doi:10.1046/j.1365-2893.1999.6120139.x

[13] F. Zoulim, M. Chevallier, M. Maynard and C. Trepo, "Clinical Consequences of Hepatitis C Virus Infection," *Reviews in Medical Virology*, Vol. 13, No. 1, 2003, pp. 57-68. doi:10.1002/rmv.371

[14] A. Bruchfeld, L. Stahle, J. Andersson and R. Schvarcz, "Ribavirin Treatment in Dialysis Patients with Chronic Hepatitis C Virus Infection—A Pilot Study," *Journal of Viral Hepatitis*, Vol. 8, No. 4, 2001, pp. 287-292. doi:10.1046/j.1365-2893.2001.00300.x

[15] G. Mazzella, E. Accogli, S. Sottili, *et al.*, "Alpha Interferon Treatment May Prevent Hepatocellular Carcinoma in HCV-Related Liver Cirrhosis," *Journal of Hepatology*, Vol. 24, No. 2, 1996, pp. 141-147. doi:10.1016/S0168-8278(96)80022-5

[16] M. Kohara, T. Tanaka, K. Tsukiyama-Kohara, *et al.*, "Hepatitis C Virus Genotypes 1 And 2 Respond to Interferon-Alpha with Different Virologic Kinetics," *Journal of Infectious Diseases*, Vol. 172, 1995, pp. 934-938. doi:10.1093/infdis/172.4.934

[17] H. Nakamura, H. Ogawa, T. Kuroda, *et al.*, "Interferon Treatment for Patients with Chronic Hepatitis C Infected with High Viral Load of Genotype 2 Virus," *Hepatogastroenterology*, Vol. 49, No. 47, 2002, pp. 1373-1376.

[18] J. Bukh, X. Forns, S. U. Emerson and R. H. Purcell, "Studies of Hepatitis C Virus in Chimpanzees and Their Importance for Vaccine Development," *Intervirology*, Vol. 44, No. 2-3, 2001, pp. 132-142. doi:10.1159/000050040

[19] D. G. Bowen and C. M. Walker, "Mutational Escape from CD8+ T cell Immunity: HCV Evolution, from Chimpanzees to Man," *The Journal of Experimental Medicine*, Vol. 201, No. 11, 2005, pp. 1709-1714. doi:10.1084/jem.20050808

[20] M. Lechmann and T. J. Liang, "Vaccine Development for Hepatitis C," *Seminars in Liver Disease*, Vol. 20, No. 2, 2000, pp. 211-226. doi:10.1055/s-2000-9947

[21] S. Okada, H. Harada, T. Ito, T. Saito and S. Suzu, "Early Development of Human Hematopoietic and Acquired Immune Systems in New Born NOD/Scid/Jak3null Mice Intrahepatic Engrafted with Cord Blood-Derived CD34 + Cells," *International Journal of Hematology*, Vol. 88, No. 5, 2008, pp. 476-482. doi:10.1007/s12185-008-0215-z

[22] S. Hattori, K. Ide, H. Nakata, *et al.*, "Potent Activity of a Nucleoside Reverse Transcriptase Inhibitor, 4'-ethynyl-2-fluoro-2'-deoxyadenosine, against Human Immunodeficiency Virus Type 1 Infection in a Model Using Human Peripheral Blood Mononuclear Cell-Transplanted NOD/SCID Janus Kinase 3 Knockout Mice." *Antimicrob Agents Chemother*, Vol. 53, No. 9, 2009, pp. 3887-3893. doi:10.1128/AAC.00270-09

[23] F. Kobune, H. Sakata and A. Sugiura, "Marmoset Lymphoblastoid Cells as a Sensitive Host for Isolation of Measles Virus," *Journal of Virology*, Vol. 64, 1990, pp. 700-705.

[24] K. Tsukiyama-Kohara, S. Tone, I. Maruyama, *et al.*, "Activation of the CKI-CDK-Rb-E2F Pathway in Full Genome Hepatitis C Virus-Expressing Cells," *The Journal of Biological Chemistry*, Vol. 279, No. 15, 2004, pp. 14531-14541. doi:10.1074/jbc.M312822200

[25] M. Satoh, M. Saito, K. Tanaka, *et al.*, "Evaluation of a Recombinant Measles Virus Expressing Hepatitis C Virus Envelope Proteins by Infection of Human PBL-NOD/Scid/Jak3null Mouse," *Comparative Immunology, Microbiology and Infectious Diseases*, Vol. 33, No. 6, 2010,pp. e81-e88. doi:10.1016/j.cimid.2010.02.006

[26] M. Yoneda, R. Miura, T. Barrett, K. Tsukiyama-Kohara and C. Kai, "Rinderpest Virus Phosphoprotein Gene is a Major Determinant of Species-Specific Pathogenicity," *Journal of Virology*, Vol. 78, No. 12, 2004, pp. 6676-6681. doi:10.1128/JVI.78.12.6676-6681.2004

[27] A. Op De Beeck, L. Cocquerel and J. Dubuisson, "Biogenesis of Hepatitis C Virus Envelope Glycoproteins," *Journal of General Virology*, Vol. 82, No. 11, 2001, pp. 2589-2595.

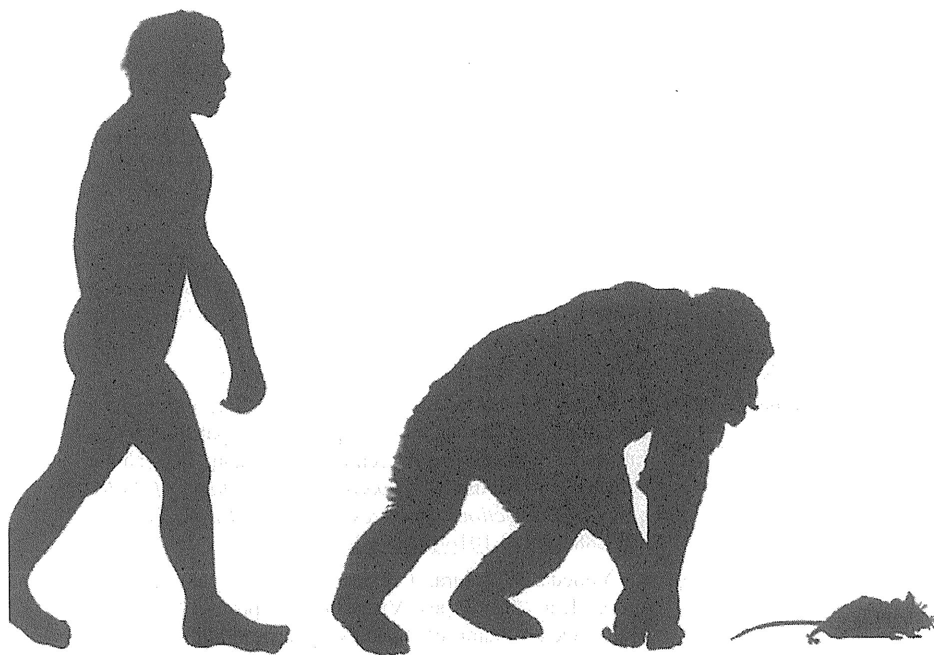
[28] E. Falkowska, F. Kajumo, E. Garcia, J. Reinus and T. Dragic, "Hepatitis C Virus Envelope Glycoprotein E2 Glycans Modulate Entry, CD81 Binding, and Neutralization," *Journal of Virology*, Vol. 81, No. 15, 2007, pp. 8072-8079. doi:10.1128/JVI.00459-07

[29] F. Helle, A. Goffard, V. Morel, *et al.*, "The Neutralizing Activity of Anti-hepatitis C Virus Antibodies is Modulated by Specific Glycans on the E2 Envelope Protein," *Journal of Virology*, Vol. 81, No. 15, 2007, pp. 8101-8111. doi:10.1128/JVI.00127-07

[30] P. Jackson, J. Petrik, G. J. Alexander, G. Pearson and J. P. Allain, "Reactivity of Synthetic Peptides Representing Selected Sections of Hepatitis C Virus Core and Envelope Proteins with a Panel of Hepatitis C Virus-Seropositive Human Plasma," *Journal of Medical Virology*, Vol. 51, No. 1, 1997, pp. 67-79. doi:10.1002/(SICI)1096-9071(199701)51:1<67::AID-JMV11>3.0.CO;2-1

[31] J. H. Hoofnagle, "Course and Outcome of Hepatitis C," *Hepatology*, Vol. 36, No. 5, 2002, pp. S21-S29. doi:10.1002/hep.1840360704

Copyright © 2011 SciRes.



RESEARCH TECHNIQUE

The murine candidate

Small animals that mimic human hepatitis C infection will help researchers pinpoint weakness in the viral life cycle.

BY ELIE DOLGIN

The hepatitis C virus (HCV) is hard to study. Most of what researchers know about how it multiplies comes from cell-culture systems. Such cellular set-ups have proven invaluable for developing new drugs, including protease inhibitors and polymerase inhibitors, which prevent the virus from replicating its components inside the cell. Yet these cell-based systems fail to capture other important parts of the viral life cycle, such as the step before replication, when the virus attaches to liver cells and gains entry. What's more, cell-culture systems cannot reproduce the interaction between the immune system and the virus nor can they recapitulate entire organs so that liver pathology can be studied. For these reasons, researchers interested in how the virus causes disease have long sought a small-animal model.

Common laboratory animals, including rodents and most primates, are not susceptible hosts for HCV. Scientists have therefore had to settle for chimpanzees, which, like humans, are vulnerable to chronic HCV infections. However, "for ethical and economic reasons, the chimp is a terrible model," says Matthew Evans, an HCV researcher at the Mount Sinai School of Medicine in New York. Research involving chimpanzees is banned in many parts of the world, including Europe. And in most places where experimentation with great apes is allowed, laws against euthanizing chimps require investigators to fund the animals' long-term care — a prohibitively expensive commitment.

That's where a colony of ordinary-looking black mice running around in cages on the fourth floor of the Rockefeller University Comparative Bioscience Center in New York comes in. These animals might not look special, but they have been engineered to express either a pair or a quartet of human genes and, as such, are the first small animals with fully functioning immune systems that are prone to HCV infection. Using these models, "you can actually now look at hepatitis C virus entry *in vivo*," says Rockefeller immunologist Alexander Ploss, who developed the animals together with Charles Rice, executive and scientific director of the Center for the Study of Hepatitis C in New York.

NATURE.COM
to read the latest research using animal models
go.nature.com/qDGI2e

These mice, and others like them, could provide a cheaper, more robust and less ethically fraught route to HCV drug and vaccine discovery.

THE HUMAN SIDE

Getting to this point has been a hard slog. In the years immediately after the virus was first described in 1989, many research teams developed transgenic mice carrying one or more genes encoding HCV proteins. Thus it was possible to study HCV-induced liver pathology without infecting mice with the virus. This approach still has some proponents. Last year, a team led by Matti Sällberg, a viral immunologist at the Karolinska Institute in Stockholm, used mice expressing the viral protease and showed that treatment with a drug targeting the cytokine tumour-necrosis factor-α led to improved liver function[1].

But the approach is highly artificial, leading to overexpression of the introduced viral genes and ignoring the rest of the viral life cycle. Over the past decade, most researchers have moved away from this set-up in favour of systems that involve infecting animals with the virus.

The first such model was reported ten years ago by a team led by transplant surgeon Norman Kneteman at the University of Alberta, in Edmonton, Canada. Kneteman's group engineered mice to express a gene that kills off the animals' own liver cells, which aren't susceptible to HCV infection; in their place they transplanted human liver cells, which are. These mice with humanized livers could be infected with HCV[2]. "This was the first [mouse] model that actually allowed HCV infection for prolonged periods of time by the normal route," says Kneteman.

These animals have proven useful for testing many candidate drugs. For example, a Japanese team led by Hiroshima University's Kazuaki Chayama treated Kneteman's liver transplant mice with a combination of new drugs: the protease inhibitor telaprevir (from Vertex Pharmaceuticals, based in Cambridge, Massachusetts) and the experimental polymerase inhibitor MK-0608 (from drug giant Merck, headquartered in Whitehouse Station, New Jersey). Late last year, the team reported that this combination eliminated the virus from the animals after a month of therapy and prevented the emergence of drug resistance, which often arises in mice and humans treated with either drug alone[3].

But to facilitate the human tissue transplant, the mice must be engineered to lack components of their immune system. The animals are thereby rendered poor models for testing drugs that alter the immune system, known as immunotherapies. Generating these mice also presents special difficulties. For one,

researchers can't breed chimaeric animals. And the mice are sickly because of the liver toxic gene.

Two recent transplant models of HCV infection provide improvements over Kneteman's mice[4,5]. Both types of mouse are less frail because of technical workarounds that allow researchers to introduce the liver deficit later in life. The model developed by Lishan Su, an immunologist at the University of North Carolina at Chapel Hill, in collaboration with Ploss and Rice at Rockefeller, also involves transplanting human blood stem cells into the animals to reconstitute a human-like immune system[5]. Of all of the published reports, says Su, "this is the only one that has both the immune system and the human liver in a chimaeric animal", creating a living platform for testing vaccines and immunotherapies in a human-like model.

Even though Su's mice generate a human T-cell response against the virus when infected, they still lack a complete immune system. "What we need now is a mouse — an immunocompetent, normal, mouse — that can be infected by a hepatitis C virus capable of replicating, spreading and initiating an immune response," says Frank Chisari, a virologist at The Scripps Research Institute in La Jolla, California. "We are light years away from that because that virus does not like to infect or replicate in mouse cells." But scientists are getting closer.

ENTRY LEVEL POSITION

To gain entry into liver cells, HCV hijacks four proteins. Although mice naturally produce these proteins, the human versions of two of them are needed for viral entry[6]. The black rodents at Rockefeller are the first animals into which the required human entry factors have successfully been introduced. "This has a lot of applications," says Ploss. "Right now, it's useful to measure HCV entry and potential entry inhibitors."

"This is a big advance," says Michael Houghton, a virologist at the University of Alberta, who co-discovered HCV more than 20 years ago. "It's been difficult to do vaccine research for hepatitis C because of the lack of an animal model other than the chimp. Now we can start using different vaccine strategies in mice to see which are best at eliciting a protective response."

Ploss's mice are the first such animals with a fully intact immune system that are susceptible to the viral infection. But the infection stops after cell entry: the virus does not seem to replicate. "You can recapitulate HCV entry," says Ploss, "but replication is still very inefficient and not detectable by conventional methods." So the big challenge now remains identifying whether additional human factors are needed to achieve the next step of the HCV life cycle in mice.

After replication comes assembly, when the viral components are gathered into new infectious particles that will be released from the cell and invade other cells. Fortunately, this final stage in the viral life cycle seems to be possible in mouse cells without introducing any human proteins, according to research presented at this year's International Liver Congress, in Berlin, by Ralf Bartenschlager, a molecular virologist at Heidelberg University in Germany. If the barriers to replication can be overcome, Bartenschlager says, it should be straightforward to get a full infection cycle going in a mouse. "We have the early steps; we have the late steps; the big black box now is the step in between."

It took more than a decade for scientists to deduce the factors needed for HCV cell entry. But Thomas Baumert, a hepatologist and virologist at the University of Strasbourg in France, is confident that the community will solve the problem of replication much faster. "We have better model systems now, so I think we can advance more rapidly." Within five years, he predicts, "it will be possible to produce transgenic mice for the entire viral life cycle".

Rice is equally confident this approach will work — but he is hedging his bets. Even such a model would have its drawbacks, he says, because the more mouse-like the model, the further removed it is from the human system. That's why even as his lab is aggressively pursuing a transgenic animal, he maintains active collaborations to develop other models, including new transplant chimaeric mice with humanized livers and immune systems. Other researchers are looking to animals that provide the natural susceptibility of primates without the ethical baggage (see 'The turn of the shrew'). "All of these things should be pursued in parallel," Rice says, "because we really don't know which of these models is going to be the best for a given application."

And so Rice and others continue to try and build a better mouse to help the research community beat a path to new HCV treatments. ■

Elie Dolgin *is a news editor with* Nature Medicine *in New York.*

THE TURN OF THE SHREW

Unusual model isn't persuading researchers of its practicality

Although most of the work developing small-animal models of hepatitis C virus (HCV) infection has focused on mice, some research teams have advanced an alternative model: the northern treeshrew (Tupaia belangeri). This squirrel-shaped animal shares a common ancestor with apes and is the only non-ape species known to be naturally susceptible to HCV. Last year, the first longitudinal analysis of HCV-infected tree shrews showed that, over the course of three years, the animals developed chronic hepatitis, fatty liver degeneration and liver cirrhosis[7]. "It's very similar to HCV infection in human beings," says study co-author Kyoko Tsukiyama-Kohara of Kumamoto University in Japan.

But few research teams have managed to establish long-term infections in the animals. And given the limited track record of tree shrews in drug discovery, most scientists agree that more traditional lab animal models of infection, such as mice, are needed. "If you're going to take a multimillion dollar drug and do your final trial before you go into humans, you need to have a reproducible model," says Robert Lanford, who has studied HCV in chimps for more than 20 years at the Texas Biomedical Research Institute in San Antonio.

NEIL BOWMAN/FLPA

The northern treeshrew, a natural host to hepatitis C virus, is proving an unpopular model of infection.

1. Brenndörfer, E. D. *et al. Hepatology* **52**, 1553–1563 (2010).
2. Mercer, D. F. *et al. Nature Med.* **7**, 927–933 (2001).
3. Ohara, E. *et al. J. Hepatol.* **54**, 872–878 (2011).
4. Bissig, K. D. *et al. J. Clin. Invest.* **120**, 924–930 (2010).
5. Washburn, M. L. *et al. Gastroenterology* **140**, 1334–1344 (2011).
6. Ploss, A. *et al. Nature* **457**, 882–886 (2009).
7. Amako, Y. *et al. J. Virol.* **84**, 303–311 (2010).

J Antimicrob Chemother 2011; **66**: 1127–1139
doi:10.1093/jac/dkr034 Advance Access publication 22 February 2011

Journal of Antimicrobial Chemotherapy

Ribavirin concentration in the later stages of 48 week pegylated interferon-α2b plus ribavirin therapy for chronic hepatitis C is useful for predicting virological response

Norihiro Furusyo*, Masayuki Murata, Eiichi Ogawa, Kazuhiro Toyoda, Takeshi Ihara, Hiroaki Ikezaki, Takeo Hayashi, Tsunehisa Koga, Mosaburo Kainuma and Jun Hayashi

Department of General Internal Medicine, Kyushu University Hospital, Fukuoka, Japan

*Corresponding author. Tel: +81-92-642-5909; Fax: +81-92-642-5916; E-mail: furusyo@gim.med.kyushu-u.ac.jp

Received 14 September 2010; returned 9 November 2010; revised 22 January 2011; accepted 23 January 2011

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-α2b 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 quasispecies 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]

© The Author 2011. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com

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-α2b 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-α2b (Pegintron; Schering-Plough, Osaka, Japan) plus ribavirin (Rebetol; Schering-Plough) for 48 weeks. PEG-IFN-α2b was administered subcutaneously once a week with the dose based on BW (60 μg for <45 kg, 80 μg for 46–60 kg, 100 μg for 61–75 kg, 120 μg for 76–90 kg and 150 μg 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 $\geq$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 μg/kg/week, to 1 μg/kg/week, then to 0.5 μg/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, 1×10^9/L or 25×10^9/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-α2b and ribavirin were calculated individually as averages on the basis of BW at baseline by reviewing the medical records: dosages of PEG-IFN-α2b are expressed in μg/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)×fasting serum insulin (μIU/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 μL 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 μg/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 ± 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 ≥80% of their BW-based assigned total cumulative PEG-IFN dosage. No significant difference was found in the frequency of patients receiving ≥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 ≥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 aminotranseferase. 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).
[a]P value for the comparison between patients with SVR, relapse, non-response by Kruskal–Wallis test.
[b]P 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.

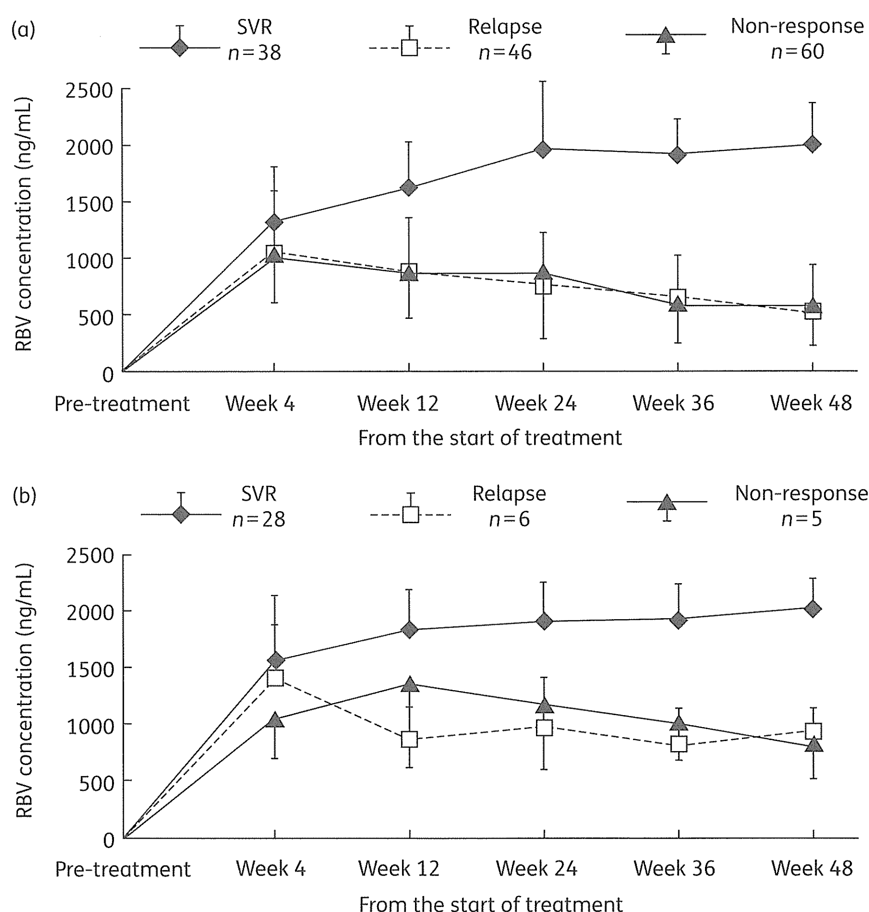


Figure 1. (a) Changes in serum ribavirin (RBV) concentration for 144 chronic hepatitis C patients with <60% of the ribavirin assigned total cumulative dosage during 48 week PEG-IFN plus ribavirin combination treatment, classified by virological response. Each point represents the mean ribavirin concentration,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

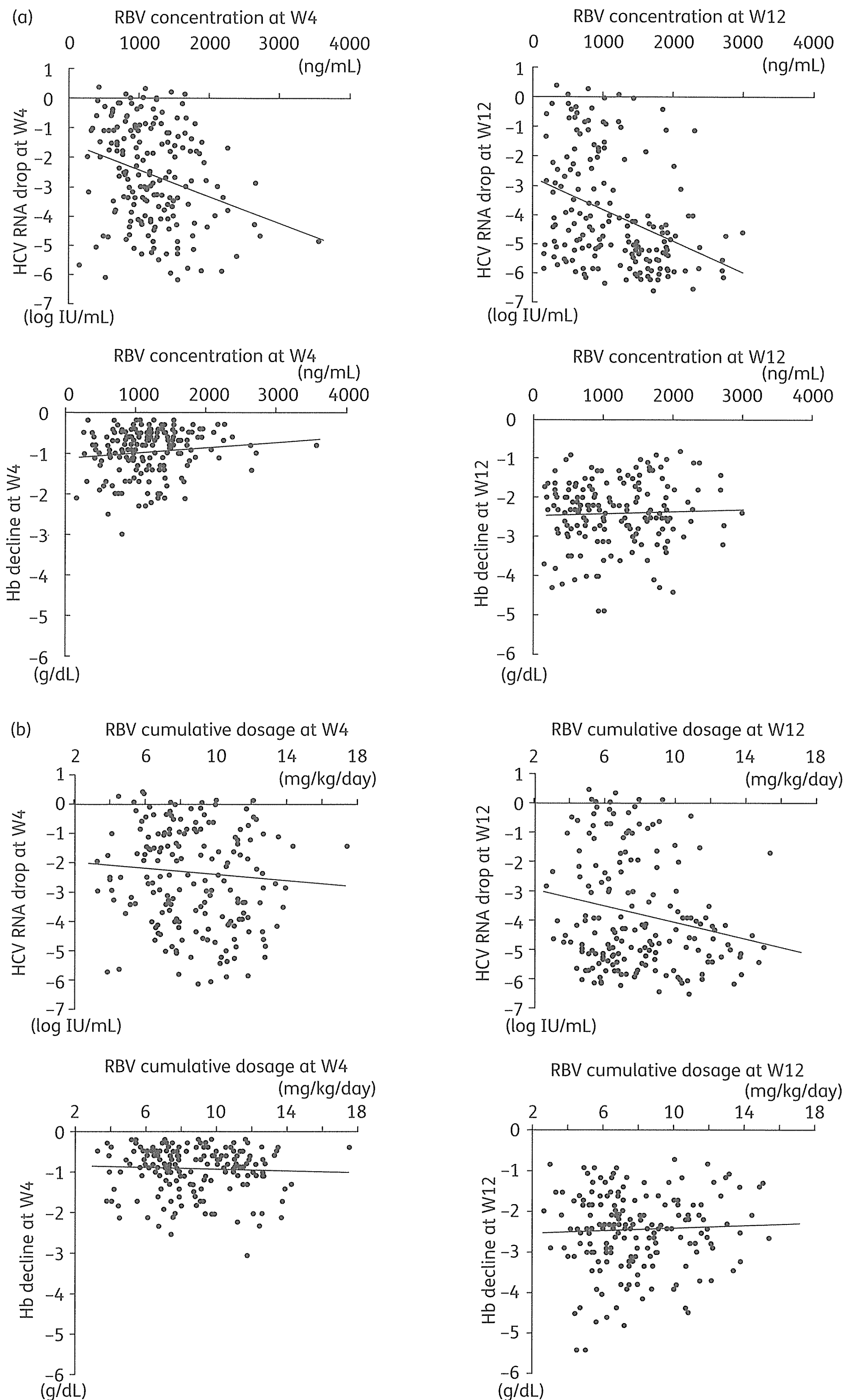


Figure 2. (a) Correlation of ribavirin (RBV) concentration with HCV RNA drop and Hb decline for chronic hepatitis C patients receiving 48 week PEG-IFN plus ribavirin combination treatment (at W4 and W12) The horizontal axis represents ribavirin concentration and the vertical axis represents HCV RNA

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 ≥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 (γGTP), 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 ≥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 γGTP (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

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

	RBV concentration					RBV cumulative dosage				
Point	Cut-off value (ng/mL)	Sensitivity	Specificity	AUC (SE)	*P* value[a]	Cut-off value (mg/kg/day)	Sensitivity	Specificity	AUC (SE)	*P* 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 findings[14–18] 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 γGTP 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 γGTP in human serum is of hepatic origin.[38] Although excessive alcohol intake may be involved, the mechanism responsible for the phenomenon of increased γGTP in chronic hepatitis C patients is uncertain. Increased γGTP 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 γGTP, serum parameters of glucose metabolism, HOMA and C-peptide, which represent insulin secretion, were higher than for patients with normal γGTP.[40] Moreover, increased γGTP 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 γGTP 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 $\log_{10}$ 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.

Acknowledgements

We are grateful to Hiroshi Takechi, PhD and Toshihiro Ueda, PhD for providing comments of statistical analysis.

Funding

This study was partially supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports Science and Technology of Japan.

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.

References

1 Marcellin P, Boyer N, Gervais A *et al.* Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. *Ann Intern Med* 1997; **127**: 875–81.

2 Hayashi J, Kishihara Y, Yamaji K *et al.* Hepatitis C viral quasispecies and liver damage in patients with chronic hepatitis C virus infection. *Hepatology* 1997; **25**: 697–701.

3 Furusyo N, Hayashi J, Ueno K *et al.* Human lymphoblastoid interferon treatment for patients with hepatitis C virus-related cirrhosis. *Clin Ther* 1997; **19**: 1352–67.

4 Hayashi J, Furusyo N, Ariyama I *et al.* A relationship between the evolution of hepatitis C virus variants, liver damage, and hepatocellular carcinoma in patients with hepatitis C viremia. *J Infect Dis* 2000; **181**: 1523–7.

5 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.

6 Grasso A, Malfatti F, De Leo P *et al.* Insulin resistance predicts rapid virological response in non-diabetic, non-cirrhotic genotype 1 HCV patients treated with peginterferon alpha-2b plus ribavirin. *J Hepatol* 2009; **51**: 984–90.

7 Cardoso AC, Moucari R, Figueiredo-Mendes C *et al.* Impact of peginterferon and ribavirin therapy on hepatocellular carcinoma: incidence and survival in hepatitis C patients with advanced fibrosis. *J Hepatol* 2010; **52**: 652–7.

8 Jacobson IM, Brown RS Jr, Freilich B *et al.* Peginterferon alfa-2b and weight-based or flat-dose ribavirin in chronic hepatitis C patients: a randomized trial. *Hepatology* 2007; **46**: 971–81.

9 Furusyo N, Kajiwara E, Takahashi K *et al.* Association between the treatment length and cumulative dose of pegylated interferon alpha-2b plus ribavirin and their effectiveness as a combination treatment for Japanese chronic hepatitis C patients: project of the Kyushu University Liver Disease Study Group. *J Gastroenterol Hepatol* 2008; **23**: 1094–104.

10 Dixit NM, Layden-Almer JE, Layden TJ *et al.* Modeling how ribavirin improves interferon response rates in hepatitis C virus infection. *Nature* 2004; **432**: 922–4.

11 Furusyo N, Kubo N, Toyoda K *et al.* Helper T cell cytokine response to ribavirin priming before combined treatment with interferon alpha and ribavirin for patients with chronic hepatitis C. *Antiviral Res* 2005; **67**: 46–54.

12 Asahina Y, Izumi N, Enomoto N *et al.* Mutagenic effects of ribavirin and response to interferon/ribavirin combination therapy in chronic hepatitis C. *J Hepatol* 2005; **43**: 623–9.

13 Liu WL, Su WC, Cheng CW *et al.* Ribavirin up-regulates the activity of double-stranded RNA-activated protein kinase and enhances the action of interferon-alpha against hepatitis C virus. *J Infect Dis* 2007; **196**: 425–34.

14 Maynard M, Pradat P, Gragnieu MC *et al.* Prediction of sustained virological response by ribavirin plasma concentration at week 4 of therapy in hepatitis C virus genotype 1 patients. *Antivir Ther* 2008; **13**: 607–11.

15 Merello J, Rodriguez-Novoa S, Jimenez-Nacher I *et al.* Usefulness of monitoring ribavirin plasma concentrations to improve treatment response in patients with chronic hepatitis C. *J Antimicrob Chemother* 2008; **62**: 1174–80.

16 Pedersen C, Alsio C, Lagging M *et al.* Ribavirin plasma concentration is a predictor of sustained virological response in patients treated for chronic hepatitis C virus genotype 2/3 infection. *J Viral Hepat* 2010; doi:10.1111/j.1365-2893.2010.01303.x.

17 Aguilar Marucco D, Gonzalez de Requena D, Bonora S *et al.* The use of trough ribavirin concentration to predict sustained virological response and haematological toxicity in HIV/HCV-co-infected patients treated with ribavirin and pegylated interferon. *J Antimicrob Chemother* 2008; **61**: 919–24.

18 Loustaud-Ratti V, Alain S, Rousseau A *et al.* Ribavirin exposure after the first dose is predictive of sustained virological response in chronic hepatitis C. *Hepatology* 2008; **47**: 1453–61.

19 Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. *Hepatology* 1994; **20**: 15–20.

20 Davis GL, Wong JB, McHutchison JG *et al.* Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645–52.

21 Cammà C, Cabibbo G, Bronte F *et al.* Retreatment with pegylated interferon plus ribavirin of chronic hepatitis C non-responders to interferon plus ribavirin: a meta-analysis. *J Hepatol* 2009; **51**: 675–81.

22 Matthews DR, Hosker JP, Rudenski AS *et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–9.

23 Tsubota A, Horose Y, Izumi N *et al.* Pharmacokinetics of ribavirin in combined interferon-alpha 2b and ribavirin therapy for chronic hepatitis C virus infection. *Br J Clin Pharmacol* 2003; **55**: 360–7.

24 Halfon P, Bourlière M, Pénaranda G *et al.* Real-time PCR assays for hepatitis C virus (HCV) RNA quantitation are adequate for clinical management of patients with chronic HCV infection. *J Clin Microbiol* 2006; **44**: 2507–11.

25 Pawlotsky JM, Dahari H, Neumann AU *et al.* Antiviral action of ribavirin in chronic hepatitis C. *Gastroenterology* 2004; **126**: 703–14.

26 Neumann AU, Lam NP, Dahari H *et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103–7.

27 McHutchison JG. Hepatitis C advances in antiviral therapy: what is accepted treatment now? *J Gastroenterol Hepatol* 2002; **17**: 431–41.

28 Preston SL, Drusano GL, Glue P *et al.* Pharmacokinetics and absolute bioavailability of ribavirin in healthy volunteers as determined by stable-isotope methodology. *Antimicrob Agents Chemother* 1999; **43**: 2451–6.

29 Shiffman ML, Ghany MG, Morgan TR *et al.* Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. *Gastroenterology* 2007; **132**: 103–12.

30 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.

31 Bronowicki JP, Ouzan D, Asselah T *et al.* Effect of ribavirin in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2a plus ribavirin. *Gastroenterology* 2006; **131**: 1040–8.

32 Glue P, Schenker S, Gupta S *et al.* The single dose pharmacokinetics of ribavirin in subjects with chronic liver disease. *Br J Clin Pharmacol* 2000; **49**: 417–21.

33 Romero-Gómez M, Del Mar Viloria M, Andrade RJ *et al.* Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; **128**: 636–41.

34 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840–8.

35 de Lucas S, Bartolome J, Carreno V. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. *J Infect Dis* 2005; **191**: 93–9.

36 Taguchi T, Nagano-Fujii M, Akutsu M *et al.* Hepatitis C virus NS5A protein interacts with 2′, 5′-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. *J Gen Virol* 2004; **85**: 959–69.

37 Furusyo N, Sawayama Y, Maeda S *et al.* High molecular weight form of adiponectin levels of Japanese patients with chronic hepatitis C virus infection. *Hepatol Res* 2007; **37**: 1052–61.

38 Huseby NE. Multiple forms of serum gamma-glutamyltransferase. Association of the enzyme with lipoproteins. *Clin Chim Acta* 1982; **124**: 103–12.

39 Benini F, Pigozzi MG, Baisini O *et al.* Increased serum gamma-glutamyl-transpeptidase concentration is associated with nonalcoholic steatosis and not with cholestasis in patients with chronic hepatitis C. *J Gastroenterol Hepatol* 2007; **22**: 1621–6.

40 Benini F, Pigozzi MG, Pozzi A *et al*. Elevation of serum gamma-glutamyltranspeptidase activity is frequent in chronic hepatitis C, and is associated with insulin resistance. *Dig Liver Dis* 2009; **41**: 586–90.

41 Kainuma M, Furusyo N, Kajiwara E *et al*. Pegylated interferon alpha-2b plus ribavirin combination treatment for older patients with chronic hepatitis C: Project of the Kyushu University Liver Disease Study Group. *World J Gastroenterol* 2010; **16**: 4400–9.

42 Thompson AJ, Fellay J, Patel K *et al*. Variants in the ITPA gene protect against ribavirin-induced hemolytic anemia and decrease the need for ribavirin dose reduction. *Gastroenterology* 2010; **139**: 1181–9.

J Infect Chemother (2011) 17:737–743
DOI 10.1007/s10156-011-0249-7

ORIGINAL ARTICLE

Abbott RealTime PCR assay is useful for evaluating virological response to antiviral treatment for chronic hepatitis C

Hiroaki Ikezaki · Norihiro Furusyo · Takeshi Ihara · Takeo Hayashi · Eiichi Ogawa · Kazuhiro Toyoda · Hiroaki Taniai · Mosaburo Kainuma · Masayuki Murata · Jun Hayashi

Received: 13 December 2010 / Accepted: 31 March 2011 / Published online: 29 April 2011
© Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases 2011

Abstract This study was done to evaluate the utility of the Abbott RealTime PCR assay (ART) for the monitoring of chronic hepatitis C patients. The serum samples of 183 patients infected with hepatitis C virus (HCV) genotype 1b who had completed a 48-week period of pegylated interferon (PEG-IFN) alpha-2b plus ribavirin treatment were prospectively analyzed. Serum HCV RNA levels were measured both by ART and by the Roche COBAS Amplicor Monitor test, version2.0 (CAM) at baseline and at weeks 4, 12, 24, 36, and 48 of treatment, and at 24 weeks after the end of treatment (EOT). A significant positive correlation of pretreatment HCV RNA levels was found between ART and CAM ($r = 0.595$, $P < 0.0001$). Of the 183 patients, 66 (36.0%) achieved a sustained virological response (SVR). The logarithmic decline of the HCV RNA level from the pretreatment level determined by ART in SVR patients was significantly higher than that in non-SVR patients at all time points tested. The logarithmic decline determined by CAM in SVR patients was significantly higher than that in non-SVR patients only at week 4, but there was no significant difference at other weeks. Of 124 patients who were HCV RNA-negative at EOT by ART, 58 (46.8%) had a relapse of viremia at 24 weeks after EOT, whereas 77 of 143 patients (53.8%) who were HCV RNA-negative at EOT by CAM had a relapse. The relapse rate was lower when determined by ART than by CAM, but not significantly so. ART is more useful than CAM for evaluating the virological response to antiviral treatment for chronic hepatitis C.

Keywords Hepatitis C · Interferon · Ribavirin · RealTime PCR

H. Ikezaki (✉) · N. Furusyo · T. Ihara · T. Hayashi · E. Ogawa · K. Toyoda · H. Taniai · M. Kainuma · M. Murata · J. Hayashi
Department of General Internal Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka 812-8582, Japan
e-mail: ikezaki@gim.med.kyushu-u.ac.jp

H. Ikezaki · N. Furusyo · T. Ihara · T. Hayashi · E. Ogawa · K. Toyoda · H. Taniai · M. Kainuma · M. Murata · J. Hayashi
Department of Environmental Medicine and Infectious Disease, Kyushu University, Fukuoka, Japan

Introduction

The number of patients infected with hepatitis C virus (HCV) is estimated to be approximately 170 million worldwide and 2.0 million in Japan. In Japan, chronic hepatitis C is the main cause of liver cirrhosis and hepatocellular carcinoma [1].

Interferon (IFN) is a cytokine produced to defend the host against infection. Type-I IFNs, including IFN α and IFN β, are induced by the activation of two pathways, Toll-like receptor 3 and cytosolic retinoic acid-inducible gene-I (RIG-I) like helicases, by which HCV is recognized [2]. All type-I IFNs bind to the same cell surface receptor, the IFN α receptor (IFNAR), consisting of two chains, and induce intracellular signaling through the Jak-signal transducer and activator of transcription (STAT) pathway. The important role of IFN α in antiviral responses is based on direct antiviral actions through the transcriptional activation of hundreds of IFN-stimulated genes (ISGs). Induction of these ISG-encoded proteins and their related pathways can lead to a block in viral transcription, degradation of viral RNA, inhibition of translation, or interference with various steps of viral replication [3, 4].

Table 1 Demographic characteristics of the183 studied patients

Characteristics	Total no. = 183	SVR no. = 66	Non-SVR no. = 117	*P* value
Male no. (%)	83 (45.4)	37 (56.1)	46 (43.9)	0.0289
Age (years)	58.7 ± 10.4	55.0 ± 10.7	60.7 ± 9.7	0.0003
Body mass index (kg/m^2)	23.2 ± 3.0	22.8 ± 3.0	23.4 ± 3.0	0.2017
Creatinine (mg/dl)	0.67 ± 0.17	0.70 ± 0.16	0.65 ± 0.18	0.1054
Creatinine clearance (ml/min)	95.1 ± 27.7	96.8 ± 27.7	94.1 ± 27.6	0.5224
Albumin (g/dl)	4.2 ± 0.4	4.2 ± 0.3	4.2 ± 0.4	0.2451
Alanine aminotranseferase (IU/l)	68.1 ± 48.8	71.7 ± 54.7	66.0 ± 45.2	0.4441
γ-Glutamyl-transpeptidase (IU/l)	46.5 ± 31.9	31.5 ± 16.7	55.0 ± 35.2	<0.0001
White blood cell count (/mm^3)	4900 ± 1367	5018 ± 1232	4833.4 ± 1438	0.3811
Hemoglobin (g/dl)	13.5 ± 1.5	13.7 ± 1.5	13.3 ± 1.4	0.0813
Platelet count (10^9/l)	16.4 ± 4.6	17.3 ± 4.7	15.9 ± 4.5	0.0671
Serum HCV RNA levels (log IU/ml)[a]	5.7 ± 0.5	5.6 ± 0.6	5.7 ± 0.5	0.1062
Histological hepatic fibrosis				
Stage 0 no. (%)	16 (8.7)	9 (13.6)	7 (6.0)	
Stage 1 no. (%)	56 (30.6)	22 (33.3)	34 (29.1)	0.0526
Stage 2 no. (%)	46 (25.1)	16 (24.2)	30 (25.6)	
Stage 3 no. (%)	23 (12.6)	3 (4.6)	20 (17.1)	
Stage 4 no. (%)	19 (10.4)	5 (7.6)	14 (12.0)	
Untested no. (%)	23 (12.6)	11 (16.7)	12 (10.2)	

Data are shown as numbers (%) or means ± SD

HCV hepatitis C virus, *SVR* sustained virological response

[a] Measured by Abbott RealTime PCR assay

The most common current antiviral treatment for chronic hepatitis C is the combination of pegylated interferon α (PEG-IFN α) and ribavirin (RBV) [5], which has brought about a higher rate of sustained virological response (SVR) than standard IFN monotherapy [6, 7]. Before initiating antiviral therapy, factors such as age, sex, HCV genotype, HCV viral load, and the stage of liver fibrosis, which influence the rate of success, must be considered. During therapy, the rate of success is influenced by adherence to the treatment regimen and viral load kinetics [8–12].

In large multicenter studies, positive and negative predictors of SVR, using viral load kinetics, have been established that are now used for recommendations on the management of antiviral treatment by the American and European international consensus conferences. Therefore, accuracy of the quantification of HCV RNA is essential for the clinical management of patients under treatment [13, 14]. Real-time polymerase chain reaction (PCR) assays have recently become available for sensitive HCV RNA quantification. In this study, we evaluated the utility of the Abbott RealTime PCR assay (ART) for the management of patients undergoing antiviral treatment by comparing it to the Roche COBAS Amplicor Monitor test, version2.0 (CAM).

Patients and methods

Patients

A total of 183 Japanese patients [83 male (45.3%), mean age 58.7 years] infected with HCV genotype-1 were enrolled for the study. Their clinical characteristics are shown in Table 1. All received therapy with PEG-IFN α2b plus RBV. Serum HCV RNA levels were prospectively analyzed by both ART and CAM at baseline, and at weeks 4, 12, 24, 36, and 48 of treatment, and at 24 weeks after the end of treatment (EOT). Written informed consent was obtained from each patient, and the study was approved by the local ethics committee in accordance with the Declaration of Helsinki.

Therapeutic protocol

All patients were treated with PEG-IFN α2b (1.5 μg/kg of body weight by subcutaneous injection once a week) plus RBV (600–1000 mg/day orally according to body weight) for 48 weeks. The duration and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. The dose of PEG-IFN α2b was reduced if patients had

adverse psychological effects or a decrease in the white blood cell and platelet counts. Likewise, the dose of RBV was reduced if the hemoglobin level decreased to under 100 g/l. Both PEG-IFN α2b and RBV were discontinued if the hemoglobin level and white blood cell and platelet counts fell below 85 g/l, 1×10^9/l, and 2.5×10^9/l, respectively.

The present study did not set a treatment discontinuation rule as was used in other studies [15, 16]. This rule recommends that antiviral therapy should be discontinued for patients whose HCV RNA levels have decreased by less than 2 log IU/ml at week 12 or those in whom HCV RNA has remained detectable after week 24.

Definition of SVR, relapse, non-responder, and virological breakthrough

SVR was defined as undetectable serum HCV RNA by ART or CAM at 6 months after EOT. Relapse was defined as serum HCV RNA becoming undetectable during the treatment and sustained until EOT, but reappearing positive after EOT. Non-responder was defined as serum HCV RNA never becoming undetectable during the treatment or after EOT. Virological breakthrough was defined as undetectable serum HCV RNA during the treatment and the reappearance of serum HCV RNA positivity during the treatment.

Determination of HCV RNA level

We prospectively analyzed the serum HCV RNA levels of all patients by both ART and CAM.

ART was carried out following the manufacturer's protocols (Abbott Molecular, Des Plaines, IL, USA). Briefly, nucleic acid was extracted from a 500-μl serum sample and amplified with a control. Quantification was performed automatically and was based on a stored calibration curve derived from replicate testing of high and low calibrators, provided by Abbott, which are standardized to the WHO reference material [17]. ART provides a lower detection limit of 1.08 log IU/ml, a specificity of more than 99.5%, and a linear amplification range from 1.08 to 8.0 log IU/ml independent of the HCV genotype [18–20].

CAM was done following the manufacturer's instructions (Roche Molecular Systems, Branchburg, NJ, USA). In brief, 100 μl of serum was subjected to chaotropic lysis in the presence of known amounts of an internal quantitation standard (QS). The target viral RNA and the QS were resuspended in Specimen Diluent (Roche), and the mixture was mixed with an equal volume of amplification ready solution (Master Mix; Roche) containing the primers KY78 (biotinylated) and KY80 (nonbiotinylated), deoxynucleoside triphosphates, AmpErase, and r*Tth* DNA polymerase. Amplification, amplicon dilution, detection, and quantitation were automatically performed by the COBAS Amplicor analyzer [21]. The dynamic range of CAM is 500 to approximately 5,100,000 IU/ml with a specificity of almost 100%, independent of the HCV genotype [22–24]. To compare serum HCV RNA levels determined by ART with those determined by CAM, we transformed the levels determined by CAM (IU/ml) into logarithmic levels (log IU/ml). Therefore, the range of CAM was 2.7 to 6.7 log IU/ml.

Dynamic changes of serum HCV RNA levels during treatment

We analyzed the serum HCV RNA levels of the patients by both ART and CAM at the same time and compared the logarithmic declines of HCV RNA levels with the pretreatment levels. Serum HCV RNA levels that were undetectable by both ART and CAM were treated as 0 log IU/ml.

Determination of HCV genotype

HCV genotype was determined using type-specific primers from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier [25].

Statistical analysis

Statistical analysis was done with BMDP statistical software for the IBM 3090 computer system (BMBD Statistical Software, Los Angeles, CA, USA). Continuous data were expressed as mean values or means ± SD. A paired *t*-test, unpaired *t*-test, Mann–Whitney *U*-test, or Kruskal–Wallis non-parametric analysis of variance was used to compare HCV dynamics. A "*P*" value of less than 0.05 was regarded as statistically significant.

Results

Correlation of ART and CAM pretreatment HCV RNA levels

Figure 1 shows the ART and CAM pretreatment HCV RNA levels for 66 SVR and 117 non-SVR patients infected with HCV genotype 1. The levels determined by ART ranged from 4.20 to 6.90 log IU/ml (median 5.66 log IU/ml) and those by CAM ranged from 4.40 to 6.75 log IU/ml (median 6.08 log IU/ml). We found a significant positive

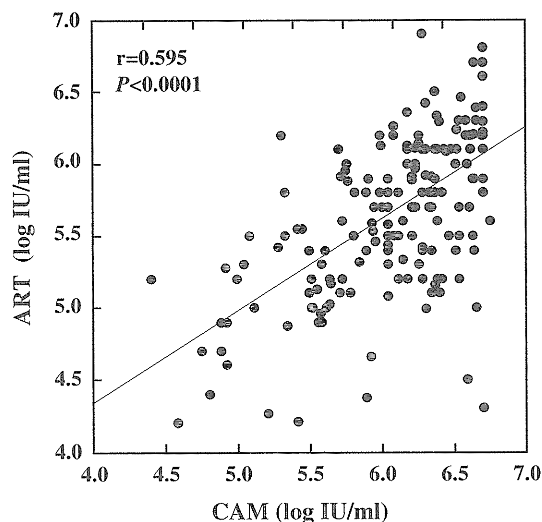


Fig. 1 Correlation of pretreatment hepatitis C virus (HCV) RNA levels determined by Abbott RealTime PCR assay (*ART*) and Roche COBAS Amplicor Monitor (*CAM*)

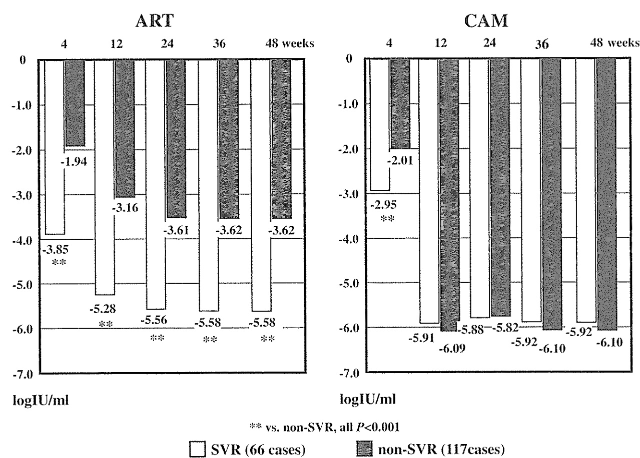


Fig. 2 The logarithmic declines from pretreatment hepatitis C virus (HCV) RNA levels determined by Abbott RealTime PCR assay (*ART*) and Roche COBAS Amplicor Monitor (*CAM*) during pegylated interferon alpha 2b plus ribavirin treatment. *White* and *black bars* indicate sustained virological response (*SVR*) and non-SVR patients, respectively

correlation between the ART and CAM pretreatment HCV RNA levels of the 183 patients ($r = 0.595$, $P < 0.0001$).

Comparison of ART with CAM in the logarithmic decline of HCV RNA levels

The logarithmic decline during treatment from the pretreatment HCV RNA level was compared between SVR and non-SVR patients (Fig. 2). The logarithmic declines of the HCV RNA levels of SVR patients determined by ART (−3.85, −5.28, −5.56, −5.58, and −5.58 at weeks 4, 12, 24, 36, and 48, respectively) were significantly higher than those of the non-SVR patients (−1.94, −3.16, −3.61, −3.62, and −3.62 at weeks 4, 12, 24, 36, and 48, respectively) (all $P < 0.001$). The logarithmic declines of the HCV RNA levels of SVR patients determined by CAM (−2.95) were significantly higher than those of the non-SVR patients (−2.01) ($P < 0.001$) at week 4, but there was no significant difference at weeks 12, 24, 36, and 48 (SVR, −5.91, −5.88, −5.92, and −5.92, vs. non-SVR, −6.09, −5.82, −6.10, and −6.10, respectively).

Virological response: comparison of ART and CAM

Of the 183 patients, 66 (36.0%) were shown to have achieved an SVR determined by both ART and CAM. By ART, 58 patients relapsed during the treatment (31.7%) and by CAM 77 (42.1%) relapsed ($P = 0.051$); 59 were non-responders (32.2%) by ART and 40 (21.8%) were non-responders by CAM ($P = 0.034$). All 19 patients whose HCV RNA was undetectable at EOT by CAM but detectable by ART had relapsed at the end of follow-up (EOF).

Comparison of ART and CAM for determining the time HCV RNA became undetectable

We analyzed the distribution of the time at which HCV RNA became undetectable by ART and by CAM for 66 SVR and relapsed patients ($n = 58$ by ART and $n = 77$ by CAM). For the 66 SVR patients, the percentages of patients for whom HCV RNA became undetectable at weeks 4, 12, and 24 were 27.2% ($n = 18$), 50.0% ($n = 33$), and 22.7% ($n = 15$) by ART and 62.1% ($n = 41$), 36.4% ($n = 24$), and 1.5% ($n = 1$) by CAM. For the 58 relapsed patients determined by ART, HCV RNA was undetectable in the serum of 5.2% ($n = 3$), 32.7% ($n = 19$), 43.1% ($n = 25$), 15.5% ($n = 9$), and 3.4% ($n = 2$) at weeks 4, 12, 24, 36, and 48, respectively. For the 77 relapsed patients determined by CAM, the percentages were 18.1% ($n = 14$), 55.8% ($n = 43$), 19.5% ($n = 15$), 1.3% ($n = 1$), and 5.2% ($n = 4$) at weeks 4, 12, 24, 36, and 48, respectively.

Comparison of the positive predictive value (PPV) and negative predictive value (NPV) rates for SVR determined by ART and CAM

The PPV and NPV rates were calculated for each treatment period (at weeks 4, 12, 24, 36, and 48) based on undetectable HCV RNA determined by ART and CAM. The PPV rates for SVR determined by ART were higher than those by CAM at all weeks (ART, 85.7, 70.8, 58.6, 57.4, and 55.9% vs. CAM, 74.5, 53.3, 47.8, 47.8, and 46.8%, respectively). There was a significant difference at week 12 ($P = 0.032$), but there were no significant differences at the other testing time points. The NPV rates for SVR