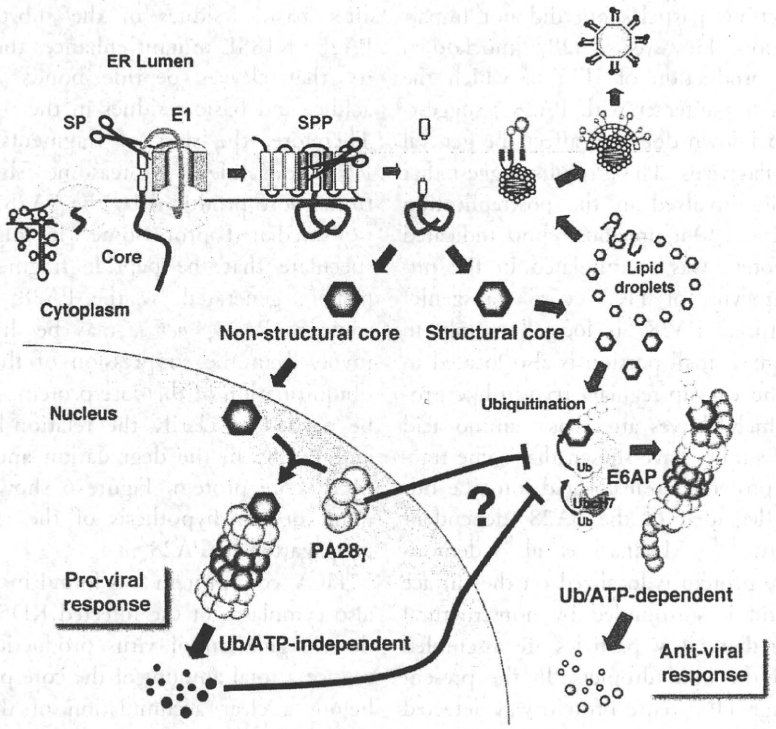


Fig. 6. Schematic diagram of the potential roles of PA28$\gamma$ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets close to the endoplasmic reticulum to form a nucleocapsid with the viral RNA genome and is incorporated into virus particles as a structural protein. In addition to the structural protein of HCV, the core protein has characteristics of a nonstructural protein. HCV core protein is degraded through ubiquitin-dependent and ubiquitin-independent proteasome pathways. E6AP catalyzes ubiquitin ligation to HCV core protein and promotes degradation in the cytoplasm, which contributes to the antiviral response. In contrast, the core protein partially migrates into the nucleus and is degraded through a ubiquitin-independent and PA28$\gamma$-dependent proteasome pathway, and the core protein fragments generated by the PA28$\gamma$ pathway or PA28$\gamma$ *per se* were suggested to participate in the suppression of E6AP-dependent ubiquitination of HCV core protein, which contributes to the proviral response.

In conclusion, in this study we demonstrated that the proteasome activator PA28$\gamma$ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28$\gamma$ plays a crucial role in the development of liver pathology induced by HCV core protein.[8] PA28$\gamma$ knockout mice exhibit only mild growth retardation.[15,16] Therefore, PA28$\gamma$ may be a novel and promising antiviral target not only for elimination of HCV from hepatitis C patients but also for intervention in the progression of liver diseases induced by chronic HCV infection.

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## References

1. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1-16.
2. Moriishi K, Matsuura Y. Host factors involved in the replication of hepatitis C virus. Rev Med Virol 2007;17:343-354.
3. Hussy P, Langen H, Mous J, Jacobsen H. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 1996;224:93-104.
4. Okamoto K, Mori Y, Komoda Y, Okamoto T, Okochi M, Takeda M, et al. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. J Virol 2008;82:8349-8361.
5. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A 1997;94:1200-1205.
6. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78:1527-1531.
7. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237-10249.
8. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661-1666.
9. Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, et al. Involvement of PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. J Virol 2007;81:1727-1735.

10. Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, et al. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. Cell 2006;124: 381-392.
11. Zhang Z, Zhang R. Proteasome activator PA28 gamma regulates p53 by enhancing its MDM2-mediated degradation. EMBO J 2008;27: 852-864.
12. Chen X, Barton LF, Chi Y, Clurman BE, Roberts JM. Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. Mol Cell 2007;26:843-852.
13. Li X, Amazit L, Long W, Lonard DM, Monaco JJ, O'Malley BW. Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGgamma-proteasome pathway. Mol Cell 2007;26:831-842.
14. Jariel-Encontre I, Bossis G, Piechaczyk M. Ubiquitin-independent degradation of proteins by the proteasome. Biochim Biophys Acta 2008; 1786:153-177.
15. Barton LF, Runnels HA, Schell TD, Cho Y, Gibbons R, Tevethia SS, et al. Immune defects in 28-kDa proteasome activator gamma-deficient mice. J Immunol 2004;172:3948-3954.
16. Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, et al. Growth retardation in mice lacking the proteasome activator PA28gamma. J Biol Chem 1999;274:38211-38215.
17. Suzuki R, Moriishi K, Fukuda K, Shirakura M, Ishii K, Shoji I, et al. Proteasomal turnover of hepatitis C virus core protein is regulated by two distinct mechanisms: a ubiquitin-dependent mechanism and a ubiquitin-independent but PA28gamma-dependent mechanism. J Virol 2009;83:2389-2392.
18. Shirakura M, Murakami K, Ichimura T, Suzuki R, Shimoji T, Fukuda K, et al. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J Virol 2007;81:1174-1185.
19. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802-1808.
20. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
21. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
22. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008-4021.
23. Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, et al. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J Virol 2005;79:3448-3458.
24. Targett-Adams P, Chambers D, Gledhill S, Hope RG, Coy JF, Girod A, et al. Live cell analysis and targeting of the lipid droplet-binding adipocyte differentiation-related protein. J Biol Chem 2003;278: 15998-16007.
25. Okamoto T, Omori H, Kaname Y, Abe T, Nishimura Y, Suzuki T, et al. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J Virol 2008;82: 3480-3489.
26. Zhang Z, Clawson A, Realini C, Jensen CC, Knowlton JR, Hill CP, et al. Identification of an activation region in the proteasome activator REGalpha. Proc Natl Acad Sci U S A 1998;95:2807-2811.
27. Li J, Gao X, Ortega J, Nazif T, Joss L, Bogyo M, et al. Lysine 188 substitutions convert the pattern of proteasome activation by REGgamma to that of REGs alpha and beta. EMBO J 2001;20:3359-3369.
28. Nikaido T, Shimada K, Nishida Y, Lee RS, Pardee AB, Nishizuka Y. Loss in transformed cells of cell cycle regulation of expression of a nuclear protein recognized by SLE patient antisera. Exp Cell Res 1989; 182:284-289.
29. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, et al. Molecular determinants for subcellular localization of hepatitis C virus core protein. J Virol 2005;79:1271-1281.
30. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089-1097.
31. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.

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# Secondary Structure of the Amino-Terminal Region of HCV NS3 and Virological Response to Pegylated Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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The aim of the study was to identify a predictive marker for the virological response in hepatitis C virus 1b (HCV-1b)-infected patients treated with pegylated interferon plus ribavirin therapy. A total of 139 patients with chronic hepatitis C who received therapy for 48 weeks were enrolled. The secondary structure of the 120 residues of the amino-terminal HCV-1b non-structural region 3 (NS3) deduced from the amino acid sequence was classified into two major groups: A and B. The association between HCV NS3 protein polymorphism and virological response was analyzed in patients infected with group A ($n = 28$) and B ($n = 40$) isolates who had good adherence to both pegylated interferon and ribavirin administration (>95% of the scheduled dosage) for 48 weeks. A sustained virological response (SVR) representing successful HCV eradication occurred in 33 (49%) in the 68 patients. Of the 28 patients infected with the group A isolate, 18 (64%) were SVR, whereas of the 40 patients infected with the group B isolate only 15 (38%) were SVR. The proportion of virological responses differed significantly between the two groups ($P < 0.05$). These results suggest that polymorphism in the secondary structure of the HCV-1b NS3 amino-terminal region influences the virological response to pegylated interferon plus ribavirin therapy, and that virus grouping based on this polymorphism can contribute to prediction of the outcome of this therapy. ***J. Med. Virol. 82:1364–1370, 2010.*** © 2010 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis C; interferon; ribavirin; interaction; polymorphism



## INTRODUCTION

Hepatitis C virus (HCV) is the major pathogen that causes chronic liver diseases with a risk of progression to cirrhosis and hepatocellular carcinoma. Currently, the standard treatment for chronic hepatitis C is antiviral therapy using pegylated interferon (Peg-IFN) plus ribavirin (RBV), and this approach is most effective for eradication of HCV viremia. However, even with the widely used treatment regimen of 48 weeks, the rate of sustained virological response (SVR), which indicates eradication of viremia, is still approximately 50% for patients infected with the therapy-resistant HCV genotype 1b (HCV-1b) with a high viral load [Manns et al., 2001; Bruno et al., 2004; Hadziyannis et al., 2004]. It would be useful to predict the virological response to this therapy and to identify patients who would obtain beneficial therapeutic effects before treatment, in order to avoid any serious side effect and to eliminate those who would not be helped by the treatment. In the future it will be important to establish a protocol of tailor-made medicine for chronic hepatitis C.

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Both the HCV genotype and pre-treatment viral load are major viral factors that influence the response to IFN-based antiviral therapy, but IFN resistance is also partly due to variation of the amino acid sequence encoded by HCV itself. Enomoto et al. [1996] proposed that variation of 40 amino acids within the NS5A region (aa 2,209–2,248), which is referred to as the IFN sensitivity-determining region (ISDR), is well correlated with IFN responsiveness. ISDR and its adjacent sequence bind and inhibit the enzymatic activity of a double-stranded RNA-activated protein kinase (PKR), which can have an antiviral effect, and therefore the combined region is referred to as the PKR-binding domain (PKR-BD) [Gale et al., 1997, 1998]. A correlation between sequence variation in the PKR-BD and IFN responsiveness has been reported [Nousbaum et al., 2000], and some reports show a correlation between IFN responsiveness and the sequence diversity of variable region 3 (V3) (aa 2,356–2,379) or surrounding regions near the carboxy terminus of NS5A [Murphy et al., 2002; Sarrazin et al., 2002; Puig-Basagoiti et al., 2005]. A high degree of amino acid substitution in the V3 and pre-V3 regions (aa 2,334–2,355) of NS5A, which is referred to as the IFN/RBV resistance-determining region (IRRDR) (aa 2,334–2,379), has been associated with SVR in Peg-IFN/RBV combination therapy for patients infected with HCV-1b [El-Shamy et al., 2007, 2008]. In addition to these findings in non-structural proteins of the virus, amino acid substitution in a structural region of HCV has been reported to be a predictive viral marker for the virological response to PegIFN/RBV therapy. Amino acid polymorphisms in the HCV core region (Arg70 vs. Gln70 and Leu91 vs. Met91) correlate with virological outcome and on-treatment viral kinetics in Peg-IFN/RBV therapy [Akuta et al., 2006, 2007], and a double wild-type HCV core (Arg70 and Leu91) may be a significant predictor of SVR in Peg-IFN/RBV therapy [Akuta et al., 2007].

Interactions between viral and host proteins in infected cells may influence therapeutic effects and the natural history of infection, since the HCV NS3 region has a significant effect on immunity. The amino-terminal part of this region encodes a serine protease, for which the minimum activity has been mapped to a region between aa 1,059 and 1,204 [Yamada et al., 1998]. The serine protease inactivates Cardif, a caspase recruitment domain (CARD)-containing adaptor protein that interacts with the RNA helicase retinoic acid inducible gene 1 (RIG-1)-dependent antiviral pathway in infected cells [Foy et al., 2003; Meylan et al., 2005; Evans and Seeger, 2006]. This action inhibits phosphorylation and subsequent heterodimerization of interferon regulatory factor-3 (IRF-3), which is essential for activation of IFN signaling through translocation of IRF-3 heterodimers into the nucleus, and eventually blocks IFN-beta production. In addition, inactivation of IRF-3 is postulated to influence the therapeutic effect of IFN-based antiviral therapy, because the IRF-3 heterodimer translocates into the nucleus to bind to the IFN-stimulated response element that produces many antiviral proteins, including 2′,5′-oligoadenylate synthetase and PKR [Nakaya et al., 2001; Grandvaux et al., 2002]. Collectively, these findings suggest that polymorphisms in HCV NS3 structure deduced from sequence variation may influence IFN-related signaling and the antiviral effect of IFN-based anti-HCV therapy.

We have focused on polymorphisms in the secondary structure of the viral polyprotein that interacts with host proteins involved in immunity, with the aim of identification of predictive viral markers for the response to Peg-IFN/RBV therapy. In this study, we examined the potential correlation between polymorphisms in the secondary structure of the HCV NS3 amino-terminal region and virological responses to Peg-IFN/RBV therapy in patients infected with HCV-1b with a high viral load.

## PATIENTS AND METHODS

### Patients and Treatment Regimen With Peg-IFN Plus Ribavirin

A total of 139 consecutive patients diagnosed with chronic hepatitis C were enrolled in the study from December 2004 to March 2007. These patients included 81 men and 58 women, and were aged from 31 to 75 years old (mean ± SD, 56.8 ± 8.7 years old). All patients were infected with HCV-1b with a high viral load of over 100 KIU/ml, and all received Peg-IFN/RBV therapy. Patients with alcoholic liver injury, autoimmune liver disease, and those who had symptoms of decompensated cirrhosis including ascites were excluded. Briefly, all patients were treated with a combination of Peg-IFN-alpha 2b (Pegintron®; Schering-Plough, Kenilworth, NJ) and RBV (Rebetol®; Schering-Plough) for 48 weeks. Peg-IFN was administered subcutaneously once a week and RBV was given orally twice a day for the total dose. The dosages were determined on the basis of body weight according to the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare, and there was a limit for calculating the optimized dose: patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given Peg-IFN at doses of 60, 80, 100, and 120 μg, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1,000 mg, respectively. The dose of Peg-IFN or RBV was reduced according to the Japanese standard criteria based on the white blood cell count, neutrophil count, hemoglobin concentration and platelet count [Hiramatsu et al., 2008].

### Virological Tests and Response to Peg-IFN Plus Ribavirin

Virological responses were evaluated at 12 weeks after the start of treatment with an early depletion of viremia referred to as an early virological response (EVR), at the end of treatment with depletion of viremia referred to as an end of treatment virological response (ETR), and at 24 weeks after completion of treatment,

with a clinical outcome of a sustained virological response (SVR) representing successful HCV eradication. All patients were negative for hepatitis B surface antigen. Quantification of serum HCV RNA was performed using an RT-PCR-based commercial kit (Amplicor HCV monitor test, ver. 2.0, Roche Diagnostics, Tokyo, Japan). This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml. SVR was determined by monitering negativity for HCV RNA monthly for 6 months. The real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics) for more precise quantitation of HCV viremia has recently become available and pre-treatment viral titers were re-evaluated using preserved serum samples. This real-time PCR assay has a lower limit of detection of 15 IU/ml. The study protocol was approved by the Ethics Committee of Yamagata University Hospital. Informed consent was obtained from all patients.

### PCR Amplification of the Amino-Terminal Region of NS3

RNA was extracted from 50 μl of serum using an RNeasy Mini kit (Qiagen, Tokyo, Japan). To amplify the region of the HCV genome encoding the amino-terminal region of NS3 (1,027–1,206), a one-step PCR was performed in a tube using the Superscript One-Step RT-PCR kit with Platinum Taq (Gibco-BRL, Tokyo, Japan) and an outer set of primers: NS3-F1 (sense primer; 5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3,295–3,316) and NS3-AS2 (antisense primer; 5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4,040–4,019), as reported previously [Ogata et al., 2002a, 2003]. PCR was initially performed at 45°C for 30 min at RT and then at 94°C for 2 min, followed by the first-round PCR for forty 3-min cycles at 94°, 55°, and 72°C for 1 min each. The second-round PCR was performed with *Pfu* DNA polymerase (Promega, Tokyo, Japan) and an inner set of primers: NS3-F3 (sense primer; 5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3,390–3,407) and NS3-AS1 (antisense primer; 5′-GCC ACT TGG AAT GTT TGC GGT A-3′; nucleotides 4,006–3,985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed amplification of the corresponding portion of the HCV genome from HCV-1b RNA-positive samples. The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced (without being subcloned) in both directions using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (Applied Biosystems, Tokyo, Japan).

### Classification of the Secondary Structure of the HCV-1b NS3 Amino-Terminal Region

The secondary structure of the amino-terminal region of HCV NS3 was predicted by computer-assisted Robson analysis [Garnier et al., 1978] with Genetyx-Mac software (ver.10.1; Software Development Co., Tokyo, Japan). Previously, the full-length secondary structure of the HCV-1b NS3 region was analyzed, and this showed that the secondary structure deduced from the carboxy-terminal 60 residues was well conserved in terms of linear structure, without any turn structure [Ogata et al., 2002a]. We have shown that the secondary structure of the 120 residues in the amino-terminal region of HCV-1b NS3 can be classified into two major groups: A and B (Fig. 1) [Ogata et al., 2002a, 2003]. Briefly, the criteria for this classification are as follows: in group A isolates, the carboxy-terminal 20 residues (aa 1,125–1,146) are oriented leftward relative to a domain composed of the remaining amino-terminal region; whereas in group B isolates, the same 20 residues are oriented rightward relative to the rest of the amino-terminal domain.

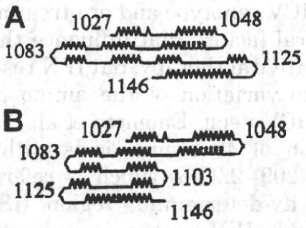


Fig. 1. Secondary structure of the 120 amino-terminal residues of HCV-1b nonstructural 3 (NS3) region classified into two major groups: A and B. The looped, zigzag, straight, and bent lines represent α-helix, β-sheet, coil, and turn structures, respectively. The numbers indicate amino acid positions. **A**: Group A, (**B**) Group B.

### Analysis of Amino Acid Substitutions in the Core Region

To amplify a region of the HCV genome encoding the core region including positions 70 and 91, reverse transcription and the first-round PCR were performed in a tube by the Superscript One-Step RT-PCR kit with Platinum *Taq* (Gibco-BRL) and an outer set of primers, followed by second-round PCR with an inner set of primers in accordance with procedures reported previously [Ogata et al., 2002b]. The sequences of the amplified fragments were determined by direct sequencing.

### Statistical Analysis

Data were analyzed by a $\chi^2$ test for independence with a two-by-two contingency table and a Student *t*-test. A *P*-value $<0.05$ was considered significant.

## RESULTS

### Virological Response *and Adherence* to the Peg-IFN Plus Ribavirin Regimen

Rates of virological responses in patients treated with PegIFN/RBV combination therapy for 48 weeks are shown in Figure 2. Of the 139 patients enrolled in the study, SVR, non-SVR and cessation of therapy occurred in 58 (42%), 62 (45%), and 19 (14%), respectively. Serious

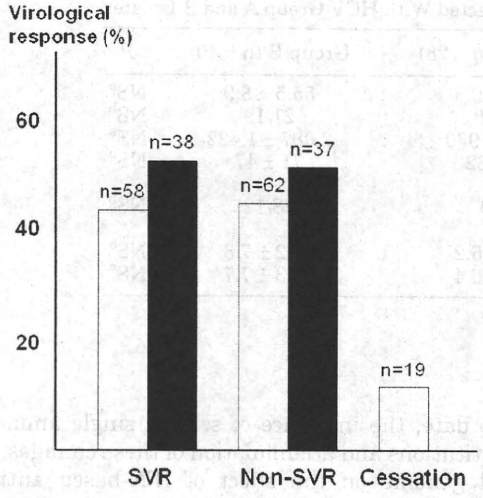


Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

adverse events that necessitated discontinuation of this therapy were depression in one patient, thyroid function disorder in 2, general itching in 2, infection in 2, anorexia in 2, occurrence of hepatocellular carcinoma in 2, and a decreased neutrophil count in 2. Six patients also terminated this therapy at their own request. Of the 139 patients, 75 (54%) received >80% of the scheduled dosage of Peg-IFN and RBV designated before treatment, and of these 75 cases SVR and non-SVR occurred in 38 (51%) and 37 (49%), respectively.

### Prevalence of Types of Secondary Structure of the Amino-Terminal Region of HCV NS3

The prevalence of the types of secondary structure of HCV NS3 in the 139 subjects is shown in Table I. Among these subjects, 43 (31%), 70 (50%), and 26 (19%) were classified into groups A, B, and others, including 3 of mixed type (A plus B) and 23 of non-A, non-B type. Of the 75 cases with good adherence to administration of >80% of the scheduled dosage, 28 (37%), 40 (53%) and 7 (9%) were classified into groups A, B, and others. The amino acid data of group A and B in the cases with good adherence to administration are available in the DDBJ/EMBL/GenBank databases with the accession numbers AB548070–AB548137. Our analysis revealed no specific correlations between amino acid sequences and the secondary structure deduced by the Robson method, as we have reported previously [Ogata et al., 2003].

TABLE I. Prevalence of the HCV NS3 Secondary Structure Type

| | Group A (%) | Group B (%) | Others (%) |
|---|---|---|---|
| Enrolled cases (n = 139) | 43 (31) | 70 (50) | 26 (19) |
| Adherent cases (n = 75) | 28 (37) | 40 (53) | 7 (9) |

### Characteristics of Adherent Patients Based on Different HCV NS3 Structure Types

The virological responses to Peg-IFN/RBV combination therapy for patients infected with group A and B isolates were assessed in the 68 subjects with good adherence to the scheduled dosage of Peg-IFN and RBV. The characteristics of patients infected with group A and B isolates are shown in Table II. Age, gender, pre-treatment level of serum HCV RNA and ALT, and frequency of fibrosis stage did not differ significantly between the two groups. Peg-IFN/RBV combination therapy was completed in all the patients, and the total administered dosages of Peg-IFN and RBV was >95% of the scheduled dosage in both groups.

### Relationship Between Virological Responses and Polymorphisms in the HCV NS3 Amino-Terminal Region

In the 68 patients who received >95% of the scheduled doses of Peg-IFN and RBV for 48 weeks, SVR and non-SVR occurred in 33 (49%) and 35 (51%), respectively. The EVR, ETR, and SVR rates in patients infected with group A and B isolates are shown in Table III. There was a significant difference in the rates of EVR between subjects infected with group A and B isolates: EVR was achieved in 19 of 28 (68%) patients with group A infection, compared to 17 of 40 (43%) with group B infection ($P < 0.05$). The final outcome also differed significantly between subjects infected with group A and B isolates: SVR was achieved in 18 of 28 (64%) patients with group A infection, compared to 15 of 40 (38%) with group B infection ($P < 0.05$).

### Polymorphisms in Core Amino Acids 70/91 and in the HCV NS3 Secondary Structure

The wild-type core sequence (Arg70, Leu91) has been associated with SVR in Peg-IFN/RBV combination therapy, while the non-double wild-type containing one or two substitutions at positions 70 and/or 91 was associated with non-SVR [Akuta et al., 2007]. Therefore, we examined substitutions at positions 70 and 91 in the HCV core region in pre-treatment serum samples of 44 cases that were available for testing. The double wild-type 70/91 sequence was found in 22 of the 44 cases (50%), of which 12 were SVR and 10 were non-SVR. Combination analysis of polymorphisms of the HCV core 70/91 positions and the NS3 amino-terminal region showed that 10 (83%) of the 12 SVR cases and only 3 (30%) of the 10 non-SVR cases with the double wild-type core had a group A polymorphism in HCV NS3 (Table IV). Thus, combination analysis of the core and NS3 regions may improve prediction of the outcome of Peg-IFN/RBV therapy.

TABLE II. Characteristics of Adherent Patients Infected With HCV Group A and B Isolates

| | Group A (n = 28) | Group B (n = 40) | P |
|---|---|---|---|
| Age (years) | 55.5 ± 9.5 | 55.5 ± 8.9 | NS[a] |
| Sex (men/women) | 18/10 | 21/19 | NS[b] |
| Pre-treatment HCV RNA (KIU/ml) | 1,635 ± 930 | 2,087 ± 1,422 | NS[a] |
| Alanine aminotransferase level (U/L) | 80 ± 62 | 71 ± 47 | NS[a] |
| Stage of liver fibrosis | | | |
| F1 or F2/F3 or F4 | 19/9 | 28/12 | NS[b] |
| Drug adherence dosage (%) | | | |
| Pegylated interferon | 97.7 ± 5.2 | 95.2 ± 7.3 | NS[a] |
| Ribavirin | 96.8 ± 6.4 | 95.3 ± 7.7 | NS[a] |

NS, not significant.
[a] $t$-test.
[b] $\chi^2$ test.

## Re-Evaluation of Pre-Treatment HCV Viremia Status Using Real-Time PCR

Since the viral titer before treatment is a major predictive marker of the outcome of Peg-IFN/RBV therapy, we re-evaluated the pre-treatment viral titers more precisely using preserved serum samples taken within 1 month before treatment, using a real-time PCR assay. The pre-treatment viral titers did not differ significantly between sera with group A and B isolates (5.98 ± 0.94 vs. 6.25 ± 0.62 logIU/ml) (Table V). The secondary structure polymorphisms of HCV NS3 were independent of the pre-treatment viral titers.

## DISCUSSION

Antiviral therapy with Peg-IFN/RBV for 48 weeks fails to eradicate HCV in about half of patients infected with a high titer of HCV genotype 1b, and the severe adverse events and high costs associated with this therapy require outcome prediction to allow targeted treatment for chronic hepatitis C. The pre-treatment viral titer, viral factors that influence the virological response to IFN-based anti-HCV therapy have been widely investigated. Viral kinetics showing prompt seronegativity after the start of treatment is a critical factor for achieving SVR, and thus the possible correlation between an early virological response and genetic sequence variation of the HCV has been studied. In particular, amino acid substitutions in the HCV core region at positions 70 and 91 or multiple mutations detected in the IRRDR of the HCV NS5A region are useful markers for predicting EVR and subsequent SVR.

TABLE III. Virological Responses in Subjects With Different Polymorphisms in the Secondary Structure of HCV NS3

| | EVR* | ETR** | SVR* |
|---|---|---|---|
| Group A (n = 28) | 19 (68%) | 23 (82%) | 18 (64%) |
| Group B (n = 40) | 17 (43%) | 25 (63%) | 15 (38%) |

EVR: early virological response at 12 weeks after the start of treatment.
ETR: virological response at the end of treatment.
SVR: sustained virological response 24 weeks after completion of treatment.
*$P < 0.05$.
**$P = 0.08$; $\chi^2$ test.

To date, the influence of several single amino acid substitutions and accumulation of these changes in the viral genome on the effect of IFN-based anti-HCV therapy has been examined. Since interactions between host and viral proteins in infected cells may influence the therapeutic effect of an antiviral agent, we focused on the association of structural polymorphism of a viral protein with the effect of Peg-IFN/RBV combination therapy in this study. Our results suggest that polymorphism analysis of secondary structure deduced from sequence variations in the HCV NS3 amino-terminal region can be used to predict viral responses to this therapy.

Amino acid sequences of the HCV NS3 amino-terminal region, which encodes a serine protease, vary greatly among HCV isolates. Interactions between HCV NS3 and host proteins may influence both oncogenesis and immunity, and thus elucidation of the biological significance of these interactions could result in a new prognostic marker for HCC or a predictive marker for anti-HCV therapy. First, HCV NS3 interacts with the p53 tumor suppressor to suppress p53-dependent apoptosis or p21 transcriptional activity [Ishido and Hotta, 1998; Kwun et al., 2001; Deng et al., 2006]. Transfection of a plasmid expressing the amino-terminal portion of HCV NS3 induces cell transformation in vitro, and transplanted cells proliferate with sarcoma-like features in vivo [Sakamuro et al., 1995]. These findings suggest that NS3 may be involved in the oncogenic pathway in HCV infection. We have shown that the secondary structure of the 120-residue amino-terminal region of NS3 (1,027–1,146) is classifiable into two major groups: A and B. This region encodes a serine protease and also includes p53-binding sites. Our

TABLE IV. Treatment Outcome of Cases With a Double Wild-Type Core Region and Different HCV NS3 Structural Polymorphism

| | Group A (%) | Group B (%) | P |
|---|---|---|---|
| SVR (n = 12) | 10 (83) | 2 (17) | 0.02[a] |
| Non-SVR (n = 10) | 3 (30) | 7 (70) | |

SVR, sustained virological response.
[a] $\chi^2$ test.

TABLE V. Pre-Treatment HCV RNA Levels Measured by Real-Time PCR for Subjects With Different HCV NS3 Structural Polymorphism

| | Group A | Group B | *P* |
|---|---|---|---|
| SVR (n = 33) | 5.78 ± 1.05 | 6.13 ± 0.71 | NS[a] |
| Non-SVR (n = 35) | 6.33 ± 0.59 | 6.32 ± 0.55 | NS[a] |
| Total (n = 68) | 5.98 ± 0.94 | 6.25 ± 0.62 | NS[a] |

SVR, sustained virological response. NS, not significant.
[a]*t* test.

previous cross-sectional studies revealed that the prevalence of group B infection is significantly higher in HCC cases than in non-HCC cases [Ogata et al., 2003], and that the group B infection is an independent risk factor for development of HCC in patients with chronic HCV infection [Nishise et al., 2007]. Second, NS3 interacts with host proteins associated with IFN signaling and thus influences cellular immunity. Since the serine protease encoded by the amino-terminal region of NS3 inhibits the IFN-signaling pathway, polymorphism of this region is likely to influence the effect of Peg-IFN/RBV combination therapy.

Several factors associated with the virological response to this therapy are well known, with adherence to both IFN and RBV strongly influencing outcome [Pearlman, 2004; Arase et al., 2005; Yamada et al., 2008]. In this study, we analyzed 75 cases in which >80% of the scheduled dosage of both drugs was administered. Of these cases, 28 (37%) and 40 (53%) were infected with group A and B isolates, respectively, which were similar rates to those for the 139 cases in the overall study. Age, gender, viral load before treatment, ALT level, proportion of fibrosis stage and adherence to Peg-IFN and RBV did not differ between the group A and B cases. However, the frequencies of SVR and EVR were significantly higher in group A, and those for non-EVR and non-SVR were significantly higher in group B. The results suggest that infection with the group B isolate, which correlates with a higher rate of HCC, is resistant to Peg-IFN/RBV therapy. The pre-treatment viremia status in the 68 cases with group A or B isolates showed no significant differences between the two groups of patients. Therefore, these results suggest that the secondary structure of the HCV NS3 amino-terminal region may be useful for prediction of the outcome of Peg-IFN/RBV combination therapy. In this initial study setting, the relationship of these polymorphisms to the frequency of rapid viral response at 4 weeks after the start of treatment was not evaluated. It will be important to assess this relationship in a future study.

The polymorphism in HCV core region (Arg70/Leu91) is a useful predictive marker for virological responses in Peg-IFN/RBV therapy [Akuta et al., 2007]. Interestingly, a combined analysis of polymorphisms of the core region (which encodes a structural protein) and HCV NS3 (a nonstructural protein) improved the prediction rate. Therefore, analysis of NS3 polymorphism in combination with the core structural polymorphism appears to improve prediction of the outcome of Peg-IFN/RBV therapy. A larger, multi-center prospective study would be necessary to validate the present results. In conclusion, the results of this study suggest that secondary structure polymorphism in the amino-terminal region of HCV NS3 is a useful predictive marker of the effect of Peg-IFN/RBV combination therapy for chronic hepatitis C. Although the present findings are clinically important, and will be helpful for predicting the outcome of Peg-IFN/RBV therapy, further in vitro studies will be needed to elucidate the molecular mechanism underlying the association of HCV NS3polymorphisms with clinical outcome.

## REFERENCES


Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2006. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 78:83–90.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46:403–410.

Arase Y, Ikeda K, Tsubota A, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Kobayashi M, Kumada H. 2005. Significance of serum ribavirin concentration in combination therapy of interferon and ribavirin for chronic hepatitis C. Intervirology 48:138–144.

Bruno S, Cammà C, Di Marco V, Rumi M, Vinci M, Camozzi M, Rebucci C, Di Bona D, Colombo M, Craxì A, Mondelli MU, Pinzello G. 2004. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: A randomized controlled trial. J Hepatol 41:474–481.

Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, Sada K, Hotta H. 2006. NS3 protein of hepatitis C virus associated with the tumor suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. J Gen Virol 87:1703–1713.

El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol 51:471–482.

El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Evans JD, Seeger C. 2006. Cardif: A protein central to innate immunity is inactivated by the HCV NS3 serine protease. Hepatology 43:615–617.

Foy E, Li K, Wang C, Sumpter R, Jr., Ikeda M, Lemon SM, Gale M, Jr. 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300:1145–1148.

Gale MJ, Jr., Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230:217–227.

Gale MJ, Jr., Korth MJ, Katze MG. 1998. Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: A potential mechanism of interferon resistance. Clin Diagn Virol 10:157–162.

Garnier J, Osguthorfe DJ, Robson B. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120:97–120.

Grandvaux N, Servant MJ, tenOever B, Sen GC, Balachandran S, Barber GN, Lin R, Hiscott J. 2002. Transcriptional profiling of interferon regulatory factor 3 target genes: Direct involvement in the regulation of interferon-stimulated genes. J Virol 76:5532–5539.

Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. Ann Intern Med 140:346–355.

Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, Oshita M, Katayama K, Yoshihara H, Imai Y, Kato M, Kawata S, Tsubouchi H, Kumada H, Okanoue T, Kakumu S, Hayashi N. 2008. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. Hepatol Res 38:52–59.

Ishido S, Hotta H. 1998. Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. FEBS Lett 438:258–262.

Kwun HJ, Jung EY, Ahn JY, Lee MN, Jang KL. 2001. p53-dependent transcriptional repression of p21(waf1) by hepatitis C virus NS3. J Gen Virol 82:2235–2241.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomized trial. Lancet 358:958–965.

Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437:1167–1172.

Murphy MD, Rosen HR, Marousek GI, Chou S. 2002. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. Dig Dis Sci 47:1195–1205.

Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, Tanaka N, Taniguchi T. 2001. Gene induction pathways mediated by distinct IRFs during viral infection. Biochem Biophys Res Commun 283:1150–1156.

Nishise Y, Saito T, Sugahara K, Ito JI, Saito K, Togashi H, Nagano-Fujii M, Hotta H, Kawata S. 2007. Risk of hepatocellular carcinoma and secondary structure of hepatitis C virus (HCV) NS3 protein amino-terminus, in patients infected with HCV subtype 1b. J Infect Dis 196:1006–1009.

Nousbaum J, Polyak SJ, Ray SC, Sullivan DG, Larson AM, Carithers RL, Jr., Gretch DR. 2000. Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy. J Virol 74:9028–9038.

Ogata S, Ku Y, Yoon S, Makino S, Nagano-Fujii M, Hotta H. 2002a. Correlation between secondary structure of an amino-terminal portion of the nonstructural protein 3 (NS3) of hepatitis C virus and development of hepatocellular carcinoma. Microbiol Immunol 46:549–554.

Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. 2002b. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol 40:3625–3630.

Ogata S, Florese RH, Nagano-Fujii M, Hidajat R, Deng L, Ku Y, Yoon S, Saito T, Kawata S, Hotta H. 2003. Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. J Clin Microbiol 41:2835–2841.

Pearlman BL. 2004. Hepatitis C treatment update. Am J Med 117:344–352.

Puig-Basagoiti F, Forns X, Furcić I, Ampurdanés S, Giménez-Barcons M, Franco S, Sánchez-Tapias JM, Saiz JC. 2005. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. J Gen Virol 86:1067–1075.

Sakamuro D, Furukawa T, Takegami T. 1995. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 69: 3893–3896.

Sarrazin C, Herrmann E, Bruch K, Zeuzem S. 2002. Hepatitis C virus nonstructural 5A protein and interferon resistance: A new model for testing the reliability of mutational analyses. J Virol 76:11079–11090.

Yamada K, Mori A, Seki M, Kimura J, Yuasa S, Matsuura Y, Miyamura T. 1998. Critical point mutations for hepatitis C virus NS3 proteinase. Virology 246:104–112.

Yamada G, Iino S, Okuno T, Omata M, Kiyosawa K, Kumada H, Hayashi N, Sakai T. 2008. Virological response in patients with hepatitis C virus genotype 1b and a high viral load: Impact of peginterferon-alpha-2a plus ribavirin dose reductions and host-related factors. Clin Drug Investig 28:9–16.

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# Analysis of Neutralizing Antibodies against Hepatitis C Virus in Patients Who Were Treated with Pegylated-Interferon *plus* Ribavirin

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**Key Words:** hepatitis C virus, neutralizing antibody, pegylated-interferon *plus* ribavirin, treatment outcome

## ABSTRACT

**The role of neutralizing antibodies (NAb) in determining responses to antiviral therapy has not been defined well. By using hepatitis C virus (HCV) cell culture system with the J6/JFH1 strain of HCV genotype 2a, we analyzed NAb responses in patients with chronic hepatitis C who received pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) antiviral therapy. A total of 65 patients chronically infected with HCV genotype 1b were enrolled in this study. Of all the 65 patients, 34 (52%) patients achieved early virological response (EVR), with the remaining 31 patients (48%) being Non-EVR. Twenty-seven patients (42%) achieved sustained virological response (SVR), with the remaining 38 patients (58%) being Non-SVR. Thus, NAb titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Rather unexpectedly, NAb titers did not significantly decrease when measured even one year after disappearance of HCV RNA. On the other hand, when change ratios of NAb titers before and after disappearance of HCV RNA were compared between patients with different treatment outcomes, we noticed that the change ratio of NAb titers of patients who achieved an EVR was significantly lower than that of Non-SVR. In conclusion, our present results suggest that NAb titers were significantly associated with clinical responses to PEG-IFN/RBV therapy.**

## INTRODUCTION

Hepatitis C virus (HCV), an enveloped, positive-stranded RNA virus, is a member of the Genus *Hepacivirus*, the Family *Flaviviridae*. The life cycle of the virus, including viral attachment and entry to the cells, genome replication, protein synthesis and virion assembly, has recently been studied using an HCV cell culture system (12). An estimated 170 million individuals are infected with HCV worldwide. The current standard therapy is based on a combination of pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) and, with this treatment

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regimen, viral eradication can be achieved in around 50% of the patients infected with HCV-1b.

Both viral and host factors play important roles in the control of viral infection. Whereas viral factors help to adjust the cellular environment to support viral replication, host factors generally function to combat the viral invasion either by actively blocking the virus replication through innate and/or acquired immune responses or by having the infected cells die out by themselves through apoptosis so that the virus can no longer replicate in the infected cells.

Acquired immune responses of the host involve cell-mediated immunity and humoral immunity. The importance of cellular immunity in combating HCV infection has been well documented (4, 14). On the other hand, humoral immune responses in protection against and/or recovery from HCV infection may be of less importance. Nevertheless, it has been reported that the neutralizing antibody (NAb) responses play an important role in the prevention of infection and in limiting viremia (10, 13, 16). Indeed, patients chronically infected with HCV were reported to possess relatively high titers of cross-reactive NAb (1). It is reported that patients with chronic hepatitis C infection also have high NAb titers to envelope protein of HCV-like particles (HCV-LPs) (2). Humoral and cellular immune responses are also important in determining response to antiviral therapy with IFN/RBV (7). We previously reported that the degree of antibody responses to the NS5A protein of HCV was correlated with early virological response after the initiation of PEG-IFN/RBV therapy (8). However, the role for NAb in determining responses to PEG-IFN/RBV antiviral therapy has not been well documented.

In the present study, we have established an experimental system to measure NAb titers using hepatitis C virus cell culture (HCVcc) model, and measured NAb titers in patients with chronic hepatitis C who were treated with PEG-IFN/RBV. Our data revealed that good treatment outcome was associated with higher NAb titers in patients chronically infected with HCV-1b.

## MATERIALS AND METHODS

### Cells

Huh-7.5 cells (3), a kind gift from Dr. C. M. Rice (Rockefeller University, New York, NY, USA), were propagated in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Biowest), 0.1 mM nonessential amino acids (Invitrogen), 100 IU penicillin per ml and 100 μg streptomycin per ml (Invitrogen). Cells were grown at 37°C in a $CO_2$ incubator.

### Virus

The J6/JFH1 strain of HCV (11) was a kindly gift from Dr. C. M. Rice. Virus stocks were produced in Huh-7.5 cells, and the viral titers were determined by focus forming units (FFU) assay in Huh-7.5 cells, as described previously (5). The viral stocks were kept at -80°C until ready for use.

### Patients and serum samples

Patients chronically infected with HCV-1b, who were treated with pegylated interferon α-2b (1.5 μg per kilogram body weight, once weekly, subcutaneously) and ribavirin (600-800 mg daily, per os), were described previously (8, 9). Sera were collected and stored at -80°C until ready for use. The sera were inactivated at 56°C for 30 min before being used for the virus neutralization test, as describe below.

### HCV focus reduction neutralization assay

An HCV focus reduction neutralization assay was performed, as described elsewhere (Sasayama *et al.*, in preparation). Briefly, one-tenth volume of serum obtained from

uninfected healthy human, which had been inactivated at 56°C for 30 min, was mixed with HCV solutions at 37°C for 1 h to avoid nonspecific inhibition. Serial 3-fold dilutions of each serum sample were mixed with pre-treated HCV solution containing $10^4$ FFU. After incubation at 37°C for 1 h, the mixtures were inoculated to naïve Huh-7.5 cells (2 x $10^5$ cells per well in 24-well plates) and incubated in a 5% $CO_2$ incubator. After 3 h of virus adsorption, the inocula were removed and fresh complete DMEM were added to the cells. At 24 h postinfection, cells were washed with PBS, fixed with 100% methanol, blocked with 5% goat serum in PBS and subjected to immunofluorescence analysis using mouse monoclonal antibody against HCV core antigen (clone 2H9; a kind gift from Dr. T. Wakita, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan) (15) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen). The immunostained cells were washed with PBS, counterstained with Hoechst 33342 solution (Invitrogen) at room temperature for 5 min, mounted on glass slides, and observed under a fluorescence microscope (BZ-9000; Keyence). The number of HCV-infected foci in each well was counted by a software BZ-H1C (Keyence). The dilution that neutralized 50% of the initial virus infectivity was calculated by curvilinear regression analysis. Each neutralization titer was determined as the logarithmic value of the reciprocal antibody dilution that reduced 50% of viral foci to the total number. Titers were expressed as logarithmic values and means ± standard deviation (SD) were calculated.

**Statistical analysis.**

The statistical significance of comparisons between the two groups of patients was determined using Student's *t*-test. In all tests, a *p*-value lower than 0.05 was considered statistically significant.

## RESULTS

**Virological response of the patients underwent treatment with PEG-IFN/RBV**

In this study, 65 patients infected with HCV-1b were enrolled. During and after PEG-IFN/RBV therapy, their clinical responses to the treatment were evaluated (Table I.). Of all the 65 patients, 34 (52%) patients achieved early virological response (EVR) by week 12, with the remaining 31 patients (48%) being Non-EVR. Twenty-seven patients (42%) achieved sustained virological response (SVR), with the remaining 38 patients (58%) being Non-SVR, which is divided into 2 categories, complete non-response (CNR) and relapse as described previously (14). CNR was observed with 16 (25%) of the 65 patients and relapse was observed with 22 patients (34%).

**Table. I.** Proportion of various virological responses of patients treated with PEG-IFN/RBV.

| Virological response | Proportion |
|---|---|
| EVR | 52% (34 / 65)* |
| Non-EVR | 48% (31 / 65) |
| SVR | 42% (27 / 65) |
| Non-SVR | 58% (38 / 65) |
| CNR | 25% (16 / 65) |
| Relapse | 34% (22 / 65) |

* Number of patients/Number of total.

**Pre-treatment NAb titers in the serum and PEG-IFN/RBV treatment outcome**

To study the possible impact of NAb on PEG-IFN/RBV treatment outcome of patients infected with HCV-1b, we measured NAb titers in patients sera using the J6/JFH1 strain of HCV. The mean dilution of antibodies required for 50% neutralization ($NAb_{50}$ titer) of HCV J6/JFH1 in patients who achieved an EVR (2.28±0.36) was significantly higher than that of non-EVR (1.97±0.38) ($p<0.01$) (Fig. 1). Also, the $NAb_{50}$ titers in patients who achieved an SVR (2.27±0.40) was significantly higher than that of non-SVR (2.04±0.37) ($p<0.05$) or CNR (1.91±0.43) ($p<0.05$) (Fig. 1). These data suggest that $NAb_{50}$ titers in the pre-treatment sera are associated with treatment outcome.

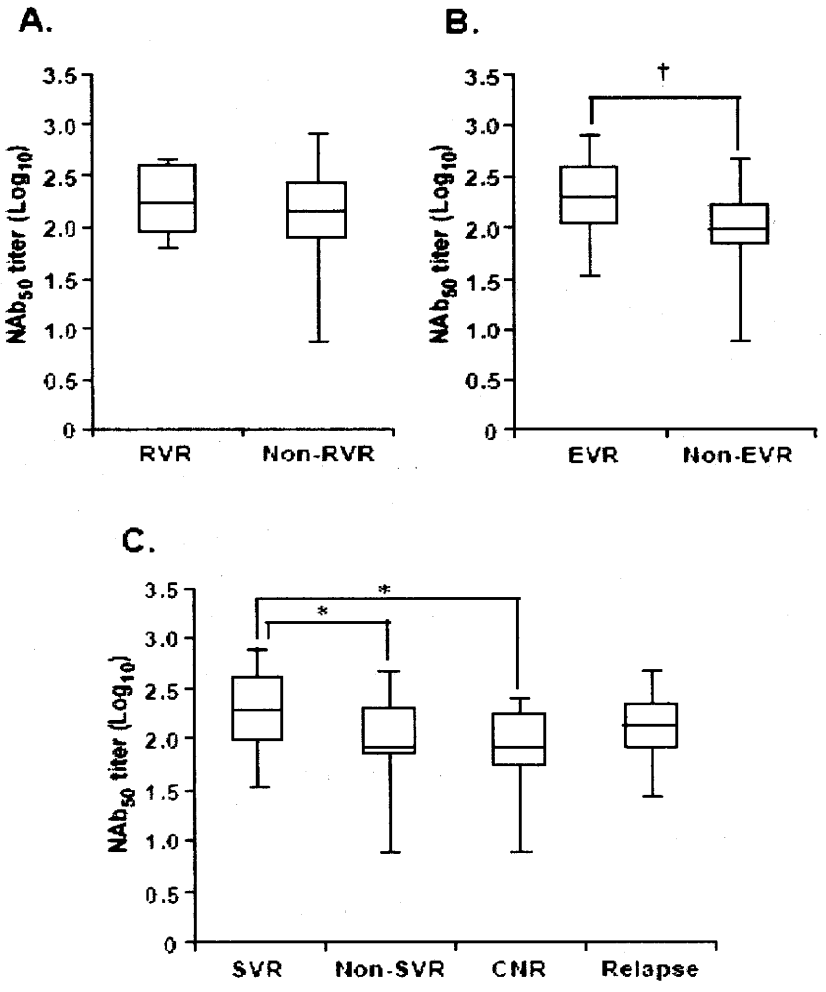


**Fig. 1.** $NAb_{50}$ titers in the sera of HCV-infected patients. $NAb_{50}$ titers in the pre-treatment sera of HCV-1b-infected patients were compared between RVR and Non-RVR (A), EVR and Non-EVR (B), SVR and Non-SVR (C), SVR and CNR (C), and SVR and Relapsers (C). *, $p<0.05$; †, $p<0.01$.

**Fate and change ratios of NAb titers before and after disappearance of HCV RNA in the serum of patients treated with PEG-IFN/RBV**

In order to investigate as to whether or not the NAb titers decrease after disappearance of HCV RNA in the serum of patients treated with PEG-IFN/RBV, we measured NAb titers in patients' sera collected at both pre-treatment (before disappearance) and after disappearance of HCV RNA in the serum. Rather unexpectedly, NAb titers did not decrease when measured even one year after disappearance of HCV RNA. On the other hand, when change ratios of NAb titers before and after disappearance of HCV RNA were compared between patients with different treatment outcomes, we noticed that the change ratio of $NAb_{50}$ titers of patients who achieved an EVR was significantly lower than that of Non-EVR (Fig. 2).

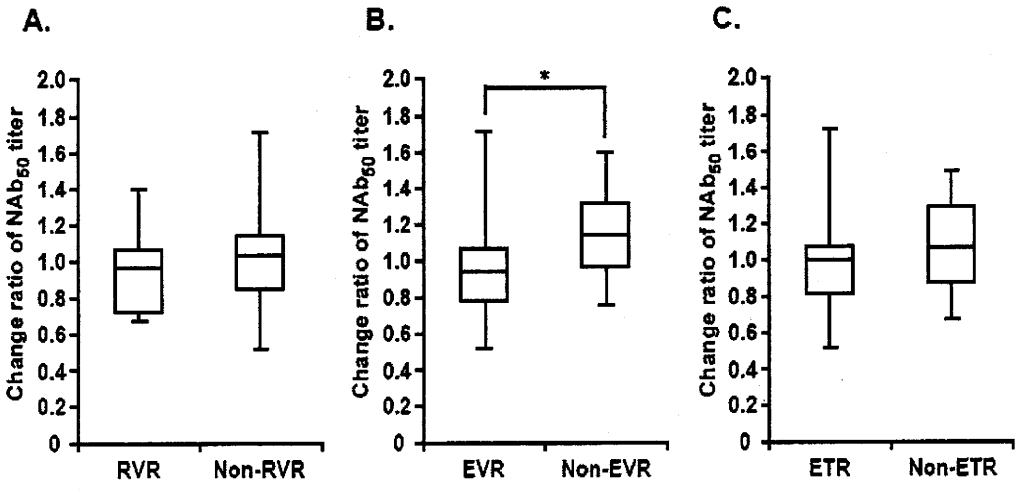


**Fig. 2.** Change ratios of NAb before and after disappearance of HCV RNA in the serum. Change ratios (post-treatment/pre-treatment) in the $NAb_{50}$ titers before and after disappearance of HCV RNA in the sera of patients who were treated with PEG-IFN/RBV were compared between RVR and Non-RVR (A), EVR and Non-EVR (B), and ETR and Non-ETR (C). *, $p<0.05$.

## DISCUSSION

We previously reported that anti-NS5A antibodies were more frequently detected in sera of patients who achieved EVR compared to Non-EVR (8). In this study, we demonstrated that $NAb_{50}$ titers in the pre-treatment patients' sera were associated with the good responses (EVR and SVR) to PEG-IFN/RBV combination therapy (Fig. 1). Consistent with our observations, it was reported that NAb titers to HCV-LPs were higher in patients who achieved an SVR with IFN/RBV therapy than in relapsers and non-responders (2). The better humoral responses, such as NAb and anti-NS5A antibodies, might be associated with better cell-mediated immune responses, which involve CD4+ and CD8+ T cells. It is well known that peripheral and intrahepatic CD8+ T cell responses, which play an important role in the control of and recovery from HCV infection, are also important in determining an SVR in response to PEG-IFN/RBV treatment (6). Thus our present results imply the possibility that individuals who can maintain harmonized good immune responses are able to achieve good responses to PEG-IFN/RBV therapy, such as EVR and SVR.

We also found that $NAb_{50}$ titers did not decrease significantly when measured even one year after disappearance of HCV RNA in the serum (Fig. 2).

Another important finding in this study is that the NAb in patients infected with HCV-1b significantly cross-reacts to HCV-2a; we observed that average NAb titers of HCV-2a-infected patients were ca. 3 times higher than those of HCV-1b-infected patients when measured with the same experimental system using the J6/JFH1 strain of HCV-2a (439±2.72 vs. 139±2.48; $p<0.0001$). This information would be helpful when considering immunological prophylaxis against HCV infection, either active or passive immunizations using vaccines and NAb.

In conclusion, $NAb_{50}$ titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Also, $NAb_{50}$ titers declined only slightly during the course of one year after disappearance of HCV RNA in the sera.

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## REFERENCES

1. **Bartosch, B., Bukh, J., Meunier, J. C., Granier, C., Engle, R. E., Blackwelder, W. C., Emerson, S. U., Cosset, F. L., and Purcell, R. H.** 2003. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci U S A. **100:** 14199-204.
2. **Baumert, T. F., Wellnitz, S., Aono, S., Satoi, J., Herion, D., Tilman, G. J., Pape, G. R., Lau, J. Y., Hoofnagle, J. H., Blum, H. E., and Liang, T. J.** 2000. Antibodies against hepatitis C virus-like particles and viral clearance in acute and chronic hepatitis C. Hepatology **32:** 610-17.
3. **Blight, K. J., MaKeating, J. A., and Rice, C. M.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. **76:** 13001-13014.
4. **Bowen, D. G., and Walker, C. M.** 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature **436:** 946-52.
5. **Bungyoku, Y., Shoji, I., Makine, T., Adachi, T., Hayashida, K., Nagano-Fujii, M., Ide, YH., Deng, L., and Hotta, H.** 2009. Efficient prodection of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J Gen Virol. **90:** 1681-91.
6. **Caetano, J., Martinho, A., Paiva, A., Pais, B., Valente, C., and Luxo, C.** 2008. Differences in hepatitis C virus (HCV)-specific CD8 T-cell phenotype during pegylated alpha interferon and ribavirin treatment are related to response to antiviral therapy in patients chronically infected with HCV. J Virol. **82:** 7567-77.
7. **Cramp, M. E., Rossol, S., Chokshi, S., Carucci, P., Williams, R., and Naoumov, N. V.** 2000. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin

treatment in chronic hepatitis C. Gastroenterology. **118:** 346-55.

8. **El-Shamy, A., Sasayama, M., Nagano-Fujii, M., Sasase, N., Imoto, S., Kim, S. R., and Hotta, H.** 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol. **51:** 471-82.
9. **El-Shamy, A., Nagano-Fujii, M., Sasase, N., Imoto, S., Kim, S. R., and Hotta, H.** 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. **48:** 38-47.
10. **Lavillette, D., Morice, Y., Germanidis, G., Donot, P., Soulier, A., Pagkalos, E., Sakellariou, G., Intrator, L., Bartosch, B., Pawlotsky, JM., and Cosset, FL.** 2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J Virol. **79:** 6023-34.
11. **Lindenbach, B. D, Evans, M. J., Syder, A. J., Wölk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A., and Rice, C. M.** 2005. Complete replication of hepatitis C virus in cell culture. Science. **309:** 623-626.
12. **Moradpour, D., Francois P. F., and Rice, C. M.** 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. **5:** 453-63.
13. **Youn, J. W., Park, S. H., Lavillette, D., Cosset, F. L., Yang, S. H., Lee, C. G., Jin, H. T., Kim, C. M., Shata, M. T., Lee, D. H., Pfahler, W., Prince, A. M., and Sung, Y. C.** 2005. Sustained E2 antibody response correlates with reduced peak viremia after hepatitis C virus infection in the chimpanzee. Hepatology. **42:** 1429-36.
14. **Takaki, A., Wiese, M., Maertens, G., Depla, E., Seifert, U., Liebetrau, A., Miller, J. L., Manns, M. P., and Rehermann, B.** 2000. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med. **6:** 578-82.
15. **Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med. 2005 **11:** 791-6.
16. **Youn, J. W., Park, S. H., Lavillette, D., Cosset, F. L., Yang, S. H., Lee, C. G., Jin, H. T., Kim, C. M., Shata, M. T., Lee, D. H., Pfahler, W., Prince, A. M., and Sung, Y. C.** 2005. Sustained E2 antibody response correlates with reduced peak viremia after hepatitis C virus infection in the chimpanzee. Hepatology. **42:** 1429-36.

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# Double-Filtration Plasmapheresis plus IFN for HCV-1b Patients with Non-Sustained Virological Response to Previous Combination Therapy: Early Viral Dynamics

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**Key Words**

Chronic hepatitis C · Double-filtration plasmapheresis · Early viral dynamics · Genotype 1b · High viral load · Interferon β · Non-sustained virological responder · Peginterferon plus ribavirin combination therapy

**Abstract**

Double-filtration plasmapheresis (DFPP) was approved in Japan in April 2008 for the retreatment of chronic hepatitis C patients with genotype 1b and high viral loads, whose hepatitis C virus was not eradicated by earlier IFN therapy or by pegylated IFN plus ribavirin (PEG-IFN/RBV) combination therapy. In this study, we assessed the early viral dynamics of 9 patients with non-sustained virological response to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction of ≥1 log in the viral load in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment. By contrast, DFPP plus consecutive intravenous IFN-β for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks. The viral load declined by ≥2 log in 50% (3/6) at 4 weeks after the start of treatment. DFPP plus consecutive intravenous IFN-β for 4 weeks is a promising treatment for non-sustained virolgical response patients.



## Introduction

Hepatitis C virus (HCV) infection is the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) in industrialized countries. HCV infection is manageable, however, and its complications can be prevented by antiviral therapy [1, 2]. Currently, the most effective treatment for chronic HCV infection is based on pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy [3]. Nonetheless, sustained

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virological response (SVR) rates for those infected with the most resistant genotypes (HCV-1a and HCV-1b) still hover around 50% [3, 4].

To surmount this SVR rate with combination therapy, several trials have been undertaken, two of which are: (1) retreatment with combination therapy and (2) double-filtration plasmapheresis (DFPP). By the protocol-defined primary analysis of the former, the SVR rate has been 16% at most, even for a 72-week induction group [5].

The use of DFPP [approved in Japan in April 2008 for the retreatment of chronic hepatitis C (CHC) patients with genotype 1b and high viral loads] together with IFN administration has produced a substantial reduction in the viral load during the early stages of treatment and has effected a high SVR [6], suggesting that this treatment is a new modality for CHC patients in difficult-to-treat states. In this study, we used DFPP plus IFN to enhance the efficacy of the treatment of CHC patients whose HCV was not eradicated by earlier PEG-IFN/RBV combination therapy, and we assessed early viral dynamics associated with SVR.

## Patients and Methods

*Patients*

Nine patients (aged 43–66 years) whose HCV had not been eradicated by earlier PEG-IFNα-2b plus RBV combination therapy carried out between 2008 and 2009 were enrolled in this study. The patients were divided into 2 groups: partial responders (PR; relapse after the end of therapy) and non-responders (NR; no disappearance of HCV RNA during therapy). All the patients were confirmed to be HCV RNA positive with high transaminase levels persisting for 6 months or longer, and with HCV RNA genotype 1b at levels exceeding $10^5$ log IU/ml in blood (as determined before the start of therapy by real-time PCR). Also, the patients were negative for hepatitis B surface antigen. Patients with platelet counts of $\leq 10 \times 10^4/\mu l$, leukocyte counts of $\leq 3{,}000/\mu l$, or hemoglobin levels of $\leq 12$ g/dl were excluded from the study.

Each patient gave written informed consent and agreed to receive concomitant DFPP, and the study was approved by the review board of the Kobe Asahi Hospital.

*DFPP and Blood Collection*

Blood collected from the peripheral vein for DFPP by a Plasmaflo™ OP-18W filter (Asahi Kasei Medical, Tokyo, Japan) was separated into plasma and cell components. The virus was then removed from the plasma by a second filter (Cascadeflo™ EC-50W; Asahi Kasei Medical) of an average pore size of 30 nm. For each session, the final volume of treated plasma was 50 ml/kg; the number of sessions was 5 over 2 weeks, and the time of DFPP, based on the reduced plasma fibrinogen levels during DFPP, was decided by the physicians and as required by the patients.

*Types of IFN for 4 Weeks with DFPP*

During DFPP, the patients were treated with different kinds of IFN: patient 1 with PEG-IFNα-2b plus RBV for 4 weeks; patients 2 and 3 with IFN-β 3 MU twice daily for 2 weeks and PEG-IFNα-2a plus RBV for 2 weeks; patients 4 and 9 with IFN-β 3 MU twice daily for 2 weeks and IFN-β 6 MU daily for 2 weeks; patient 5 with IFN-β 3 MU twice daily for 10 days and IFN-β 6 MU daily for 18 days, and patients 6, 7 and 8 with IFN-β 3 MU twice daily for 4 weeks. The dose of PEG-IFNα-2b was 1.5 μg/kg and 180 μg of α-2a per week. The RBV dose was 800 mg/day with α-2b and 600–800 mg/day with α-2a. After DFPP plus IFN treatment for 4 weeks, all patients were scheduled to receive PEG-IFN/RBV combination therapy (patient 1: PEG-IFNα-2b 1.5 μg/kg per week plus RBV 800 mg/day; patients 2–9: PEG-IFNα-2a 180 μg per week plus RBV 600–800 mg/day).

*Amino Acid Substitutions in the Core Region (aa 30 and aa 91) and Number of IFN Sensitivity-Determining Region Mutations*

We measured pre-treatment factors such as prediction of clinical outcome of therapy, amino acid sequence variation in the NS5A region (referred to as IFN sensitivity-determining regions) and in the core protein regions (aa 70 and aa 91) of HCV with a given genotype, and the viral load.

*HCV RNA Measurement*

The quantity of HCV RNA was measured by real-time PCR (detection limit 1.2 log IU/ml), by HCV core antigen (detection limit 20 fmol/l), and by RT-PCR (Amplicor HCV monitor v 2.0; Roche; detection limit 50 IU/ml).

*Virus Removal at Second Filter Inlet and Outlet*

Plasma was collected twice from the inlet and outlet of the second filter during 1 session of DFPP: once when the treated plasma volume reached half of the target quantity, and once when DFPP was completed. The change in the quantity of HCV RNA was evaluated through the plasma samples collected.

*Viral Reduction and Viral Response Rate*

The quantity of HCV RNA was converted to a log value at the beginning of the treatment (A) and at each of the virus measurement points (B). Δlog was then calculated: $\Delta\log = \log A - \log B = \log(A/B)$.

*Evaluation of DFPP Safety*

The subjective and objective adverse events of DFPP were observed, and five clinical factors were measured (platelet and lymphocyte counts, and hemoglobin, albumin and fibrinogen levels) before the first session of DFPP, before successive sessions on the second, third, fourth, fifth and sixth days, and 2 weeks after the last session.

*Statistical Analysis*

Statistical analysis consisted of analysis of variance for patient background factors, and the paired t test for quantities of HCV RNA at the second filter inlet during DFPP. The t test was used for viral load reductions and Fisher's exact test for viral response rates among the groups. The t test was 2-tailed, and differences of $p < 0.05$ were considered significant.

**Table 1.** Early viral dynamics with DFPP plus IFN treatment

| Case | Age/ sex | Type of IFN for 4 weeks with DFPP | Viral dynamics after DFPP+IFN | | | | | | Viral dynamics of previous treatment (PEG-IFN/RBV) | | | | Viral mutation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | before treatment | log drop | | | unit | | before treatment | log drop | unit | outcome | aa 70 | aa 91 | ISDR |
| | | | | 24 h | 1 wk | 2 wks | 4 wks | | | 4 wks | | | | | |
| 1 | 66/M | PEG-IFNα-2b/RBV 4 wks | 6,510 | 0.5 | 0.6 | 0.6 | 1.1 | fmol/l | 452 | 0.7 | KIU/ml | NR | wild | wild | 0 |
| 2 | 65/F | IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks | 7.5 | 0.4 | 1.3 | 2.6 | 1.0 | log IU/ml | 2,800 | ND | KIU/ml | PR | wild | wild | 0 |
| 3 | 52/F | IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks | 5.8 | 0.4 | 1.0 | 1.6 | +0.2 | log IU/ml | 6.3 | 0.2 | log IU/ml | NR | wild | wild | 1 |
| 4 | 47/F | IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks | 6.8 | 0.6 | 0.3 | 0.4 | 0.4 | log IU/ml | 2,900 | 0.3 | KIU/ml | NR | mutant | mutant | 1 |
| 5 | 52/F | IFN-β (3 MU 2/day) 10 days → IFN-β (6 MU 1/day) 18 days | 5.5 | 1.4 | 1.5 | 1.2 | 1.9 | log IU/ml | 782 | 0.6 | fmol/l | NR | wild | mutant | 1 |
| 6 | 61/F | IFN-β (3 MU 2/day) 4 wks | 6.5 | 1.2 | 3.4 | 5.0 | 4.8 | log IU/ml | 8,450 | 2.6 | fmol/l | NR | wild | wild | 0 |
| 7 | 66/F | IFN-β (3 MU 2/day) 4 wks | 5.3 | 0.0 | 0.8 | 1.2 | 1.3 | log IU/ml | 11,500 | 0.8 | fmol/l | NR | mutant | wild | 1 |
| 8 | 43/F | IFN-β (3 MU 2/day) 4 wks | 3,460 | 0.5 | 0.2 | 1.3 | 2.2 | fmol/l | 745 | 0.1 | fmol/l | NR | wild | mutant | 1 |
| 9 | 43/M | IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks | 7.2 | 0.6 | 1.4 | 2.5 | 2.9 | log IU/ml | 426 | 0.1 | KIU/ml | NR | wild | wild | 0 |

PEG-IFN/RBV: PEG-IFNα-2a (180 μg per week) plus RBV (600–800 mg/day) or PEG-IFNα-2b (1.5 μg/kg per week) plus RBV (800 mg/day). IFN-β: 3 MU twice daily or 6 MU daily. ND = Not done; aa = amino acid; ISDR = interferon sensitivity-determining region.

## Results

Of the 9 patients, 1 was PR and 8 were NR. Virus mutation in the core region was as follows: wild type (7 patients) and mutant type (2 patients) at aa 70; wild type (6 patients) and mutant type (3 patients) at aa 91. IFN sensitivity-determining regions demonstrated mutation 1 (5 patients) and mutation 0 (4 patients), while mutation 2 was not seen in any patient. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction in the viral load of ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The early viral dynamics after DFPP plus consecutive intravenous IFN-β treatment for 4 weeks showed a reduction in the viral load of ≥1 log in 33% (2 of 6 patients), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The reduction of the viral load by ≥2 log was observed in 50% (3 of 6 patients) at 4 weeks after the start of treatment (table 1).

## Discussion

New drugs to replace IFN as well as drugs that can be used in combination with IFN are being actively developed. Also, attempts are being made to find ways to physically remove HCV particles from the blood. Granulocyte apheresis, plasma exchange and hemofiltration have been applied to HCV-infected patients for the treatment of cryoglobulinemia and vasculitis, modalities which have been shown to reduce HCV RNA in the blood during treatment [6–11]. The mechanisms of the clinical results of plasmapheresis have been described, whereby HCV in the blood is related to the effects of IFN therapy that could be enhanced by removing the virus from blood [12–14]. Low-density lipoprotein-cholesterol apheresis and plasma exchange in hypercholesteremic patients with HCV infection reduces the quantity of HCV RNA in the blood of some patients [15]. Hemodialysis, hemofiltration and peritoneal dialysis in chronic dialysis patients infected with HCV significantly lower HCV RNA levels in the blood [16]. Combined granulocyte apheresis with IFN therapy for CHC [17–19] and the prerequisite for early reduction of the virus in the treatment of CHC [20, 21] are essential. Thus, the potential effectiveness of IFN therapy combined with early physical removal of the virus is of particular interest.

Asahina et al. [22] studied HCV dynamics in both serum and peripheral blood mononuclear cells in 44 patients, with HCV genotype 1b and high viral loads, randomly assigned to 4 treatment groups: (1) combination therapy with 6 MU daily of IFNα-2b plus 800 mg of RBV; (2) monotherapy with 6 MU daily of IFNα-2b; (3) monotherapy with twice-daily intravenous administration of 3 MU of IFN-β, and (4) monotherapy with daily intravenous administration of 6 MU of IFN-β. HCV RNA levels measured serially by highly sensitive real-time PCR and HCV dynamics in both serum and peripheral blood mononuclear cells have demonstrated a 'biphasic' pattern. The exponential decay slopes of the second phase have been significantly higher in the combination or the twice-daily dose regimen groups than in group 2 or 4 ($0.10 \pm 0.08$ vs. $0.02 \pm 0.09$ or $0.16 \pm 0.09$ vs. $0.02 \pm 0.04$ $day^{-1}$; $p < 0.05$ and $p < 0.0005$, respectively) [22]. Kim et al. [23] observed that a daily dose of IFN-β 6 MU for 4 weeks effects a 2 log decrease in the HCV RNA load in 7 patients with genotype 1b and high viral loads.

In this study, early viral dynamics were assessed in the 9 patients non-SVR to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks reduced the viral load by ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively.

The prerequisite for early virological response (EVR; indicating negative HCV RNA at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those who do not reach EVR fail to respond to further therapy. Treatment discontinued in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is highly desirable [24].

To be able to predict SVR with PEG-IFN/RBV treatment, reduction of the HCV RNA viral load by week 4 is considered essential. A 2 log reduction in the HCV RNA viral load by week 4 is a prerequisite to achieving SVR with PEG-IFN/RBV treatment [25]. In our study of DFPP plus consecutive intravenous IFN-β treatment for 4 weeks, a reduction in the viral load of ≥2 log was achieved in 50% (3 of 6 patients) at 4 weeks after the start of treatment.

From the above considerations, DFPP plus consecutive intravenous IFN-β treatment for 4 weeks is a promising regimen for non-SVR patients with genotype 1b and high viral loads, previously treated with PEG-IFN/RBV therapy. Further study is needed to elucidate the SVR rate in a larger number of patients given DFPP plus IFN treatment, especially with consecutive intravenous IFN-β.


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## Disclosure Statement

No conflict of interest exists.

## References


1 Hoofnagle JH, Seeff LB: Peginterferon and ribavirin for chronic hepatitis C. N Engl J Med 2006;355:2444–2451.

2 Pawlotsky JM: Therapy of hepatitis C: from empiricism to eradication. Hepatology 2006; 43:S207–S220.

3 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, 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;358:958–965.

4 Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002; 347:975–982.

5 Jensen DM, Freilich B, Andreone P, et al: Pegylated interferon alfa-2A (40KD) plus ribavirin (RBV) in prior non-responders to pegylated interferon alfa-2B (12KD)/RBV: final efficacy and safety outcomes of the repeat study. Hepatology 2007;46(suppl 1):291–292.

6 Fujiwara K, Kaneko S, Kakumu S, et al: Double filtration plasmapheresis and interferon therapy for chronic hepatitis C patients with genotype 1 and high viral load. Hepatol Res 2007;37:701–710.

7 Fabrizi F, Martin P, Dixit V, et al: Biological dynamics of viral load in hemodialysis patients with hepatitis C virus. Am J Kidney Dis 2000;35:122–129.

8 Manzin A, Candela M, Solforosi L, Gabrielli A, Clementi M: Dynamics of hepatitis C viremia after plasma exhange. J Hepatol 1999; 31:389–393.

9 Ramratnam B, Bonhoeffer S, Binley J, et al: Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. Lancet 1999;354: 1782–1785.

10 Schettler V, Monazahian M, Wieland E, Thomssen R, Muller GA: Effect of heparin-induced extracorporeal low-density lipoprotein precipitation (HELP) apheresis on hepatitis C plasma virus load. Ther Apher 2001;5: 384–386.