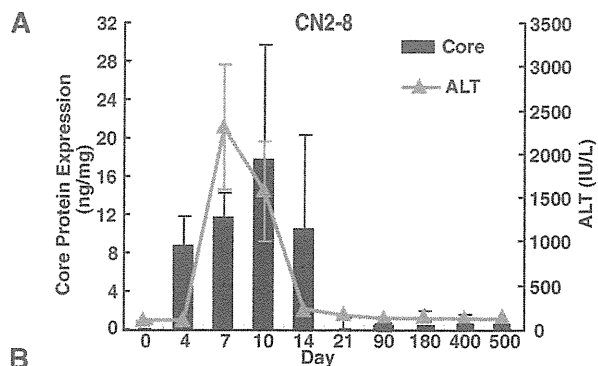
Overexpression of *bcl-2* in B-lymphoid cells causes autoimmune disease,³⁵ and *bcl-2* immunoglobulin-transgenic mice develop follicular lymphoproliferation.³⁶ Therefore, the aberrant expression of *bcl-2* in CN2 lymphocytes may be another factor that inhibits activation-induced cell death, leading to a predisposition for lymphoproliferative disorders. In support of this notion, HCV-infected patients express elevated levels of Bcl-2³⁷ and frequently carry the t(14;18) chromosomal translocation.³⁸ Suppression of cell death by full-length Bcl-2 requires phosphorylation of Ser70^{39,40}; in line with this, our Western blot analysis revealed 2 Bcl-2 species in the enlarged lymph nodes of the HCV protein-expressing mice. Bcl-2 and Fas/APO-1 regulate distinct pathways related to lymphocyte apoptosis.³⁵ Thus, both mitochondrion-dependent and mitochondrion-independent pathways exist for the induction of apoptosis. The dominant pathway differs depending on the tissue type.^{13,35} Therefore, Bcl-2 expression and *irf-1* disruption may synergistically inhibit the apoptotic response in lymphocytes. Indeed, the expression of HCV proteins, chronic inflammation, and predisposition to lymphoproliferative disorders were all enhanced in CN2 mice upon *irf-1* disruption. Collectively, these results suggest that HCV stimulates oncogenesis by multiple mechanisms. Although the loss of the *irf-1* alleles does not result in spontaneous tumor development in mice, it enhances dramatically the risk of tumors caused by the *c-Ha-ras* transgene or by *p53* nullizygosity,⁴¹ suggesting that the loss of *irf-1* confers susceptibility to tumor formation.

It has been proposed that HCV-infected lymphocytes induce t(18;14) translocation, which may result in the overexpression of Bcl-2, and a second mutation (in the *myc* oncogene), which leads to the development of lymphoma.^{42,43} Because the promoter region of Bcl-2 includes SP1, Cre, nuclear factor κ B,⁴⁴ and Akt/protein kinase B,⁴⁵ the combination of core expression and IL-10 and/or IL-2 may up-regulate these transcription factors by integrating several different pathways.

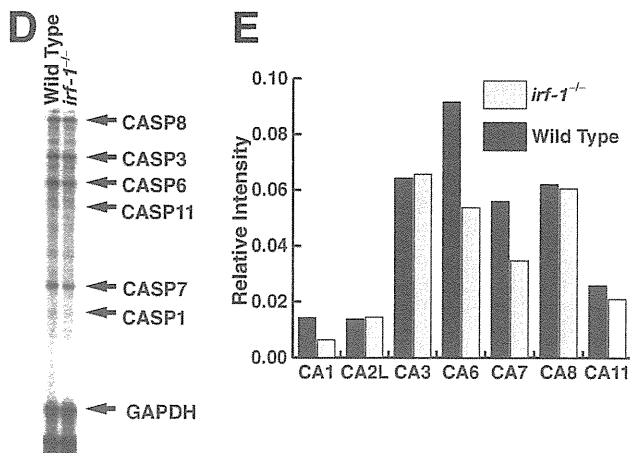
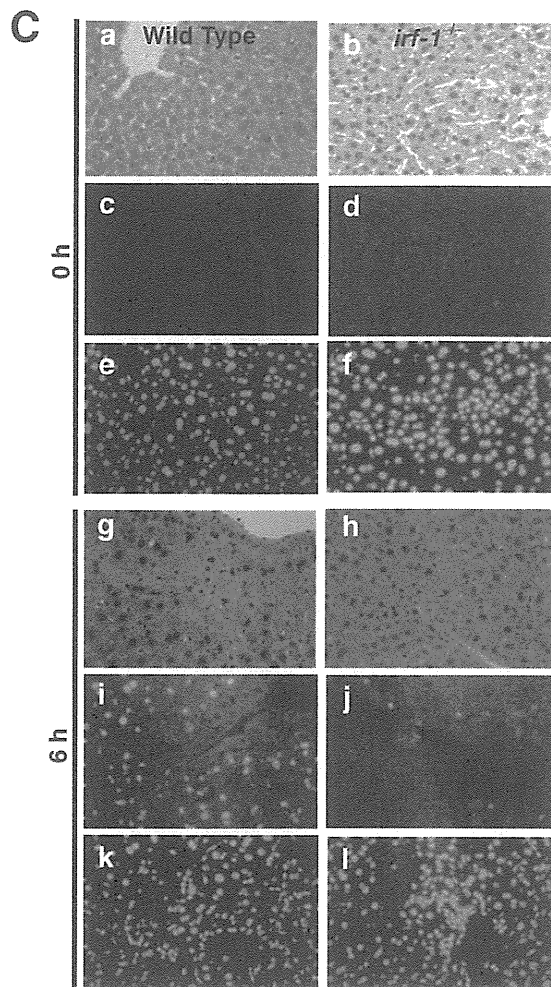
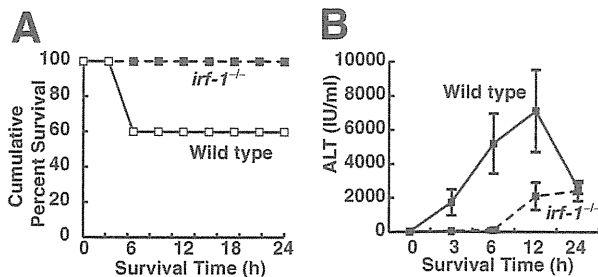
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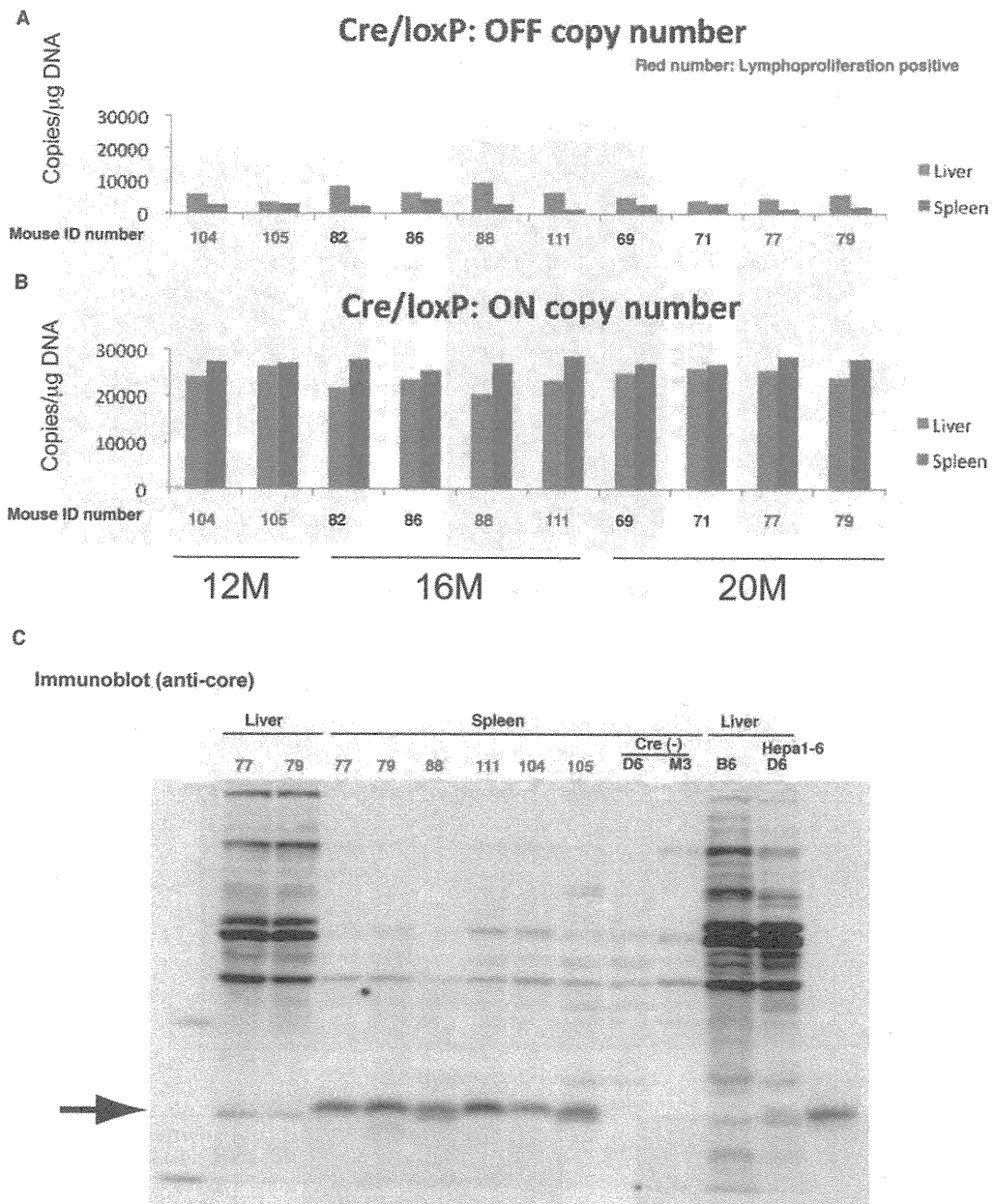


Supplementary Figure 1. (A and B) Levels of serum ALT and core protein expression in the hepatocytes of (A) CN2-8 and (B) *irf-1*^{-/-} CN2-8 mice following the administration of AxCANCre (n = 225 for *irf-1*^{-/-}; n = 75 for *irf-1*^{-/-} CN2-29; n = 150 for *irf-1*^{-/-} CN2-8; n = 225 for WT; n = 75 for CN2-29; and n = 150 for CN2-8; total of 900 mice). (C) Expression levels of the HCV core gene in the liver, splenocytes, and peripheral blood lymphocytes of the WT, *irf-1*^{-/-}, *irf-1*^{-/-} CN2, and CN2 mice, as determined by PCR.

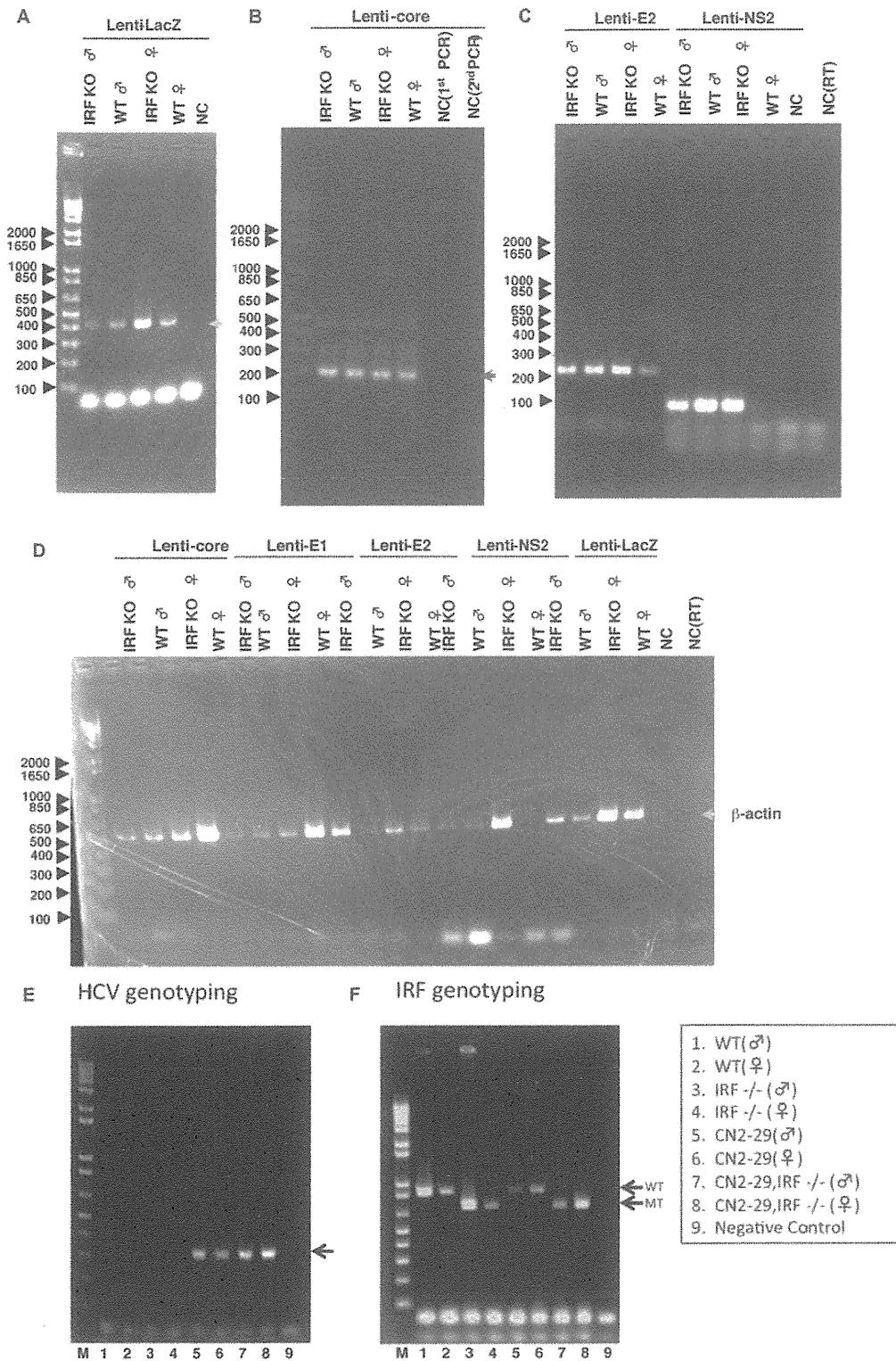


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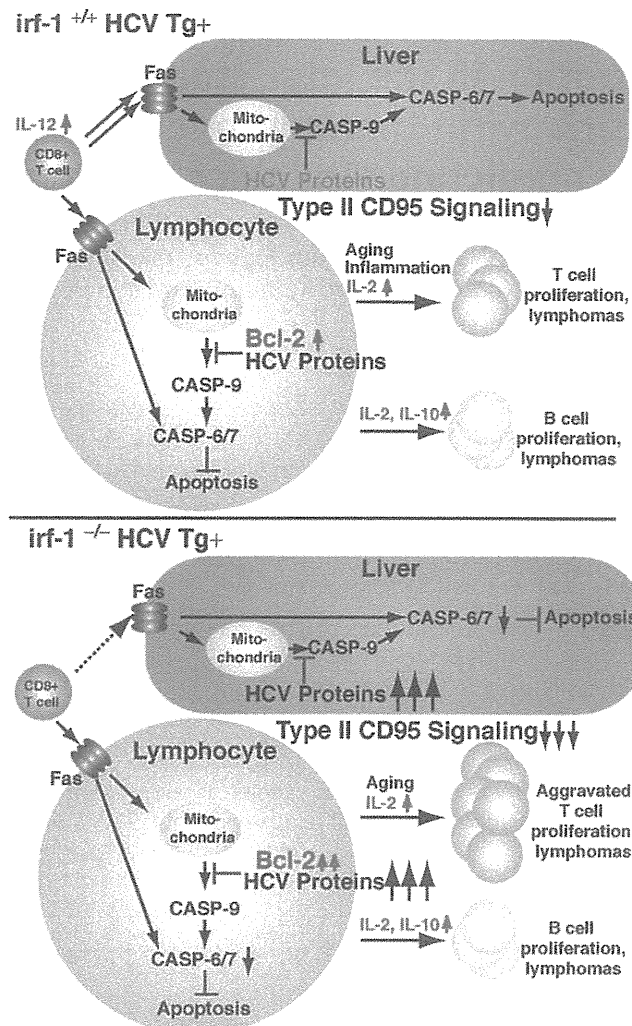
Supplementary Figure 2. Disruption of *irf-1* allows persistent expression of HCV proteins and inhibits Fas-induced apoptosis. The percentages of survivors 24 hours after injection of the anti-Fas monoclonal antibody into *irf-1*^{+/+} and *irf-1*^{-/-} mice. Serum ALT levels in *irf-1*^{-/-} and *irf-1*^{+/+} mice following anti-Fas monoclonal antibody injection. Histology of livers from *irf-1*^{-/-} CN2 and *irf-1*^{+/+} (WT) mice following injection of the anti-Fas monoclonal antibody. (a, b, g, and h) Liver sections stained with H&E 6 hours after injection of the anti-Fas monoclonal antibody (original magnification 40×). (c, d, i, and j) Apoptotic cells in the liver tissues identified by TUNEL staining 6 hours after injection of the anti-Fas monoclonal antibody. (e, f, k, and l) Nuclear staining with 4',6-diamidino-2-phenylindole of liver sections from WT and *irf-1*^{-/-} mice. The levels of expression of caspases -1, -2A, -3, -6, -7, -8, and -11 and control (glyceraldehyde-3-phosphate dehydrogenase) transcripts were determined in an ribonuclease protection assay using hepatocyte RNA species from WT and *irf-1*^{-/-} mice. Relative intensities of caspase mRNA expression in the hepatocytes of the WT and *irf-1*^{-/-} mice, with normalization for the expression of *GAPDH*.



Supplementary Figure 3. Switching efficiency for the expression of the HCV transgene using quantitative real-time PCR. (A and B) Viral gene expression levels were examined by quantitative real-time PCR before and after poly(I:C) injection. (C) Representative immunoblot for the detection of HCV core protein expression in murine livers and spleens.



Supplementary Figure 4. Real-time PCR for HCV transgene expression. (A–D) The HCV gene expression levels in splenocytes transfected with lentiviral vectors were examined by real-time PCR. Lentiviruses that expressed (A) LacZ, (B) core, and (C) E2 and NS2 were used to infect the splenocytes. The mRNA expression levels were detected by real-time PCR. (D) The real-time PCR of β -actin serves as a control. (E) Representative agarose gel images for the genotyping of HCV transgenes and *irf-1*.¹⁷



Supplementary Figure 5. Hypothetical model for the triggering of oncogenic potential by disruption of *irf-1* in synergy with HCV transgene expression. The levels of Bcl-2 and cytokines, as well as aging and inflammation, may enhance the development of lymphoproliferative disorders caused by HCV proteins. Disruption of *irf-1* enables the persistent expression of HCV proteins, leading to lymphoproliferative disease due to reduced apoptosis (ie, lower expression of caspase-1, -6, and -7). HCV CN2 transgenic (Tg+) mice are resistant to Fas-induced apoptosis due to inhibition of cytochrome *c* release from the mitochondria.⁵ Mice with disruption of the *irf-1* gene have several defects of the innate and adaptive immunity systems, such as lineage-specific defects in thymocyte development, whereby immature T cells can develop into mature CD4⁺ cells but not into CD8⁺ T cells.^{19,46} IRF-1 controls the positive and negative selection of CD8⁺ thymocytes.⁴⁷ IRF-1 is required for the development of the Th1-type immune response, and its absence leads to the induction of the Th2-type immune response.^{19,20} The number of natural killer cells is dramatically reduced in the *irf-1*^{-/-} mice.¹⁹ Expression of the IL-12 p40 subunit is defective in *irf-1*^{-/-} mice.¹⁹ In the present study, mice with persistent expression of HCV proteins had elevated serum levels of IL-2, IL-10, and IL-12 and higher levels of Bcl-2 protein in enlarged lymph nodes, and these phenomena were more pronounced in the absence of IRF-1.

Supplementary Table 1. Mouse Strains and Viral Protein Functions

Viral protein	Function	CN2-8 mouse strain		CN2-29 mouse strain	
Core	Nucleocapsid	Cre/loxP	Expressed	Cre/loxP	Expressed
E1	Envelope	Switching	Expressed	Switching	Expressed
E2	Envelope	On/off	Expressed	On/off	Expressed
NS2	Proteinase	Regulation	Expressed	Regulation	Expressed

Ribavirin concentration in the later stages of 48 week pegylated interferon- α 2b plus ribavirin therapy for chronic hepatitis C is useful for predicting virological response

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Objectives: The current standard of care for chronic hepatitis C patients is a pegylated interferon- α plus ribavirin combination treatment. This study was carried out to determine the relationship between ribavirin concentration in the later stages of treatment and virological relapse.

Patients and methods: Serum ribavirin concentration of 183 chronic hepatitis C patients (genotype 1) treated with pegylated interferon- α 2b plus ribavirin for 48 weeks was prospectively measured by HPLC at weeks 4, 12, 24, 36 and 48. Patients with undetectable serum hepatitis C virus (HCV) RNA 24 weeks after the end of treatment were designated as having sustained virological response (SVR). Patients with undetectable HCV RNA during the treatment but with virological relapse after the end of treatment were designated relapse.

Results: The mean ribavirin concentration at each testing point of patients with SVR (1401, 1725, 1803, 1811 and 1901 ng/mL) was significantly higher than that of relapse patients (998, 704, 607, 643 and 654 ng/mL) at weeks 4, 12, 24, 36 and 48, respectively (all $P < 0.001$). Multivariate regression analysis for relapse extracted ribavirin concentration at week 36, but not cumulative ribavirin dosage. The cut-off value by receiver operating curve analysis for predicting a relapse was 1503 and 1562 ng/mL at weeks 36 and 48, respectively.

Conclusions: Ribavirin concentration in the later stages of treatment is an important marker of viral relapse.

Keywords: HCV, pharmacokinetics, relapse, virological breakthrough

Introduction

Hepatitis C virus (HCV) infection can cause both acute and chronic hepatitis, leading to end-stage liver disease and hepatocellular carcinoma.^{1–4} The current standard of care for chronic hepatitis C patients is a pegylated interferon (PEG-IFN)- α plus ribavirin combination treatment.^{5–7} The main predictive factors of a poor sustained virological response (SVR) are HCV genotype other than 2 or 3 and a high baseline viral load.⁵ HCV genotype 1-infected patients with a high serum HCV RNA level are difficult to treat. The other significant factors for poor SVR are older age, higher body weight (BW), presence of bridging fibrosis, cirrhosis and significant steatosis.^{6,7} Ribavirin enhances SVR and the efficacy of ribavirin has been clearly proved.⁵ BW-adjusted dosages of ribavirin appear crucial to optimizing virological response, and ribavirin dosage reduction or discontinuation brings a significant decline in SVR.^{8,9} Therefore, ribavirin is a key element in treatments that promote SVR in chronic hepatitis C patients.

Ribavirin decreases HCV infectivity of newly produced virus, although this impact is only seen in those patients in whom the antiviral effect of interferon in blocking viral production is limited.¹⁰ Immunomodulation by ribavirin may be through an action on CD4 T cells by T cell differentiation towards T helper 1,^{11,12} which is associated with the clearance of HCV. Ribavirin increases mutation rates in non-structural 5A (NS5A) and 5B of the HCV genome,¹³ and thus may affect the proportion of defective quaspecies viruses, which are unable to infect new cells. Moreover, ribavirin may act synergistically with interferon by up-regulating host antiviral proteins or enhancing interferon signalling, thus leading to suppression of HCV replication.¹⁴

Preliminary pharmacological studies of ribavirin have been published.^{14–18} Previous studies of patients with chronic hepatitis C who were receiving PEG-IFN plus ribavirin combination treatment have shown no correlation between BW-adjusted ribavirin dose and single ribavirin time-point serum concentrations at week (W) 4 or W12 after the start of treatment, suggesting a poor dose–concentration relationship in the early stages.^{16,17}

However, a low ribavirin serum concentration was associated with a low SVR rate, suggesting a better concentration–effect relationship.^{16,17} Moreover, ribavirin exposure in the very early stages of PEG-IFN plus ribavirin treatment (at day 0) was significantly related to SVR in patients with chronic hepatitis C.¹⁸ These studies showed ribavirin pharmacokinetic predictors of SVR only in the early stages of treatment. To our knowledge, no studies have specifically evaluated the relationship between ribavirin concentration in the later stages of PEG-IFN plus ribavirin combination treatment and the rates of virological response of difficult-to-treat patients with chronic hepatitis C genotype 1. The aim of the present study was to evaluate ribavirin dosing strategies and ribavirin concentration profiles over 48 weeks of PEG-IFN- α 2b plus ribavirin combination treatment in order to establish the relationship between virological response and systemic ribavirin exposure.

Patients and methods

Patients

All participants enrolled in this study were chronic hepatitis C patients of HCV genotype 1b treated at seven participating hospitals (Kyushu University Hospital, Mitsutake Hospital, Yokota Hospital, Okabe Hospital, Kyushu Chuoh Hospital, Hara-Doi Hospital and Hara-Sanshin Hospital). Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease, hepatocellular carcinoma, kidney disease, a previous diagnosis of severe depression or other psychiatric disease, significant cardiac disease, seizure disorders or pregnancy.

Enrolment began in December 2004 and the study ended in December 2007. Of 224 patients screened, 202 were assigned to the study protocol. Of those 202, 19 (9.4%) patients withdrew from treatment within 36 weeks because of treatment-related severe adverse effects (giant urticaria at W4, $n=1$, severe anaemia between W12 and W36, $n=17$, and severe depression at W12, $n=1$) and were excluded from the pharmacological study, leaving the data of the 183 patients (90.6%) who completed the 48 weeks of the combination treatment available for analysis. Because the study was pharmacological, antiviral response analyses were performed in the per-protocol population. Demographic characteristics of the 183 studied patients are shown in Table 1. Patients weighing <60 kg formed the majority (114/183, 62.3%), and the percentage of patients weighing >75 kg was only 7.7% (14/183). Of those 14, six were >80 kg weight (80.1–90.4 kg). Serum levels of creatinine were normal for all patients. Liver biopsy was performed for 160 (87.4%) of the 183 patients by experienced hepatologists. The histological fibrosis score was estimated according to the METAVIR scoring system.¹⁹ Written informed consent was obtained from each patient and the study was approved by the local Ethics Committee of each hospital in accordance with the 1975 Declaration of Helsinki, as revised in 1983.

Treatment protocol, dose reduction and discontinuation of treatment

All patients received a combination treatment of PEG-IFN- α 2b (Pegintron; Schering-Plough, Osaka, Japan) plus ribavirin (Rebetol; Schering-Plough) for 48 weeks. PEG-IFN- α 2b was administered subcutaneously once a week with the dose based on BW (60 μ g for <45 kg, 80 μ g for 46–60 kg, 100 μ g for 61–75 kg, 120 μ g for 76–90 kg and 150 μ g for 91–120 kg). Ribavirin was given orally twice a day at a total dose based on BW (600 mg/day for <60 kg, 800 mg/day for 60–79.9 kg and 1000 mg/day for ≥ 80 kg). The above durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare.

The present study did not set the same treatment discontinuation rules as were used in other studies.^{20,21} Treatment was discontinued if there was not at least a 2 \log_{10} drop in HCV RNA at W12.

In the event of a serious adverse event developing during the course of treatment, we discontinued or modified the dosage of PEG-IFN and ribavirin until the adverse event abated or decreased in severity. Dose reduction of PEG-IFN was accomplished in a two-step process from the original starting dose of 1.5 μ g/kg/week, to 1 μ g/kg/week, then to 0.5 μ g/kg/week, if needed. If a >2 g/dL decrease in haemoglobin (Hb) was observed during any 4 week period of treatment, the first dose reduction of ribavirin was 200 mg/day, and the second dose reduction was by an additional 200 mg/day. If patients had Hb <8.5 g/dL after these dose reductions, administration of ribavirin was discontinued. Both PEG-IFN and ribavirin were discontinued if the Hb, white blood cell count or platelet count fell below 8.5 g/dL, $1 \times 10^9/L$ or $25 \times 10^9/L$, respectively. Also, the combination treatment was discontinued for patients with severe general fatigue, autoimmune hyperthyroidism, decrease or loss of vision, cardiovascular events, interstitial pneumonia or severe neuropsychiatric events including suicide attempts, suicidal and homicidal ideation, depression or aggressive behaviour towards others.

Assessment of drug exposure

The mean dosages of PEG-IFN- α 2b and ribavirin were calculated individually as averages on the basis of BW at baseline by reviewing the medical records: dosages of PEG-IFN- α 2b are expressed in μ g/kg/week and those of ribavirin are expressed in mg/kg/day. The cumulative dosage of the drugs from the start of treatment to each ribavirin concentration testing point and over the full treatment period was also evaluated for each patient to investigate the relationship with SVR and relapse.

Sample acquisition

In the morning after 12 h overnight fasting, blood samples were collected from each patient at enrolment and at regular intervals during the treatment period (at W4, W12, W24, W36 and W48) and at the end of the follow-up period (24 weeks after the end of treatment). Serum samples were frozen to -80°C within 2 h of collection for follow-up analysis. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by the following formula: fasting plasma glucose (mg/dL) \times fasting serum insulin (μ IU/mL)/405.²² HbA1c concentrations were measured on fresh whole blood samples with an HPLC assay at a clinical laboratory testing company (SRL, Inc., Tokyo, Japan). The concentrations were originally calculated by the method of the Japanese Diabetes Society/Japanese Society for Clinical Chemistry (JDS/JSCC) and converted to the US National Glycohemoglobin Standardization Program (NGSP) for use in this study.

Ribavirin concentration assay

Serum ribavirin concentration was measured using a previously published method in an assay consisting of phenylboronic acid solid phase extraction followed by HPLC at a commercial laboratory (SRL, Inc.).²³ In brief, the ribavirin concentration in a 200 μ L sample was measured by validated HPLC with column switching. Serum samples deproteinized with perchloric acid were injected into the column, and ribavirin was detected by ultraviolet absorption at 215 nm. The calibration curve was linear in the range 50–20000 ng/mL. A set of calibration standards at 0, 5, 10, 25, 50, 100, 250, 500, 1000, 2000 and 5000 μ g/L ribavirin was prepared, extracted and analysed with each series, together with internal quality controls at three levels. The above method was validated over the concentration range 10–5000 ng/mL for sensitivity, linearity, reproducibility, stability and recovery rate. Lack of adverse matrix effect and carry-over was also demonstrated. The intra-day and inter-day precision and

accuracy of the quality control samples were <9.0% relative standard deviation (RSD) and 10.6% bias for ribavirin, and 10.1% RSD and 10.6% bias for ribavirin base. The within-day and between-day coefficients of variation and bias were <2.5% over the range 10–5000 ng/mL. The recovery rate of the complete method ranged from 98.0% to 100.4%.

HCV RNA detection and HCV genotyping

HCV RNA was isolated by the Abbott m2000sp instrument from a 500 µL serum sample and detected by the m2000rt instrument. HCV RNA was extracted and quantified using a validated real-time reverse transcriptase-PCR assay with a range of detection from 12 IU/mL ($1.08 \log_{10}$ IU/mL) to 10^8 IU/mL ($8.0 \log_{10}$ IU/mL) (Abbott RealTime HCV assay; Abbott Molecular, Inc., Des Plaines, IL, USA).²⁴ HCV genotype determination was performed by means of sequence determination in the 5'-non-structural region of the HCV genome followed by phylogenetic analysis, as recently described.⁹

Virological response

Patients with undetectable serum HCV RNA 24 weeks after the end of treatment were designated SVR. Patients exhibiting a virological response during the 48 week treatment but with virological relapse 24 weeks after the end of treatment were designated relapse. Patients who were HCV RNA positive throughout the treatment period were designated non-response.

During treatment, we defined the following responses: rapid virological response (RVR), an undetectable HCV RNA at W4; complete early virological response (cEVR), detectable HCV RNA at W4 but undetectable HCV RNA at W12; complete late virological response (cLVR), detectable HCV RNA at W4 and W12 but undetectable from W24 to W48. Virological breakthrough meant reappearance of serum HCV RNA positivity among patients with undetectable HCV RNA during the treatment.

Statistical analysis

Statistical analysis was performed using SAS version 9.2 (Statistical Analysis System, SAS Institute Japan Ltd). A two-tailed *P* value of <0.05 was considered significant. Quantitative variables were expressed as mean, standard deviation (SD) of mean, standard error (SE) of mean, median value, range or 95% confidence interval (CI). Threshold values were obtained using receiver operating characteristic (ROC) curve analysis, and the results are presented as odds ratio (OR) with 95% CI. The unpaired *t*-test, Mann-Whitney *U*-test or Kruskal-Wallis test was used for non-parametric analysis. Spearman's rank correlation coefficient analysis and linear correlation analysis were carried out for two-factor relationships. Multiple logistic regression analysis was used to investigate the multivariate association of relapse with PEG-IFN plus ribavirin treatment, with the concentrations at W4, W12, W24 or W36 analysed separately.

Results

Virological outcome and response during treatment

By per-protocol analysis, SVR, relapse and non-response were found for 66 (36.1%), 52 (28.4%) and 65 (35.5%) of 183 studied patients, respectively. The percentage of patients for whom HCV RNA disappeared (SVR and relapse) was 64.5% (118 of 183). RVR, cEVR and cLVR were seen in 21 (11.5%), 52 (28.4%) and 45 (24.6%) of the 183 patients. Significant differences were found in the rates of RVR, cEVR and cLVR patients

achieving an SVR: 85.7% (18 of 21), 63.5% (33 of 52) and 33.3% (15 of 45), respectively ($P < 0.001$) (Table 1).

Changes in serum ribavirin concentration

For all patients, mean ribavirin concentration \pm SD was 1190.8 ± 519.3 (median 1145, range 167–3569) ng/mL, 1164.9 ± 635.4 (median 1022, range 161–2998) ng/mL, 1205.4 ± 762.9 (median 1020, range 111–5157) ng/mL, 1129.0 ± 690.8 (median 944, range 51–2946) ng/mL and 1142.0 ± 740.9 (median 932, range 60–3749) ng/mL at W4, W12, W24, W36 and W48, respectively. The coefficients of variation (CVs) of ribavirin concentration at each point were as follows: 43.6%, 54.5%, 63.3%, 61.3% and 64.9% at W4, W12, W24, W36 and W48, respectively.

Stratification by treatment outcome revealed differences between SVR ($n=66$) and non-SVR ($n=117$) patients (52 relapse and 65 non-response patients) at each testing point for ribavirin concentration. At each testing point, the mean ribavirin concentration (ng/mL) of SVR patients (1401, 1725, 1803, 1811 and 1901) was significantly higher than that of relapse and non-response patients: 998, 704, 607, 643 and 654 and 968, 786, 728, 636 and 654 at W4, W12, W24, W36 and W48, respectively (all $P < 0.001$). No significant difference in ribavirin concentration was found at any testing point between relapse and non-response patients ($P=0.801$ at W4, $P=0.975$ at W12, $P=0.603$ at W24, $P=0.664$ at W36 and $P=0.766$ at W48).

The mean ribavirin concentration at W4 of RVR ($n=21$) (1401 ng/mL) was not significantly higher than that of non-RVR ($n=162$) (1105 ng/mL) ($P=0.820$) patients. However, ribavirin concentrations at W4 (1581 ng/mL) and W12 (1534 ng/mL) of patients with RVR and cEVR ($n=73$) were significantly higher than those (1017 ng/mL and 839 ng/mL, respectively) of patients without RVR and cEVR ($n=110$) (all $P < 0.001$). These findings suggested that ribavirin concentration was related to EVR, but not to RVR.

Daily ribavirin BW-based doses were calculated by the cumulative dosage from the start of treatment to each point (W4, W12, W24, W36 and W48) and BW at pre-treatment. There were significant correlations between the ribavirin cumulative dosage and ribavirin concentration at each testing point (all $P < 0.001$), suggesting that ribavirin adherence (ribavirin daily dose) apparently affects virological response.

PEG-IFN and ribavirin dose reduction

Very few patients needed an extremely reduced PEG-IFN dosage: 83.6% of the studied patients received $\geq 80\%$ of their BW-based assigned total cumulative PEG-IFN dosage. No significant difference was found in the frequency of patients receiving $\geq 80\%$ PEG-IFN dose administration between patients with SVR (80.3%), relapse (92.3%) and non-response (80.0%) ($P=0.490$).

An extreme reduction in the ribavirin dosage to <60% of the total BW-based assigned total cumulative dosage was prescribed for 144 (78.7%) patients, mainly due to anaemia ($n=138$) and rarely general fatigue ($n=8$), and the remaining 39 (21.3%) had no reduction or a small reduction in ribavirin dosage, within $\geq 60\%$ of the assigned total cumulative dosage. Extreme reduction was done for 2 patients at W2, 38 between W3 and W7, 73 between W8 and W11, 12 between W12 and W23, 12 between W24 and W35, and 7 between W36 and

Table 1. Demographic characteristics of the 183 studied patients

Characteristic	All patients (n=183)	SVR (n=66)	Relapse (n=52)	Non-response (n=65)	P value ^a
Male, n (%)	83 (45.4)	37 (56.1)	21 (40.4)	25 (38.5)	0.081
Age, years (range)	60 (20–79)	56 (20–71)	58 (31–79)	64 (24–79)	<0.001
Body mass index (kg/m ²)	23.1 (16.2–30.0)	23.1 (16.2–30.0)	23.0 (17.1–29.8)	23.4 (17.3–29.5)	0.378
Prior IFN monotherapy, n (%)	67 (36.6)	20 (30.3)	20 (38.5)	27 (41.5)	0.410
Prior non-PEG-IFN plus RBV treatment, n (%)	16 (8.7)	5 (7.6)	3 (5.8)	8 (12.3)	0.277
Serum creatinine (mg/dL)	0.7 (0.2–1.3)	0.7 (0.2–1.2)	0.6 (0.3–1.1)	0.6 (0.3–1.3)	0.088
Creatinine clearance (mL/min)	91.5 (41.7–218.2)	93.8 (53.7–218.2)	95.4 (50.5–190.8)	85.1 (41.7–160.8)	0.286
Serum albumin (g/dL)	4.2 (3.5–5.3)	4.2 (3.8–5.0)	4.3 (3.5–5.3)	4.1 (3.5–4.9)	0.165
ALT (IU/L)	52 (12–267)	52.5 (12–267)	51 (16–132)	57 (15–256)	0.480
γGTP (IU/L)	34 (10–237)	26 (10–87)	32 (15–147)	63 (26–237)	<0.001
White blood cells (/mm ³)	4700 (1960–8700)	4940 (2390–8680)	4475 (2520–8700)	4700 (1960–8010)	0.281
Hb (g/dL)	13.2 (9.2–17.3)	13.5 (9.6–17.3)	13.0 (11.1–16.4)	13.2 (9.2–17.2)	0.304
Platelet count (×10 ⁹ /L)	160 (76–305)	172 (76–305)	161 (98–300)	151 (91–252)	0.116
Fasting plasma glucose (mg/dL)	96 (69–158)	92 (75–123)	99 (78–158)	98 (69–143)	0.117
Fasting serum insulin (μU/mL)	10.3 (2.8–33.7)	7.6 (2.8–21.3)	11.4 (3.2–19.9)	14.9 (3.9–33.7)	<0.001
HOMA-IR	2.6 (0.7–8.7)	1.7 (0.7–5.8)	2.7 (0.8–6.1)	3.5 (0.9–8.7)	<0.001
HbA1c (%)	5.5 (4.3–8.0)	5.2 (4.3–7.3)	5.5 (5.1–8.0)	5.6 (4.9–7.5)	0.698
Serum HCV RNA level (log IU/mL)	5.7 (4.2–7.9)	5.8 (4.2–7.7)	5.8 (4.7–7.8)	5.7 (4.5–7.9)	0.453
Histological hepatic fibrosis, n (%)					
stage 0	16 (8.7)	9 (13.6)	5 (9.6)	2 (3.1)	0.977
stage 1	56 (30.6)	22 (33.3)	17 (32.7)	17 (26.2)	
stage 2	46 (25.1)	16 (24.2)	16 (30.8)	14 (21.5)	
stage 3	23 (12.6)	3 (4.5)	7 (13.5)	13 (20.0)	
stage 4	19 (10.4)	5 (7.6)	2 (3.8)	12 (18.5)	
untested	23 (12.6)	11 (16.7)	5 (9.6)	7 (10.8)	
PEG-IFN initial dose (μg/kg/week)	1.5 (1.1–1.8)	1.5 (1.2–1.7)	1.5 (1.1–1.7)	1.5 (1.1–1.8)	0.306
Assigned total cumulative PEG-IFN dosage [≥80%, n (%)]	153 (83.6)	53 (80.3)	48 (92.3)	52 (80.0)	0.490
RBV initial dose (mg/kg/day)	11.0 (9.9–17.0)	12.3 (9.9–17.0)	10.6 (9.9–13.2)	10.1 (9.9–14.5)	<0.001
Assigned total cumulative RBV dosage [≥60%, n (%)]	39 (21.3)	28 (42.4)	6 (11.5)	5 (7.7)	<0.001
Virological response (RVR/cEVR/cLVR), n	21/52/45	18/33/15	3/19/30	0/0/0	<0.001 ^b

IFN, interferon; RBV, ribavirin; ALT, alanine aminotransferase. We defined the following responses: RVR, undetectable HCV RNA at W4; cEVR, detectable HCV RNA at W4 but undetectable HCV RNA at W12; cLVR, detectable HCV RNA from W4 to W12 but undetectable from W24 to W48. Data are shown as n (%) or median (range).

^aP value for the comparison between patients with SVR, relapse, non-response by Kruskal–Wallis test.

^bP value for the comparison between patients with SVR and relapse by Fisher's exact test.

W48. Of the 144 patients with extreme ribavirin dose reduction, 113 (78.5%) were within the first 12 weeks of treatment. The SVR rate of the patients without extreme reduction (28 of 39, 71.8%) was significantly higher than that of those with extreme reduction (38 of 144, 26.4%) ($P<0.001$).

In 144 patients with extreme reduction of ribavirin (Figure 1a), classified by virological response, the mean ribavirin concentration (ng/mL) at each testing point of SVR patients ($n=38$) (1377, 1698, 1940, 1900 and 1977) was significantly higher than that of relapse ($n=46$) and non-response ($n=60$) patients: 1027, 810, 752, 682 and 630; and 1005, 807, 806, 650 and 652 at W4, W12, W24, W36 and W48, respectively (all $P<0.001$). The findings suggested that SVR patients were able to maintain a

higher ribavirin concentration, even under low drug exposure, than those with relapse and non-response.

In 39 patients with ≥60% of the assigned total cumulative dosage (Figure 1b), classified by virological response, the mean ribavirin concentration (ng/mL) at each testing point of SVR patients ($n=28$) (1573, 1823, 1870, 1905 and 2011) was significantly higher than that of relapse ($n=6$) and non-response ($n=5$) patients: 1404, 886, 979, 872 and 920; and 1083, 1308, 1191, 1040 and 811.8 at W4, W12, W24, W36 and W48, respectively ($P<0.05$, except at W4). The findings suggested that patients with relapse and non-response were not able to maintain an adequate ribavirin concentration, even when they were given sufficient dosage.

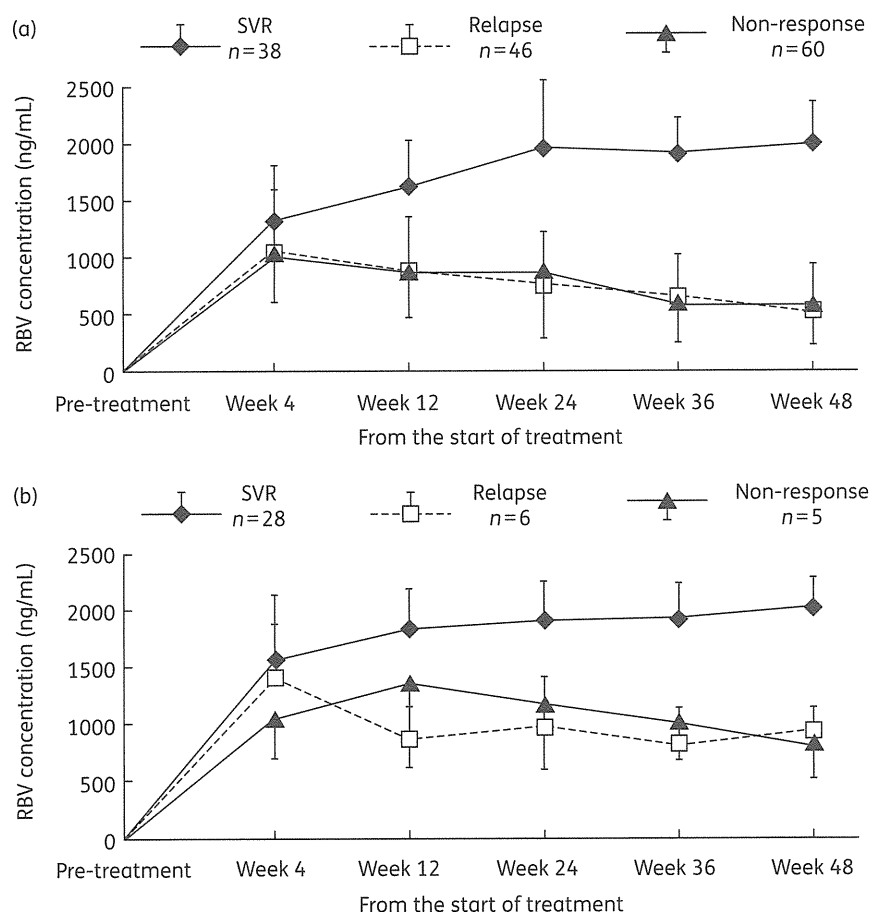


Figure 1. (a) Changes in serum ribavirin (RBV) concentration for 144 chronic hepatitis C patients with <60% of the ribavirin assigned total cumulative dosage during 48 week PEG-IFN plus ribavirin combination treatment, classified by virological response. Each point represents the mean ribavirin concentration, and the vertical bar represents the standard deviation of the mean. (b) Changes in serum ribavirin concentration for 39 chronic hepatitis C patients with ≥60% of the ribavirin assigned total cumulative dosage during 48 week PEG-IFN plus ribavirin combination treatment, classified by virological response. Each point represents the mean ribavirin concentration, and the vertical bar represents the standard deviation of the mean.

Table 2. Correlation of ribavirin (RBV) concentration with first timing of HCV RNA negativity and virological breakthrough during 48 week PEG-IFN-α2b plus RBV treatment in 52 relapse patients

First timing of HCV RNA negativity at	Assigned total cumulative RBV dosage, n (%)	Median RBV concentration (ng/mL)					Virological breakthrough, n (%)	
		W4	W12	W24	W36	W48		
W4 (n=3)	≥60%	0	—	—	—	—	—	
	<60%	3 (100)	398	353	500	577	820	0
W12 (n=19)	≥60%	1 (5.3)	1281	923	801	652	655	0
	<60%	18 (94.7)	1085	311	372	405	576	6 (31.6)
W24 (n=19)	≥60%	4 (21.1)	1456	1084	1280	954	1080	1 (5.2)
	<60%	15 (78.9)	907	771	553	673	612	5 (26.3)
W36 (n=9)	≥60%	1 (11.1)	1387	877	830	974	853	0
	<60%	8 (88.9)	1007	761	845	642	631	1 (11.1)
W48 (n=2)	≥60%	0	—	—	—	—	—	—
	<60%	2 (100)	1308	581	618.5	877.5	453	0
Total (n=52)	≥60%	6 (11.5)	1404	886	979	872	920	1 (1.9)
	<60%	46 (88.5)	1027	810	752	622	630	12 (23.0)

Virological breakthrough means a reappearance of serum HCV RNA positivity among these patients with undetectable HCV RNA during the treatment.

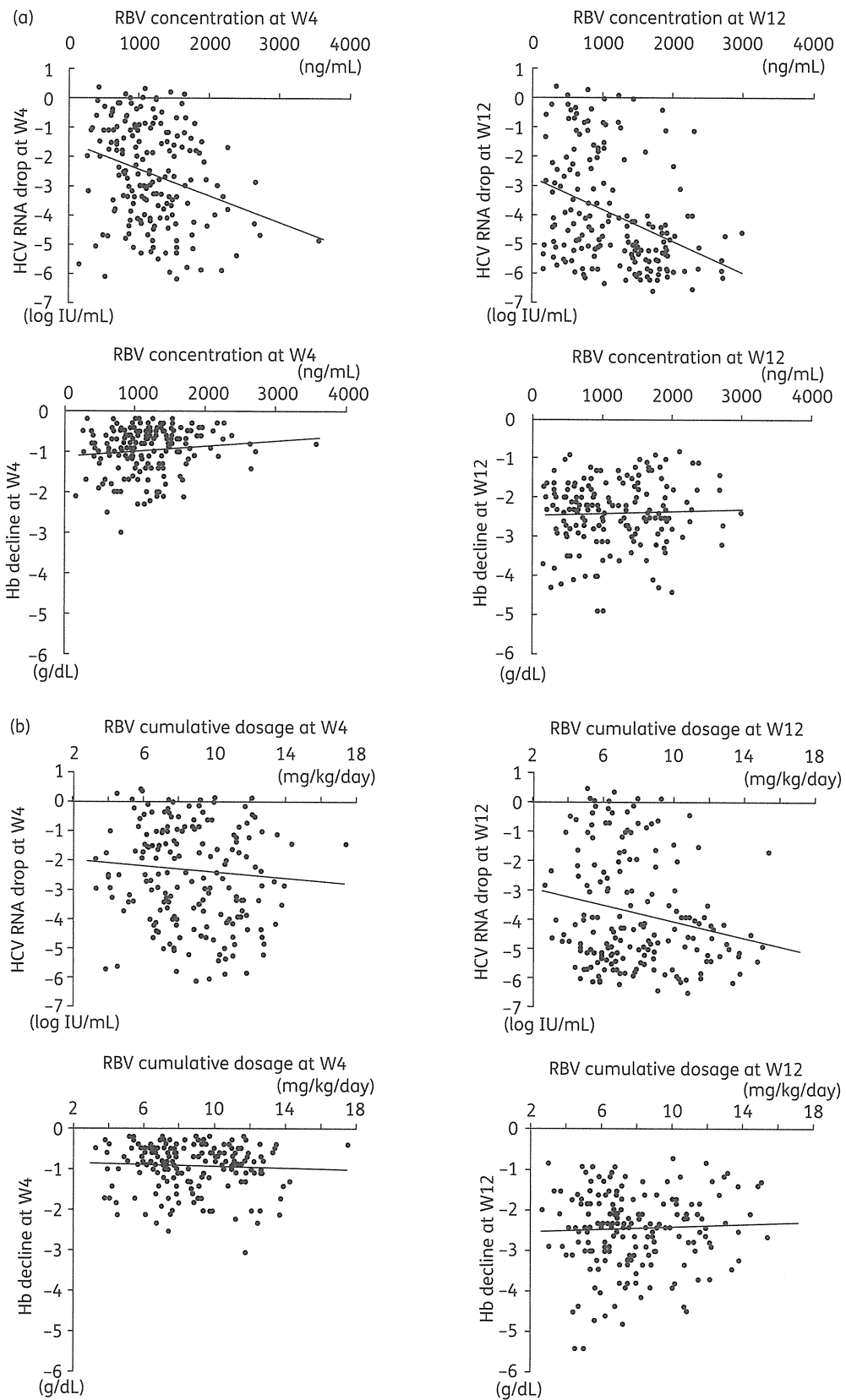


Figure 2. (a) Correlation of ribavirin (RBV) concentration with HCV RNA drop and Hb decline for chronic hepatitis C patients receiving 48 week PEG-IFN plus ribavirin combination treatment (at W4 and W12) The horizontal axis represents ribavirin concentration and the vertical axis represents HCV RNA

For relapse ($n=52$), we analysed the correlation between ribavirin concentration and virological breakthrough by the timing of first HCV RNA negativity and extreme reduction in ribavirin dosage (Table 2). Of the 52 relapse patients, only 6 (11.5%) were able to continue at $\geq 60\%$ of the BW-based assigned total ribavirin cumulative dosage. Higher virological breakthrough was found in patients with an extreme ribavirin dose reduction than those without the reduction. However, the median ribavirin concentrations were very low and decreased with the treatment period in both groups. Classified by extreme ribavirin dose reduction, the patients of both groups had low ribavirin concentration. Moreover, 13 (25.0%) had virological breakthrough. The findings suggest that inadequate ribavirin concentration induces the reappearance of HCV viraemia.

Different correlations of ribavirin concentration between HCV RNA drop and Hb decline

Figure 2(a) shows the correlation of ribavirin concentration with HCV RNA drop and Hb decline. HCV RNA drop and Hb decline were measured by fluctuation from the pre-treatment levels of each patient. There were significantly negative correlations between ribavirin concentration and HCV RNA drop at W4 and W12 (both $P<0.001$). Also, we found significant correlation between ribavirin concentration and HCV RNA drop at W24, W36 and W48 (data not shown). However, there was no significant correlation between ribavirin concentration and Hb decline at W4 ($P=0.097$) or W12 ($P=0.971$).

Figure 2(b) shows the correlation of cumulative ribavirin dosage with HCV RNA drop and Hb decline. Although there was significant correlation between cumulative ribavirin dosage and HCV RNA drop only at W12 ($P=0.029$), there was no significant correlation between cumulative dosage and HCV RNA drop only at W4 ($P=0.055$), or cumulative dosage and Hb decline at W4 ($P=0.242$) or W12 ($P=0.820$).

Correlation between serum ribavirin concentration and pre-treatment factors

Spearman's rank correlation coefficient analysis for correlation between ribavirin concentration and quantitative variables is shown in Table 3. Age, γ -glutamyl-transpeptidase (γ GTP), serum fasting insulin, HOMA-IR and ribavirin initial dose were significantly correlated with serum ribavirin concentration at each testing point, especially after W12 of treatment. The remaining factors had no clinical meaning in relation to the concentration because they had very small r (<3).

ROC curve analysis for predicting relapse

Table 4 shows ROC curve analysis to evaluate the ability of serum ribavirin concentration and cumulative ribavirin dosage to predict

relapse. The areas under the curves (AUCs) for serum ribavirin concentration were 0.742, 0.922, 0.951, 0.987 and 0.994 at W4, W12, W24, W36 and W48, respectively, suggesting that the predictive values for relapse were sufficient; the values after W12 were satisfactory and significantly increased. By comparison among the AUCs for serum ribavirin concentration, that at W36 significantly differed from those at W4 ($P<0.001$), W12 ($P=0.0031$), W24 ($P=0.0101$), but not from that at W48 ($P=0.3405$). However, the AUCs for cumulative ribavirin dosage were 0.685, 0.741, 0.760, 0.765 and 0.766 at W4, W12, W24, W36 and W48, respectively. The AUCs of serum ribavirin concentrations for relapse were superior at all points to those of cumulative ribavirin dosages.

The AUCs of serum ribavirin concentration at W36 for predicting relapse, classified by the timing of the absence of serum HCV RNA, were 0.722 for RVR patients ($n=21$), 1.000 for cEVR patients ($n=52$) and 1.000 for cLVR patients ($n=45$), suggesting that ribavirin concentration at W36 did not affect the SVR of RVR patients.

The optimal serum ribavirin concentration cut-off value was estimated for all patients by ROC curve analysis at each testing point. Between the SVR and relapse groups, the optimal serum ribavirin concentration cut-off values at each testing point during the treatment were found to be 1027, 1318, 1229, 1503 and 1562 ng/mL at W4, W12, W24, W36 and W48, respectively (Table 4). Setting a serum ribavirin concentration threshold of 1500 ng/mL, we found extremely high sensitivity of 0.970 (95% CI 0.895–0.996), specificity of 0.983 (95% CI 0.940–0.998), positive predictive value of 97.0% and negative predictive value of 98.3% for relapse at W36, which were similar to the values at W48.

The frequency of patients with a ribavirin concentration of ≥ 1500 ng/mL at W36 significantly increased with the cumulative ribavirin target dosage from the start of treatment to W36: 16 (20.2%) of 79 with $<40\%$ of the dosage, 19 (32.2%) of 59 with 41%–59%, 21 (61.8%) of 34 with 60%–79% and 10 (90.9%) of 11 with $\geq 80\%$ ($P<0.001$).

Predictive factors correlated with relapse

Multivariate regression analysis (Table 5) identified three pre-treatment factors that independently influenced relapse: female sex (OR 2.861, $P=0.018$), higher γ GTP (OR 1.280 for each 10 IU/L, $P=0.024$) and lower ribavirin initial dose (OR 0.708 for each 1 mg/kg/day, $P<0.001$). Adding the treatment parameters to the analysis, the analysis identified two parameters, lower ribavirin initial dose (OR 0.662 for each 100 ng/mL, $P=0.044$) and ribavirin concentration at W36 (OR 0.314 for each 100 ng/mL, $P<0.001$). The mean dosage of ribavirin and cumulative ribavirin dosage were not found to be significant factors that influence a relapse.

drop or Hb decline from the baseline. The line represents linear correlation between the two parameters. There is significant correlation between ribavirin concentration and HCV RNA drop at both W4 and W12 (all $P<0.001$), but not between ribavirin concentration and Hb decline (at W4, $P=0.097$ or at W12, $P=0.971$). (b) Correlation of cumulative ribavirin dosage with HCV RNA drop and Hb decline for chronic hepatitis C patients receiving 48 week PEG-IFN plus ribavirin combination treatment (at W4 and W12). The cumulative ribavirin dosage was calculated from the start of treatment through each point. The horizontal axis represents cumulative ribavirin dosage and the vertical axis represents HCV RNA drop or Hb decline from the baseline. The line represents linear correlation between the two parameters. There is significant correlation between cumulative ribavirin dosage and HCV RNA drop at W12 ($P=0.029$), but not between cumulative ribavirin dosage and HCV RNA drop at W4 ($P=0.055$), or between cumulative ribavirin dosage and Hb decline (at W4, $P=0.242$ and at W12, $P=0.820$).

Table 3. Correlation between serum ribavirin concentration at each testing point and pre-treatment factors of the 183 studied patients by Spearman's rank correlation coefficient analysis

Pre-treatment characteristics		Serum RBV concentration (ng/mL) at				
		W4	W12	W24	W36	W48
Age (years)	<i>r</i>	-0.252	-0.306	-0.347	-0.306	-0.321
	<i>P</i> value	<0.001	<0.001	<0.001	<0.001	<0.001
Body mass index (kg/m ²)	<i>r</i>	-0.084	0.034	-0.038	0.002	-0.048
	<i>P</i> value	0.253	0.644	0.603	0.977	0.512
Serum creatinine (mg/dL)	<i>r</i>	0.195	0.280	0.230	0.267	0.248
	<i>P</i> value	0.008	<0.001	0.002	<0.001	<0.001
Creatinine clearance (mL/min)	<i>r</i>	0.019	0.042	0.068	0.102	0.066
	<i>P</i> value	0.797	0.573	0.363	0.169	0.377
Serum albumin (g/dL)	<i>r</i>	0.073	0.098	0.104	0.112	0.139
	<i>P</i> value	0.327	0.188	0.161	0.130	0.059
ALT (IU/L)	<i>r</i>	-0.085	-0.016	0.048	0.018	-0.039
	<i>P</i> value	0.255	0.830	0.514	0.805	0.597
γGTP (IU/L)	<i>r</i>	-0.185	-0.278	-0.253	-0.319	-0.319
	<i>P</i> value	0.012	<0.001	<0.001	<0.001	<0.001
White blood cells (/mm ³)	<i>r</i>	0.089	0.087	0.133	0.180	0.159
	<i>P</i> value	0.231	0.243	0.070	0.014	0.030
Hb (g/dL)	<i>r</i>	0.122	0.190	0.210	0.258	0.172
	<i>P</i> value	0.098	0.009	0.004	<0.001	0.001
Platelet count (×10 ⁹ /L)	<i>r</i>	0.203	0.183	0.128	0.177	0.225
	<i>P</i> value	0.005	0.012	0.008	0.016	0.002
Fasting plasma glucose (mg/dL)	<i>r</i>	-0.146	-0.235	-0.143	-0.086	-0.118
	<i>P</i> value	0.055	0.001	0.061	0.259	0.123
Fasting serum insulin (μU/mL)	<i>r</i>	-0.163	-0.278	-0.283	-0.287	-0.325
	<i>P</i> value	0.036	<0.001	<0.001	<0.001	<0.001
HOMA-IR	<i>r</i>	-0.179	-0.308	-0.284	-0.277	-0.325
	<i>P</i> value	0.021	<0.001	<0.001	<0.001	<0.001
HbA1c (%)	<i>r</i>	-0.113	-0.142	-0.073	-0.046	-0.065
	<i>P</i> value	0.174	0.087	0.376	0.582	0.433
Serum HCV RNA level (log IU/mL)	<i>r</i>	-0.067	0.023	0.030	-0.020	-0.017
	<i>P</i> value	0.365	0.756	0.686	0.779	0.814
PEG-IFN initial dose (μg/kg/week)	<i>r</i>	-0.004	0.105	0.155	0.057	0.121
	<i>P</i> value	0.951	0.156	0.035	0.439	0.100
RBV initial dose (mg/kg/day)	<i>r</i>	0.293	0.446	0.459	0.418	0.446
	<i>P</i> value	<0.001	<0.001	<0.001	<0.001	<0.001

RBV, ribavirin; ALT, alanine aminotransferase.

Table 4. Comparison of ROC at each treatment time for the prediction of relapse by serum ribavirin (RBV) concentration and cumulative dosage

Point	RBV concentration					RBV cumulative dosage				
	Cut-off value (ng/mL)	Sensitivity	Specificity	AUC (SE)	<i>P</i> value ^a	Cut-off value (mg/kg/day)	Sensitivity	Specificity	AUC (SE)	<i>P</i> value ^a
W4	1027	0.564	0.436	0.742 (0.036)	<0.001	7.79	0.818	0.530	0.685 (0.041)	0.035
W12	1318	0.924	0.863	0.922 (0.021)	0.003	7.33	0.621	0.778	0.741 (0.039)	0.204
W24	1229	0.985	0.838	0.951 (0.015)	0.010	5.98	0.697	0.769	0.760 (0.039)	0.599
W36	1503	0.970	0.983	0.987 (0.008)		5.92	0.606	0.829	0.765 (0.038)	
W48	1562	0.985	0.974	0.994 (0.004)	0.340	4.67	0.803	0.632	0.766 (0.038)	0.985

Ribavirin cumulative dosage means the cumulative dosage from the start of treatment through each ribavirin concentration-measured point.
^a*P* value was calculated in comparison with W36 value.

Table 5. Multivariate regression analysis for relapse

Factor (category)	Analysis of baseline factors			Analysis of baseline and treatment-related factors		
	OR	95% CI	P value	OR	95% CI	P value
Sex (female to male)	2.861	1.190–6.878	0.018			
γGTP (by 10 IU/L)	1.280	1.032–1.589	0.024			
RBV initial dose (by 1 mg/kg/day)	0.708	0.589–0.837	<0.001	0.662	0.443–0.991	0.044
RBV concentration at W36 (by 100 ng/mL)				0.314	0.172–0.571	<0.001

RBV, ribavirin.

Table 6. Multivariate regression analysis for ribavirin concentration at W36 of ≥1500 ng/mL

Factor (category)	Analysis of baseline factors			Analysis of baseline and treatment-related factors		
	OR	95% CI	P value	OR	95% CI	P value
Baseline HCV RNA level (by 1 log IU/mL)	0.508	0.259–0.995	0.048	0.468	0.219–1.001	0.050
γGTP (by 10 IU/L)	0.783	0.653–0.939	0.008			
HOMA-IR (by 1)	0.679	0.492–0.936	0.018	0.735	0.522–1.033	0.076
HCV RNA drop from the baseline through W4 (by 1 log IU/mL)				1.832	1.372–2.447	<0.001
Ribavirin initial dose (by 1 mg/kg/day)	1.247	1.068–1.457	0.005			
(mg/kg/day)				1.657	1.308–2.099	<0.001

Similarly, multivariate regression analysis identified four pre-treatment factors that independently influenced SVR, including male sex ($P=0.008$), lower γGTP ($P=0.004$), lower HOMA-IR ($P=0.028$) and higher ribavirin initial dose ($P<0.001$). Adding the treatment parameters to the analysis, the analysis identified only two parameters: higher ribavirin initial dose ($P=0.010$) and ribavirin concentration at W36 ($P<0.001$).

Predictive factors correlated with ribavirin concentration >1500 ng/mL at W36

Multivariate analysis identified four pre-treatment factors that independently influenced ribavirin concentration >1500 mg at W36, including lower serum HCV RNA level ($P=0.048$), lower γGTP ($P=0.008$), lower HOMA-IR ($P=0.018$) and higher ribavirin initial dose ($P=0.005$) (Table 6). Adding the treatment parameters to the analysis, an additional two factors, dynamics of HCV RNA drop at W4 from the baseline ($P<0.001$) and ribavirin dose at W36 ($P<0.001$) were identified.

Discussion

In the treatment of chronic hepatitis C, the magnitude of serum HCV RNA decline induced by ribavirin in the early stages is significantly smaller than that induced by interferon: interferon can induce a decline of several orders of magnitude (logs), while ribavirin induces a <0.5 log decline in HCV RNA levels.^{25,26} In this study, the steady-state concentrations of ribavirin in the later stages of PEG-IFN plus ribavirin treatment, especially at W36, were significantly linked to relapse during a 48 week PEG-IFN

plus ribavirin combination treatment. Ribavirin exposure after the first dose was significantly linked to SVR, as well as RVR and, to a lesser extent, to EVR in another study.¹⁸ To our knowledge, this is the first study to propose such later stage ribavirin pharmacokinetic predictors of relapse. A minimum serum ribavirin concentration threshold of 1500 ng/mL at W36 of the combination treatment is predictive of relapse in these difficult-to-treat patients.

A well-known predictor of SVR is the achievement of RVR and EVR in the initial 12 week PEG-IFN plus ribavirin treatment period.^{5,25,26} Following the start of interferon treatment, a rapid first-phase decline in HCV viral load is observed for 1–2 days. This decline is attributed to a reduction in production and/or release of new virions from infected cells due to interferon action.²⁶ If ribavirin were to inhibit viral replication, a similar decline in HCV viral load would be expected following the onset of ribavirin administration. In fact, a higher cumulative dose of ribavirin during the initial 12 weeks was reported to be predictive of SVR.²⁷ Therefore, a very early dosing adjustment of ribavirin is effective for achieving SVR. The ROC curve analysis of our study showed that the concentration of ribavirin in the early period was correlated with relapse, but that the impact of ribavirin concentration in the later stages, especially at W36 had significantly more influence on relapse than was found for the earlier periods. Our study showed that W36 was the most useful time to check ribavirin concentration for the prediction of relapse.

Following multiple dosing, ribavirin gradually accumulates in plasma, reaching a maximum asymptotic concentration in ~4 weeks.^{14,28} Ribavirin is reported to have a large distribution volume and large inter-individual variability in concentration.¹⁴ Individual factors such as age, sex, BW, renal function, liver

function and gastrointestinal (GI) tract absorption probably influence ribavirin pharmacokinetics.¹⁴ In our study, the CV of ribavirin concentration increased with the treatment period and there was a wide distribution of ribavirin concentration among patients at each testing point. The ribavirin concentration of some of our patients increased to the end of treatment. Pharmacological theory cannot in all cases account for the concentration of ribavirin.

As treatment-related factors, BW-based dosing and dose reduction have been associated with SVR.^{5,8,9,29-31} BW-adjusted ribavirin dose has been approved for use in combination with PEG-IFN. The present study also showed that the initial BW-based dose of ribavirin influenced viral response. Adherence to the prescribed treatment dosages appears to be an important determinant of SVR. A reduction in the dose of PEG-IFN during the first 20 weeks reduced SVR, while reducing the ribavirin dose did not affect SVR as long as patients remained on full-dose PEG-IFN.²⁹ However, patients with dosages of PEG-IFN and ribavirin reduced to <80% of the original dose or who discontinued treatment prior to completing 80% of the planned 48 weeks of treatment had lower SVR than those who were compliant and received at least 80% of their PEG-IFN and at least 80% of their ribavirin for >80% of the planned 48 weeks of treatment (SVR 52% versus 63%).³⁰ Moreover, the timing of dose reduction can affect SVR. Patients who had the dose reduced or who discontinued treatment in the first 12 weeks had lower SVR than the others.³¹ Thus, dose reduction and timing of the two drugs are complicatedly associated with determinants of SVR. In the present study, the cumulative target dosage of ribavirin, based on the BW-adjusted dosage, during treatment was shown to correlate with ribavirin concentration, leading to the achievement of SVR.

The percentage of our patients who weighed >75 kg was only 7.7%, with patients who weighed <60 kg forming the majority (62.3%). There is a difference in the recommended ribavirin dosages between Japan and other countries, including Europe and the USA: 600 mg/day for <60 kg, 800 mg for >60 and <80 kg, and 1000 mg/day for >80 kg in Japan; while the dosages are 800 mg/day for <65 kg, 1000 mg/day for >65 and <80 kg, 1200 mg/day for >80 kg and <105 kg, and 1400 mg/day for >105 kg in the USA and Europe.^{9,18,21,29} Calculating the BW-based dosages, the minimum dosages are much lower in Japan (~10 mg/day/kg, e.g. 600 mg for 59.9 kg) than would be calculated in other countries (~11.5 mg/day/kg, e.g. 1200 mg for 104.9 kg). These differences could possibly influence SVR and ribavirin concentration. Our patients weighing 55–59 kg (600 mg/day) and 75–79 kg (800 mg/day) received comparatively lower BW-based ribavirin initial doses than the others (<55 kg and 60–74.9 kg) at the start of treatment. The initial doses significantly influenced relapse in the present study. Also, dose reduction influenced efficacy. When dose reduction due to adverse effects was needed for patients who started with a ribavirin dose of 600 mg/day, the dose reduction rates were 33% and 66% for 200 mg and 400 mg dose reductions, respectively. In the cases of the USA and Europe, the maxima are 25% and 50% for 200 mg and 400 mg dose reduction for patients <65 kg and an initial dose of 800 mg. The dose reduction rate by Japanese standards may have influenced SVR differently than would have been seen in other countries.

The present study showed that a minimum serum ribavirin concentration threshold of 1500 ng/mL at W36 of the combination treatment is predictive of relapse. The threshold and the consequent concentrations of ribavirin appeared to be lower than those shown by other published findings¹⁴⁻¹⁸ in which cut-off ribavirin concentrations were reported to be 1600–2860 ng/mL for EVR and SVR. Also, these data were based only on the early stages, W4–W12, not on the later stages as was done in the present study. On the other hand, our ROC analysis showed that the later stages of ribavirin concentration addressed efficacy more than other factors, although these BW-based dosages and the dose reduction were associated with ribavirin concentration and influenced relapse. Therefore, we put special emphasis on ribavirin concentration for discriminating viral response.

Ribavirin clearance is dependent on renal function, as determined by the glomerular filtration rate, and it has been recommended that the ribavirin dosage mainly be adjusted to the creatinine clearance rate and not, as is done in practice, to BW.^{15,16} Estimation of serum creatinine showed the renal function of our patients to be close to normal. However, age may have had a trifling influence on renal function in this study, because it included relatively older patients in Japan than were included in the studies of other countries. Patients with global exposure to ribavirin were widely distributed, despite dose adjustment for BW; the AUC:dose ratio was highly variable (range 1.3×10^{-3} – 8.7×10^{-3} ng h/L).¹⁸ Therefore, our directly measured ribavirin concentrations were the most predictive markers for relapse, beyond the BW-based dosage.

It is possible that the liver plays a role in the pharmacokinetics of ribavirin.²⁸ Oral administration of 600 mg of ribavirin yielded a mean plasma C_{max} (%CV) of 643 (37) ng/mL for healthy adult volunteers, significantly increasing to 886 (43), 1046 (26) and 1273 (33) ng/mL for chronic hepatitis patients with Child–Pugh Classes A, B and C, respectively.³² There were very few patients with cirrhosis and no patients with Child–Pugh Classes B and C in this study. No difference in ribavirin concentration was found among our patients classified by histological finding.

The GI tract is thought to be the major site of ribavirin first-pass elimination.¹⁵ None of our patients had a known GI tract disease that could have influenced abnormal GI absorption or metabolism of ribavirin. Also, none of our patients had portal hypertension leading to gastropathy. High-fat meals can increase ribavirin bioavailability by 46% compared with the fasting intake of ribavirin.²⁵ In this study, we cannot address whether or not there was a GI influence on ribavirin concentration because we did not carefully monitor the patients' meals. Therefore, it is possible that there might be a correlation between food intake and ribavirin concentration.

Our study showed that lower HOMA-IR and lower γ GTP level were significant factors for SVR and relapse and that serum insulin and HOMA-IR also influenced ribavirin concentration at each testing point during the treatment. Insulin resistance is commonly seen in patients with hepatitis C.³³ Host-related factors, including overweight, decreased physical activity, older age and diets high in saturated and trans-fatty acids or fructose, are thought to contribute to insulin resistance. However, certain protein components of HCV, especially the core and NS5A proteins, induce insulin resistance directly and this occurs early in the course of infection.³⁴ Moreover, the HCV core protein

reduces transcription of the interferon-activated antiviral genes 2'-5' oligoadenylate synthetase (2-5OAS), myxovirus resistance-A and RNA-dependent protein kinase.³⁵ 2-5OAS interacts with HCV protein NS5A to inhibit the antiviral activity of interferon.³⁶ From a different point of view, hypoadiponectinaemia is associated with increased risk of diabetes mellitus type 2 and the degree of insulin resistance and hyperinsulinaemia, rather than with the degree of adiposity and glucose intolerance. We previously reported that insulin resistance in patients with chronic HCV infection was related to adiponectin secretion and that hypoadiponectin was related to high HCV RNA levels.³⁷ The only detectable γ GTP in human serum is of hepatic origin.³⁸ Although excessive alcohol intake may be involved, the mechanism responsible for the phenomenon of increased γ GTP in chronic hepatitis C patients is uncertain. Increased γ GTP in chronic hepatitis C patients who abstain or have moderate alcohol intake has been reported to be associated with hepatic steatosis and fibrosis,³⁹ thus indicating more advanced liver disease. For chronic hepatitis C patients with elevated γ GTP, serum parameters of glucose metabolism, HOMA and C-peptide, which represent insulin secretion, were higher than for patients with normal γ GTP.⁴⁰ Moreover, increased γ GTP predicts poor viral response to antiviral treatment of chronic hepatitis C patients.³³ These reports explain how the serum parameters of glucose metabolism, HOMA-IR and insulin secretion, and γ GTP were independently associated with both SVR and ribavirin concentration in the present study.

It is recommended that HCV genotype 1 interferon-naive patients receiving PEG-IFN plus ribavirin combination treatment have treatment discontinued if there is not at least a 2 log₁₀ drop in HCV RNA at 12 weeks of treatment or whose HCV RNA levels remain detectable after 24 weeks of treatment.^{20,21} Regardless of genotype, previously treated patients who have detectable HCV RNA at 12 or 24 weeks are highly unlikely to achieve SVR; therefore, such rules for stopping treatment should be considered.²¹ The present study did not have such rules for stopping treatment. Moreover, most of our patients strongly hoped to complete the scheduled treatment despite non-virological response. In fact, our previous multicentre study showed that only 5.9% of 273 patients, when given the option of stopping treatment because of non-virological response, rejected the combination treatment.⁴¹ Thus, we had the data of non-responders during the course of treatment. Our findings of ribavirin concentrations can corroborate such an application of the stopping rule to non-responders.

Of the 52 who relapsed after treatment, three were negative for HCV RNA at W4, but still relapsed. We believe their early response was not caused by ribavirin but mainly by PEG-IFN, and that continuously lower ribavirin concentrations led to the relapse. In fact, our continuous monitoring of ribavirin showed that lower ribavirin concentration at each point was related to relapse. Moreover, the other patients negative for HCV RNA during the treatment period had viral breakthrough due to low ribavirin concentration.

Our findings showed no correlation between ribavirin concentration and Hb decline, or ribavirin cumulative dosage and that decline. These results are inconsistent with previously published data.¹⁵ It may be explainable because our Japanese ribavirin doses differ from those of other countries, as mentioned above. However, another reason might be inosine

triphosphatase (ITPase) deficiency, which can protect against ribavirin-induced anaemia.⁴² Thompson *et al.*⁴² reported that *ITPA* gene (encoding ITPase) polymorphisms were associated with Hb decline and ribavirin dose reduction in patients with chronic hepatitis C receiving PEG-IFN plus ribavirin treatment. According to the report, patients with *ITPA* minor variant A have an advantage in PEG-IFN plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate. Therefore, it is clinically important to investigate a relationship between *ITPA* polymorphisms and ribavirin concentration.

We conclude that ribavirin concentration in the later stages of treatment is an important marker for discriminating relapse. We propose a serum ribavirin concentration threshold of 1500 ng/mL at W36 of this combination treatment as useful for predicting relapse.

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Transparency declarations

None to declare.

Author contributions

N.F. designed and wrote the study. E.O., K.T., T.I., H.I., T.H., T.K. and M.K. selected the patients and collected clinical data. N.F. carried out the statistical analysis. M.M. and J.H. critically revised the manuscript and gave the final approval for the submission.

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