

**Table 5.** Clinical Background of 27 Patients Who Underwent Resection and 13 Patients Who Underwent Radiofrequency Ablation

Factors	Resection Group, n = 27	RFA Group, n = 13	P
Age, y*	60 (35-73)	67 (50-78)	.006
Sex (men:women)	23:4	10:3	NS
HBV:HCV:others	13:12:2	1:9:3	.015
Habitual alcohol intake, yes: no	3:24	2:11	NS
Diameter of HCC, mm*	22 (14-30)	22 (10-30)	NS
No. of HCC, single:multiple	26:1	9:4	.031
Tumor vascularity, present:absent	27:0	12:1	NS
Albumin, g/dL*	3.8 (3.3-4.3)	3.4 (2.6-4.1)	.003
Bilirubin, mg/dL*	0.9 (0.4-1.9)	0.9 (0.4-1.8)	NS
AST, IU/L*	38 (16-240)	49 (17-145)	NS
Platelets, $\times 10^4/\text{mm}^3$ *	15.1 (6.0-24.5)	10.5 (4.5-24.6)	.025
Prothrombin time, %*	94 (79-112)	86 (73-110)	NS

\* Data are expressed as median (range).

**Table 6.** Results of Univariate and Multivariate Analyses of Factors Associated With Survival Among Patients With Serum Des-gamma-carboxy Prothrombin Level  $\geq 100$ 

Variable	No.	% 5-Year Survival Rate	Hazard Ratio (95% CI)	P
<b>Univariate analysis</b>				
Age, y				.026
<65	25	87.7	—	
$\geq 65$	15	45.5	—	
Albumin, g/dL				.024
$\geq 3.8$	12	100	—	
<3.8	28	58.4	—	
Treatment procedure				.012
Resection	27	83.4	—	
RFA	13	33.6	—	
<b>Multivariate analysis</b>				
Treatment procedure				.020
Resection or RFA			1.26 (1.04-1.53)	

CI indicates confidence interval; RFA, radiofrequency ablation.

albumin level, platelet count, prothrombin time, and presence of multiple tumors were associated with recurrence-free survival. Alternatively, in RFA patients, in addition to platelet count, which indicates severity of portal hypertension, DCP levels were significant predictors of survival. Likewise, DCP levels were also significant predictors in recurrence-free survival. It is noteworthy that both survival and disease-free survival rates of patients who undergo RFA, but not resection, are correlated with DCP levels by multivariate analysis.

It is difficult to explain why DCP influenced survival and disease-free survival in the RFA group but not the resection group. We speculate that a high level of serum tumor marker reflects a high tumor malignant potential. Therefore, for a biologically aggressive tumor like HCC, resection is recommended over RFA because the radical nature of surgical resection may be superior to RFA.

According to previous reports, DCP is related to histological features of HCC.<sup>21,22</sup> Shirabe et al<sup>21</sup> examined

218 HCC patients who underwent surgical resection for HCC and concluded that serum DCP level is a predictor of microvascular invasion. They identified microvascular invasion in 44% of their patients with DCP  $\geq 100$  AU/L, but in only 16% of patients with DCP  $< 100$  AU/L. Shimada et al<sup>22</sup> examined explanted liver transplants and reported that serum DCP level is associated with vascular invasion and HCC recurrence. When we evaluated the relationship between clinicopathological features and serum DCP level in the resection group, microvascular invasion was found in 11 (44.0%) of 25 patients with DCP  $\geq 100$  AU/L, but in only 22 (13.6%) of 162 patients with DCP  $< 100$  AU/L, which was similar to the results in Shirabe et al.<sup>21</sup>

Unfortunately, in the RFA group, histological examination was performed only on nodules that showed atypical image findings. Moreover, it is sometimes difficult to judge microscopic vascular invasion in small specimens obtained by needle biopsy. However, we cannot deny the presence of microscopic vascular invasion in patients with high levels of tumor markers who have been treated with RFA. Furthermore, high levels of DCP are not only a marker of malignancy, but also indicate the biologic aggressiveness and progression of the HCC tumor. Hence, HCCs with high levels of DCP have greater chances of hypervascularity and early infiltration than do HCCs with lower levels of DCP.

In general, microscopic vascular invasion or intrahepatic metastasis is a poor prognostic factor for survival and recurrence-free survival even in patients who undergo surgical resection.<sup>23-25</sup> Why was survival and recurrence-free survival not different among the resection group in our study? One of the reasons is that we included patients with an HCC of a maximum diameter of  $\leq 3$  cm. In contrast, most previous studies included HCC as large as 5 cm in diameter.<sup>23-25</sup> The biological features of malignancy might be worse in such large tumors. We speculate that in HCC measuring  $\leq 3$  cm (median 2.0 cm), minimal microscopic vascular invasion or intrahepatic metastasis adjacent to the main tumor can be curatively resected by surgery, whereas these sometimes become incompletely necrotic even when a sufficient surrounding margin is obtained from treatment with RFA.

Analysis of factors associated with survival in patients with DCP of  $\geq 100$  AU/L showed that the type of treatment procedure (eg, hepatectomy) significantly

influenced outcome. In contrast, no such relationship was found in patients with DCP of  $< 100$  AU/L. These results indicate that DCP is an important factor in selecting treatment procedure for patients with HCC measuring  $\leq 3$  cm and numbering  $\leq 3$  tumors.

In conclusion, DCP levels were significant predictors of both survival and recurrence-free survival in the RFA group. Hence, when the level of DCP is high, hepatic resection should be the treatment of choice even if the maximum tumor diameter is  $\leq 3$  cm and there are  $\leq 3$  tumors. If the level is low, RFA should be considered, because it is less invasive.

Because the current study was retrospective in nature, it has certain limitations and potential biases. The baseline characteristics of the 2 groups were quite different. Although we enrolled only Child-Pugh A patients, the resection group was younger and had better liver function. However, etiology of the liver disease was also different; the overall survival rates were not significantly based on etiology in either the resection or the RFA group. Therefore, we believe that the etiology of liver disease could be ignored in these patients. Our study did not uncover the reason for the high risk of mortality and tumor recurrence in patients in the RFA group with high levels of DCP. A cohort validation study is needed to confirm our results. In addition, clinicopathological and molecular analyses are also needed to define the biological significance of the biomarker.

### Conflict of Interest Disclosures

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# An Open Pilot Study Exploring the Efficacy of Fluvastatin, Pegylated Interferon and Ribavirin in Patients with Hepatitis C Virus Genotype 1b in High Viral Loads

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## Key Words

Hepatitis, chronic · Virus, hepatitis C · Statin · Interferon, pegylated · Ribavirin

## Abstract

**Objective:** Response to pegylated (PEG) interferon (IFN) and ribavirin is achieved only in 40–50% of patients infected with hepatitis C virus (HCV) of genotype 1 in high viral loads, which needs to be improved. **Methods:** In an open-label pilot study, fluvastatin (HMG-CoA reductase inhibitor), 20 mg daily, was given along with PEG-IFN/ribavirin to 21 patients with chronic hepatitis C. They were followed for HCV RNA in serum. **Results:** During treatment for 48 weeks, HCV RNA was lost from serum in 93% of the patients. In the 15 patients who received 48-week therapy, a sustained virological response (SVR) with loss of HCV RNA 24 weeks after completion was achieved in 10 (67%), including 7 of the 9 (78%) male and 3 of the 6 (50%) female patients. In the remaining 6 patients who received 72-week therapy, SVR was gained in 4 (67%), including 1 of the 2 male and 3 of the 4 female patients aged 56, 58 and 62 years, respectively. **Conclusion:** Fluvastatin could be used safely to increase the response to PEG-IFN and ribavirin, especially in aged women who respond poorly to combined PEG-IFN/ribavirin.

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## Introduction

Over the world approximately 190 million people are persistently infected with hepatitis C virus (HCV) [1], and about 30% of them develop serious liver disease such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [2]. Interferon (IFN) is the only drug that can resolve HCV infection. However, using the most advanced treatment currently available, with pegylated (PEG)-IFN and ribavirin for 24–48 weeks, a sustained virological response (SVR) defined by the loss of HCV RNA from serum 24 weeks after completion of therapy is achieved in 40–80% of the patients [3–5]. The response rate is influenced by host factors such as sex, age and ethnicity [6–8], as well as virological factors including genotypes and viral loads [9]. It remains unsatisfactorily low in patients infected with HCV genotype 1 at merely 40–50%, and dismal in women aged 50 years or older at merely 22% [10]. Hence, there is an imminent need to improve the efficacy of antiviral treatment for terminating HCV infection in these patients.

Efforts have been devoted toward increasing the efficacy to PEG-IFN/ribavirin therapy. Inhibitors of viral protease, alone or in combination with the IFN-based treatment, have been found effective in preliminary studies [11, 12]. Recently, drugs that can inhibit the key enzyme

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for controlling the synthesis of cholesterol, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, have gained attention due to their potential to decrease the replication of HCV in clinical and experimental settings [13, 14].

More than three quarters of Japanese patients are infected with HCV genotype 1b in high viral loads. They are much older than Western patients owing to the widespread HCV infection which struck Japan about 20 years ago [15]. Owing to this, the response to PEG-IFN/ribavirin in Japanese patients with chronic hepatitis C is poorer than that in Western countries. In an open-label pilot study, 21 patients with chronic hepatitis C, who were infected with HCV-1b at high viral loads, received triple treatment with fluvastatin, PEG-IFN and ribavirin. On-treatment viral dynamics and SVR achieved by the triple therapy could offer hope for improving the response in patients infected with HCV-1 in high viral loads.

### Patients and Methods

From December 2005 to March 2006, 21 patients with chronic hepatitis C agreed to receive fluvastatin, in addition to standard PEG-IFN/ribavirin treatment, at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. They all were: (1) positive for antibody to HCV (anti-HCV) and HCV RNA of genotype 1b, and not co-infected with HCV of the other genotypes; (2) negative for hepatitis B surface antigen or antibody to human immunodeficiency virus type-1 (HIV-1); (3) confirmed within the past 2 months to have high HCV RNA levels of  $\geq 100$  KIU/ml, which is the Japanese definition of high viral loads [16, 17]; (4) platelet counts  $>80 \times 10^3/\text{mm}^3$  and without cirrhosis diagnosed by ultrasonography; (5) body weight  $>40$  kg and not pregnant or lactating; (6) a total alcohol intake of  $<500$  g during the past; (7) without HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; (8) without antiviral or immunosuppressive treatment during the previous 3 months, and (9) with the wish to comply to the treatment protocol for 48–72 weeks.

They were followed for liver function and virological markers at least monthly during treatment and until 24 weeks after completion of triple treatment. Informed consent was obtained from each patient, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

#### Markers of HCV Infection

Anti-HCV was determined by third-generation enzyme-linked immunosorbent assay (ELISA) using commercial kits (Ortho HCV Ab ELISA Test 3; Chiron Cooperation, Emeryville, Calif., USA). HCV RNA was determined quantitatively by polymerase chain reaction (PCR; Cobas Amplicor HCV Monitor ver. 2.0, Roche Diagnostics, Tokyo, Japan), with a dynamic range from 5 to 5,000 KIU/ml, in sera diluted 10-fold at baseline, as well as every 2 weeks until 8 weeks after initiation of therapy and monthly thereafter. Sera negative for HCV RNA ( $<5$  KIU/ml) by the

quantitative assay were tested by qualitative PCR (Amplicor, Roche Molecular Systems, Inc., Branchburg, N.J., USA) with the detection limit at 100 copies/ml.

#### Fluvastatin Added to Combined PEG-IFN and Ribavirin

Patients received subcutaneous PEG-IFN- $\alpha 2b$  (PEG-Intron, Schering-Plough Corp., Kenilworth, N.J., USA) weekly at a median dose of 1.5 (range 1.3–1.7)  $\mu\text{g}/\text{kg}$ , along with oral ribavirin daily at a median dose of 12.3 (range 10.2–13.7)  $\text{mg}/\text{kg}$  for 48–72 weeks. The dose of ribavirin was adjusted according to body weight: 600 mg for patients weighing  $\leq 60$  kg; 800 mg for those between  $>60$  and  $<80$  kg, and 1,000 mg for those  $\geq 80$  kg. In addition, the patients received 20 mg oral fluvastatin daily for 48–72 weeks.

### Results

#### Patients Who Received Triple Treatment with Fluvastatin, PEG-IFN and Ribavirin

The baseline characteristics of the 21 patients infected with HCV-1b at a high viral load and who received triple therapy with fluvastatin, PEG-IFN and ribavirin are listed in table 1. Their median age was 56 years, and 11 (52%) were men. Their hematological and biochemical values including serum lipids were within normal limits, except for elevated levels of AST and ALT. A lack of mutations affecting the response to IFN in the core protein, i.e., mutations at positions 70 and 91 [16], and amino acid substitutions in the IFN-sensitivity determining region [18] were detected in HCV RNA from 29 and 35% of the patients.

#### Loss of HCV RNA from Serum in Patients with Triple Treatment

Figure 1 illustrates the cumulative loss of HCV RNA from the serum in 21 patients infected with HCV-1b at high loads. HCV RNA was cleared in 52% of the patients during the first 12 weeks. It decreased slowly thereafter, and was lost in 93% of the patients at 48 weeks. Triple treatment was continued in 6 of the 21 patients for an additional 24 weeks. HCV RNA was cleared from the serum in 5 (83%) of them at completion of the 72-week treatment.

#### Sustained Virological Response in Patients Receiving Triple Treatment

Of the 21 patients given triple treatment, 19 received it for 48 weeks. Skin rashes and general malaise developed in 2 of them at weeks 17 and 24, respectively, and fluvastatin was withdrawn while PEG-IFN and ribavirin were continued. Six patients continued to receive triple treatment for an additional 24 weeks. Therefore, the loss of HCV RNA from the serum 24 weeks after completion of

**Table 1.** Baseline characteristics of patients (n = 21) with chronic hepatitis C who received triple treatment with fluvastatin, PEG-IFN and ribavirin

	Normal ranges	Patients
Age, years	NA	56 (32–63)
Men	NA	11 (52%)
Albumin, g/dl	3.9–5.2	3.8 (3.4–4.2)
Hemoglobin, g/dl	11.3–17.0	14.4 (12.1–16.4)
Platelets, × 10 <sup>3</sup> /mm <sup>3</sup>	141–350	190 (119–240)
AST, IU/l	13–33	50 (21–208)
ALT, IU/l	6–42	64 (23–391)
γ-GTP	9–109	36 (11–301)
ICG <sub>15</sub> , %	<10	12 (2–27)
HCV RNA, KIU/ml	NA	2,000 (14– >5,000)
Past treatments	NA	8 (36%)
Ribavirin dose, mg/kg	NA	12.3 (10.2–13.7)
Serum lipids		
Total cholesterol, mg/dl	122–240	163 (124–273)
LDL-C, mg/dl	86–160	99 (59–187)
HDL-C, mg/dl	35–75	46 (28–73)
Mutations in HCV RNA		
Wild-type at aa70	NA	12 (57%)
Wild-type at aa91	NA	10 (48%)
Double wild, aa71/aa90	NA	6 (29%)
Mutations in ISDR	NA	6/17 (35%)

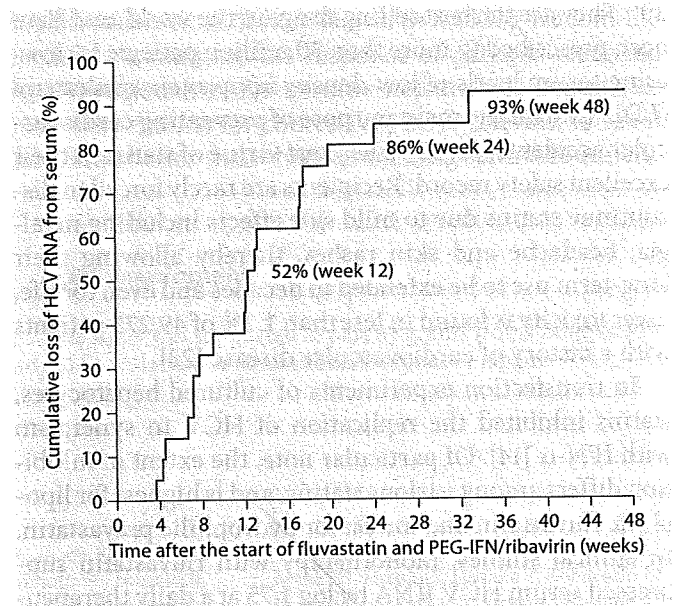
Data are expressed as the number of patients with percentages in parentheses or median values with ranges in parentheses. AST = Aspartate aminotransferase; ALT = alanine aminotransferase; γ-GTP = γ-glutamyltranspeptidase; ICG<sub>15</sub> = retention of indocyanine green at 15 min; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; NA = not applicable.

therapy was assessed in the remaining 15 patients. Figure 2 shows the SVR in 15 patients. SVR was accomplished in 10 (67%) of them, including 7 of 9 (78%) male patients and 3 of 6 (50%) female patients. Of the 5 female patients aged >50 years, 2 (40%) gained SVR.

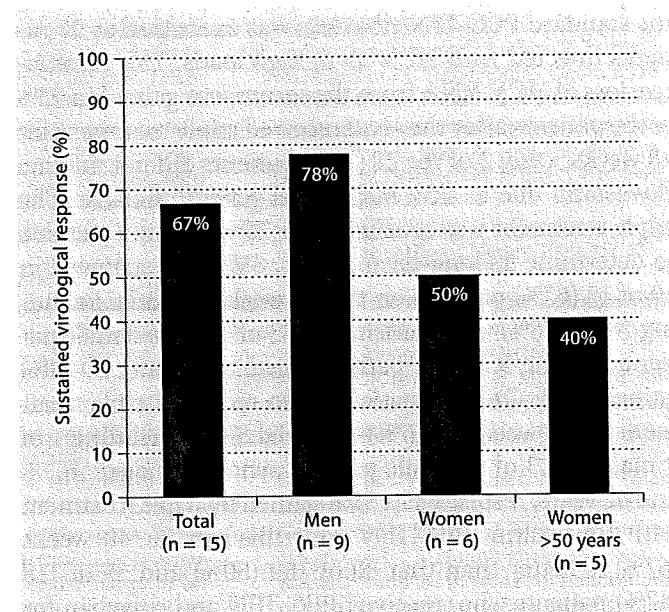
Of the 6 patients who received triple treatment for 72 weeks, SVR was accomplished in 4 (67%), including 1 of 2 male and 3 of 4 female patients who were aged 56, 58 and 62 years, respectively.

## Discussion

Statins comprise a group of drugs capable of inhibiting HMG-CoA reductase and can regulate the synthesis of cholesterol by competing with the authentic substrate [19,



**Fig. 1.** Kaplan-Meier table illustrating the cumulative loss of HCV RNA from the serum in patients with chronic hepatitis C. The patients received fluvastatin in addition to PEG-IFN/ribavirin and were followed during 48 weeks of treatment.



**Fig. 2.** Sustained virological response in patients with chronic hepatitis C to the combined treatment with fluvastatin, PEG-IFN and ribavirin for 48 weeks. SVR was compared among the total, male and female patients, as well as the female patients aged more than 50 years.

20]. They are the best selling drugs in the world, and have been prescribed to more than 30 million patients for lowering serum levels of low-density lipoprotein cholesterol (LDL-C) with the main purpose of preventing cardiovascular accidents [21, 22]. The great virtue of statins is their excellent safety record. Recipients are rarely forced to discontinue statins due to mild side effects including myalgia, headache and skin rashes, thereby allowing their long-term use to be extended to decades and even for life. Liver toxicity is found in less than 1.2% of 49,275 patients with a history of cardiovascular disease [23].

In transfection experiments of cultured hepatocytes, statins inhibited the replication of HCV in synergism with IFN- $\alpha$  [14]. Of particular note, the extent of inhibition differs among various statins, and is highest for lipophilic fluvastatin and lowest for hydrophilic pravastatin. In clinical studies, monotherapy with fluvastatin suppressed serum HCV RNA by log 1.75 at a daily therapeutic dose of <80 mg [13], while no such effects were gained with 20 mg atorvastatin daily [24]. Hence, the ability to inhibit HCV replication may differ among diverse statins through mechanisms not directly associated with lowered levels of LDL-C; fluvastatin decreases total cholesterol and LDL-C levels to a lesser extent than the other statins in current use.

In this open-label pilot study, the response to triple treatment with fluvastatin 20 mg daily supplementing the standard PEG-IFN/ribavirin was examined in 21 patients infected with HCV-1b in high loads. The cumulative loss of HCV RNA from the serum was gained in 93% of the patients after they had received triple treatment for 48 weeks. Only 2 of the 21 (10%) patients did not tolerate fluvastatin due to skin rashes and general malaise. The triple treatment was continued for 72 weeks in 6 patients to determine its long-term effects. SVR was achieved in 10 of 15 (67%) patients on the 48-week schedule, including 7 of 9 (78%) male patients and 3 of 6 (50%) female patients. Of the 5 female patients aged >50 years, 2 (40%) gained SVR. Of the 6 patients who received triple treatment for 72 weeks, 4 (67%) achieved SVR, including 1 of 2 male and 3 of 4 female patients who were aged 56, 58 and 62 years, respectively. SVR gained by triple treatment with fluvastatin, PEG-IFN and ribavirin for 48 weeks (67%) is better than that 80 of 160 (50%) and 55 of 118 (47%) patients who received PEG-IFN and ribavirin for 48 weeks [16, 25]; they all were infected with HCV-1b at high loads.

Sex and age are the two principal host factors influencing the response to IFN-based treatment. Although women respond better to combined IFN and ribavirin than

men in large-scale multinational studies in Western countries [8, 26], target patients were rather young with the median age of about 40–41 years. Sex-dependent responsiveness to IFN is reversed with age, however, and the response to PEG-IFN/ribavirin is much poorer in female than male patients aged 50 years or older (22/100 [22%] vs. 50/94 [53%],  $p < 0.001$ ) [10]. In view of the estradiols that can prohibit fibrosis in experimental cirrhosis induced in rats [27], levels of female sex hormones, which are lower in women than men older than 50 years [28], could be responsible for the sex difference in response to IFN of patients with chronic hepatitis C. Supplementation of aged female patients with estradiols may increase the response, but it can accelerate physiological osteoporosis [29, 30]. Since statin therapy does not increase the risk of severe hepatotoxicity in patients with chronic hepatitis C [31], fluvastatin may provide aged women with an advantage in gaining SVR to PEG-IFN/ribavirin. Furthermore, fluvastatin does not augment the side effects of other drugs, such as cyclosporine A and warfarin, because it is metabolized by CYP2C9 in the liver, unlike other statins that are disposed by CYP3A4 as other drugs [32].

Should statins interfere with the replication of HCV, it remains an open question how they do it. At least three mechanisms may be conceived for the decrease in HCV replication in patients receiving statins. HCV circulates in association with LDL-C [33], and on that basis, the LDL-C receptor has been proposed for infection of hepatocytes [34–36]. HCV RNA replicates in association with lipid droplets in hepatocytes [37, 38], and therefore, the synthesis of cholesterol blocked by statins can reduce the basis on which HCV thrives. However, these two scenarios seem to stand at odds with the response to IFN being better in patients with higher LDL-C levels [39, 40]. Statins prohibit the synthesis of mevalonate that is modified into geranylgeranyl (containing 20 carbons) and then farnesyl (15 carbons) [19]. They both prenylate most cellular proteins to make them lipophilic toward associations with membranes expressing their biological activities [41]. As an extension to these, the geranylgeranylation of host proteins, which may be required for the replication of HCV, is implicated in the capacity of statins to downregulate HCV infection [42–44]. For that matter, most pleiotropic effects of statins, including a decreased incidence of dementia and Alzheimer disease [45–47], could be associated with the inhibition of prenylation rather than lowered levels of LDL-C in the serum [48].

This study is not without limitations. The number of patients receiving fluvastatin along with PEG-IFN and ribavirin is small and fails to gain statistical power in any comparison with those given PEG-IFN/ribavirin in previous studies. The ability of other statins in improving the response to PEG-IFN/ribavirin has not been examined to discover the mechanism of how fluvastatin improves the response to antiviral treatments. The promising results obtained in this study hopefully will invite further interest for planning clinical trials with extended numbers of patients and targeting elderly women in par-

ticular. Meanwhile, it may be worth looking back in the database to examine the response of patients receiving PEG-IFN/ribavirin for chronic hepatitis C, simultaneously with statins for controlling LDL-C levels, to see if they fared better than those not taking statins routinely.

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## Poor Response to Pegylated Interferon and Ribavirin in Older Women Infected with Hepatitis C Virus of Genotype 1b in High Viral Loads

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**Abstract** *Background* Response to treatment in patients with chronic hepatitis C, with reference to age and gender, has not been examined fully. *Aim* The influence of gender and age on treatment with pegylated interferon (PEG-IFN) and ribavirin was evaluated in a retrospective study. *Methods* PEG-IFN and ribavirin were given for 48 weeks to 179 men and 121 women infected with hepatitis C virus (HCV) of genotype 1b in high viral loads (>100 kIU/ml). *Results* Sustained virological response at 24 weeks after treatment was poorer in women than men who were aged  $\geq 50$  years (22% vs 53%,  $P < 0.001$ ). Among the patients aged  $\geq 50$  years who had received  $\geq 80\%$  of the doses of PEG-IFN, ribavirin, or both, women responded less often than men (26% vs 64%,  $P < 0.001$ ; 33% vs 61%,  $P = 0.022$ ; and 32% vs 63%,  $P = 0.016$ ; respectively). In multivariate analysis, male gender, retention of indocyanine green, ribavirin dose and compliance with therapy increased sustained virological response. *Conclusions* Response to combined PEG-IFN and ribavirin is poorer in female than male patients with hepatitis C who are aged  $\geq 50$  years, irrespective of compliance with treatment. Low estrogen levels in older women could be responsible for their impaired response to PEG-IFN and ribavirin.

**Keywords** Aging · Women · Chronic hepatitis C · Genotypes · Interferon · Ribavirin

### Introduction

There are an estimated 170 million people worldwide that are chronically infected with hepatitis C virus (HCV) [1]. HCV can persist in 70–80% of individuals who have been exposed to it, and it can induce chronic liver disease, through cirrhosis to hepatocellular carcinoma (HCC) in approximately 30% of them until 30–40 years after they were infected [2–4]. A number of viral and host factors influence the velocity of fibrosis progression in chronic hepatitis C. Thus, stage and grade of hepatitis are more severe in patients who are infected with HCV genotype 1 in high viral loads [5–7]. Male gender, age and intake of alcohol accelerate fibrosis, as well [8–10].

Interferon (IFN) combined with ribavirin has been the most effective and favored treatment of chronic hepatitis C to date. The combined treatment with the standard IFN can terminate HCV-1 infection with high viral loads in approximately 20% [11], and that with pegylated IFN (PEG-IFN) in >40% [12]. Owing to hemolytic side effects, however, women are less tolerant to ribavirin [13]. Although the response to combined treatment has been shown to be better in women than in men in previous studies, there remains a possibility that it could be influenced by age. Hence, there is a need for the comparison of the response between men and women in different age groups.

Virological response to PEG-IFN and ribavirin at the end of a 48-week treatment (ETR), as well as sustained virological response (SVR) 24 weeks after the completion of therapy, was compared between 179 men and 121

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women who were infected with HCV-1b in high viral loads. In patients aged  $\geq 50$  years, both ETR and SVR were poorer in women than in men, irrespective of the total dose of IFN, ribavirin or both.

## Methods

### Study Population

From December 2001 to February 2006, 490 consecutive patients with chronic hepatitis C received combination therapy with PEG-IFN and ribavirin at the Department of Hepatology in the Toranomon Hospital in Metropolitan Tokyo. The following inclusion criteria were met by 300 (61%) patients: they were (1) positive test results for antibodies to HCV (anti-HCV) and for HCV RNA genotype 1b by qualitative methods, and not co-infected with HCV of other genotypes; (2) negative test results for hepatitis B surface antigen or antibodies to human immunodeficiency virus type-1 (HIV-1); (3) confirmed findings of high HCV RNA levels  $\geq 100$  kIU/ml, which is the Japanese definition of high viral loads [14, 15], within the past 2 months; (4) no cirrhosis diagnosed by laparoscopy and ultrasonography, and with platelet counts  $>80 \times 10^3/\text{mm}^3$ ; (5) body weight  $\geq 40$  kg and not pregnant or lactating; (6) total alcohol intake  $<500$  g in the past; (7) no HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; (8) no treatment with antivirals or immunosuppressants during the previous 3 months; and (9) with the wish to comply with the treatment protocol for 48 weeks. None of them received growth factors before or during the study period.

The 300 patients, comprising 179 men and 121 women, received PEG-IFN and ribavirin for 48 weeks and were followed for at least 24 weeks after completion of this combination therapy. Informed consent was obtained from each patient, and the study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

### Serum Markers of HCV Infection

Anti-HCV was determined by third-generation enzyme-linked immunosorbent assay (ELISA) by commercial kits (Ortho HCV Ab ELISA Test 3; Chiron Cooperation, Emeryville, CA, USA). HCV RNA was determined quantitatively by polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver. 2.0, Roche Diagnostics, Tokyo, Japan) in serum diluted tenfold at the baseline, as well as at least monthly during and after treatment; it has a dynamic range between 5 kIU/ml and 5,000 kIU/ml. Sera

negative for HCV RNA ( $<5$  kIU/ml) by quantitative assay were tested by qualitative PCR (Amplicor, Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a detection limit at 100 copies/ml.

### Combined PEG-IFN and Ribavirin Therapy

Patients underwent subcutaneous administration of PEG-IFN- $\alpha 2b$  (PEG-Intron, Schering-Plough Corp, Kenilworth, NJ, USA), weekly, at a median dose of 1.4  $\mu\text{g}/\text{kg}$  (range 0.8–1.9  $\mu\text{g}/\text{kg}$ ), together with ribavirin orally, at a median daily dose of 11 mg/kg (range 3.7–14.2 mg/kg) for 48 weeks. The dose of ribavirin was adjusted by body weight: 600 mg for patients weighing  $\leq 60$  kg; 800 mg for those between  $>60$  kg and  $<80$  kg; and 1,000 mg for those  $\geq 80$  kg. It was tapered in the 99 (33%) patients in whom hemoglobin levels decreased below 10 g/dl during the combination therapy.

### Statistical Analysis

Variables were compared between groups by the chi-square test, Fisher's exact probability test and the Mann-Whitney U test. Differences in the loss of HCV RNA from the serum between groups was evaluated with the Kaplan-Meier life table with use of the log rank test. The influence of various factors on the response to PEG-IFN/ribavirin was evaluated by logistic regression in univariate and multivariate analyses. Analysis of all data was performed with the computer program SPSS software (SPSS Inc., Chicago, IL, USA), and a *P* value less than 0.05 was considered significant.

## Results

### Baseline Characteristics of Male and Female Patients Infected with HCV-1b in High Loads

PEG-IFN and ribavirin were given for 48 weeks to 179 men and 121 women who had been infected with HCV-1b in high viral loads ( $>100$  kIU/ml). Table 1 compares baseline characteristics between them. Women were older, had lower hemoglobin values and platelet counts, and lower levels of albumin, gamma-glutamyl-transpeptidase ( $\gamma$ -GTP) and ferritin, than men. The stage of fibrosis was lower in women than in men, although their alanine aminotransferase (ALT) levels were comparable. Three months before the start of combination therapy, IFN had been given to 75 (42%) of the 179 male patients, comparably frequently to 40 of the 121 (33%) female patients. Age distribution for men and women is shown in Fig. 1. The proportion of patients  $\geq 60$  years was higher in women than in men (39% vs 19%, *P* < 0.001).

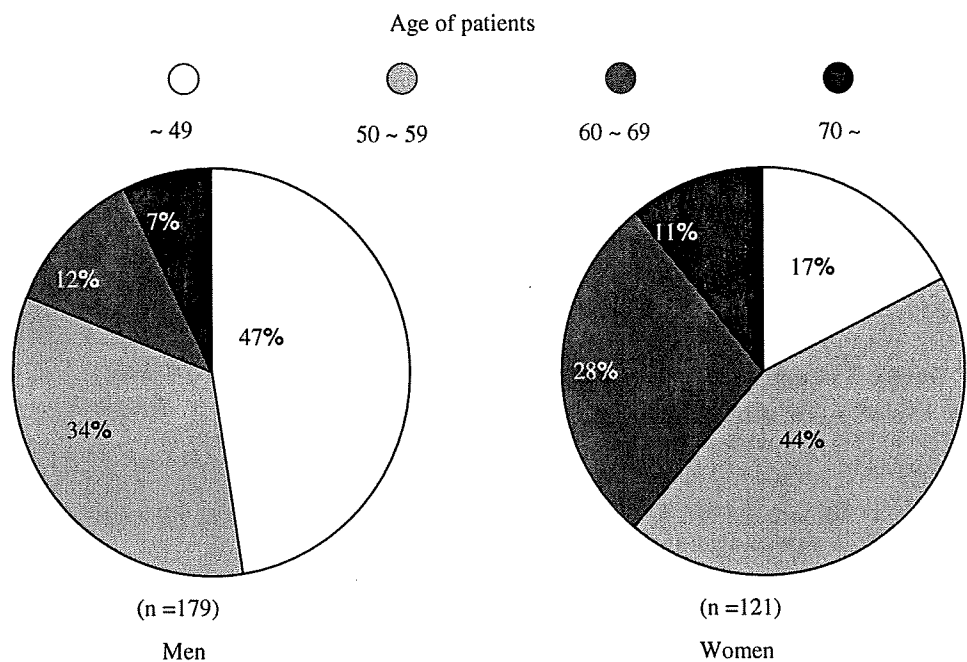
**Table 1** Baseline characteristics of 300 patients with chronic hepatitis with high-titers of HCV-1b RNA who had received PEG-IFN and ribavirin for 48 weeks and were followed for 48 weeks or longer

Characteristic <sup>a</sup>	Men (n = 179)	Women (n = 121)	Differences P
Age (years)	50 (19–66)	57 (30–69)	<0.001
Previous IFN treatment	75 (42%)	40 (33%)	0.146
Hemoglobin (g/dl)	15.2 (11.5–17.8)	13.5 (11.2–15.1)	<0.001
Platelets ( $\times 10^3/\text{mm}^3$ )	176 (88–366)	165 (91–331)	0.025
Albumin (g/dl)	3.9 (3.2–4.6)	3.8 (3.0–4.6)	0.004
ALT (IU/l)	77 (23–504)	68 (19–391)	0.078
$\gamma$ -GTP (IU/l)	78 (14–409)	37 (11–171)	0.011
LDL (mg/dl)	98 (50–176)	99 (57–168)	0.920
Ferritin (mg/l)	186 (<10–1,327)	95 (<10–4 42)	<0.001
ICG <sub>15</sub> (%)	14 (4–41)	13 (2–31)	0.969
Stage (F0-1/F2-3)	80/66 (50 unknown)	42/55 (57 unknown)	0.050

ALT alanine aminotransferase,  $\gamma$ -GTP gamma-glutamyl transpeptidase, LDL low density lipoprotein, ICG<sub>15</sub>, retention of indocyanine green at 15 min

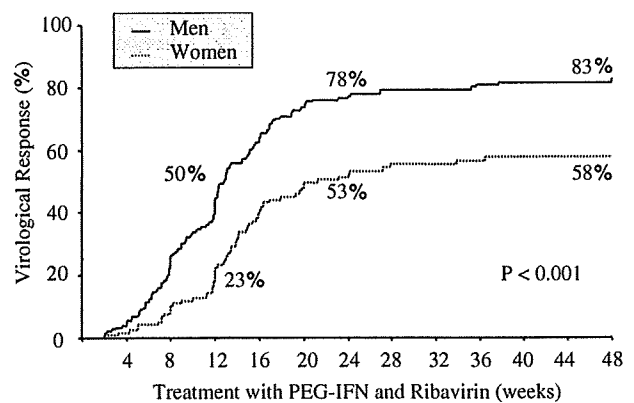
<sup>a</sup> The means (ranges) are given

**Fig. 1** Distribution of ages in the male and female patients with chronic hepatitis C who were infected with HCV-1b in high loads



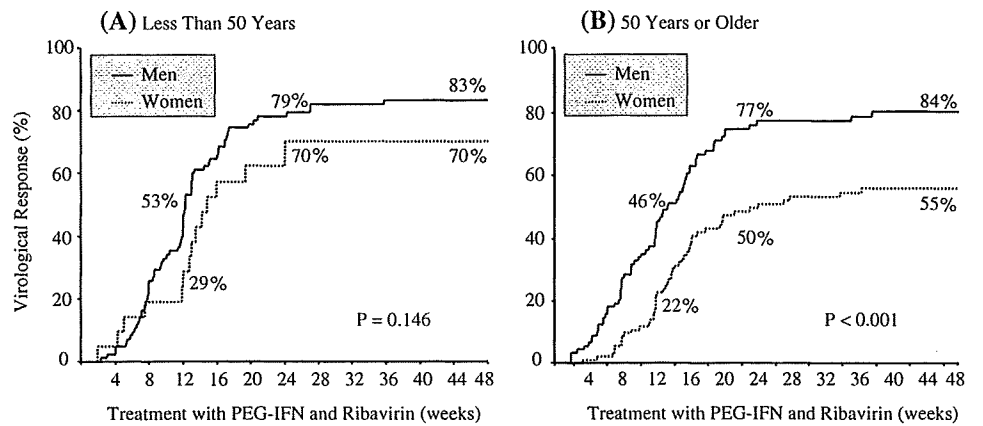
**Virological Response During the 48-Week Treatment with PEG-IFN and Ribavirin**

On-treatment response to the combined treatment is compared between men and women in Fig. 2. Through 48 weeks on treatment, women gained a virological response less frequently than did men. ETR was achieved by 58% of women as against 83% of men ( $P < 0.001$ ). Restricted to the patients who gained ETR, women lost HCV RNA from serum later than men did [median (range) 24.1 (2.0–36.4) vs 13.0 (2.0–48.0) weeks,  $P < 0.001$ ]. Figure 3 depicts the on-treatment virological response in patients <50 years and those  $\geq 50$  years separately. The virological response was no different between men and women <50 years. However, it was poorer in women than



**Fig. 2** On-treatment virological responses to PEG-IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads

**Fig. 3** On-treatment virological responses to combined IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads who were less than 50 years (a) or 50 years or older (b)

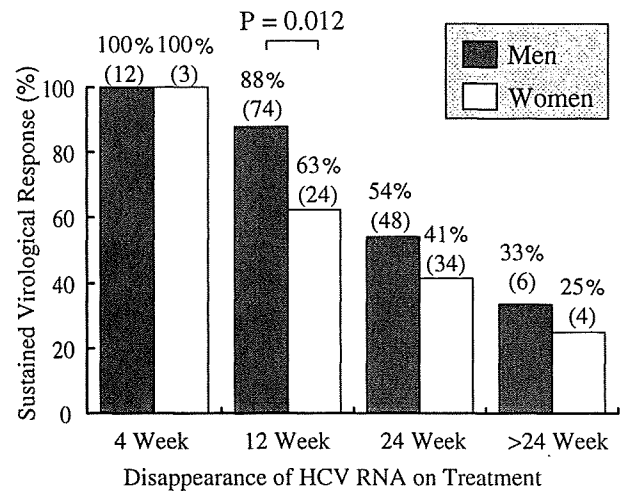


in men  $\geq 50$  years. Differences between men and women in total (Fig. 2), therefore, were attributed to a poorer response of women  $\geq 50$  years to the combined treatment.

**Sustained Virological Response to the 48-Week Treatment with PEG-IFN and Ribavirin**

Sustained virological response 24 weeks after the completion of combined treatment was accomplished much less frequently in women than in men [33/121 (27%) vs 105/179 (59%),  $P < 0.001$ ]. SVR was influenced by age both in men and in women (Fig. 4). It was found significantly less often in women than men who were 50 years or older.

Figure 5 illustrates the relationship between the earliest on-treatment virological response and SVR in men and women. Patients with a virological response at 4 weeks gained SVR invariably. However, in the patients with virological response in later weeks, SVR was achieved less frequently in women than in men. In the patients who had lost HCV RNA from the serum at 12 weeks, in particular, SVR was achieved significantly less often in women than in men (63% vs 88%,  $P = 0.012$ ). The relationship



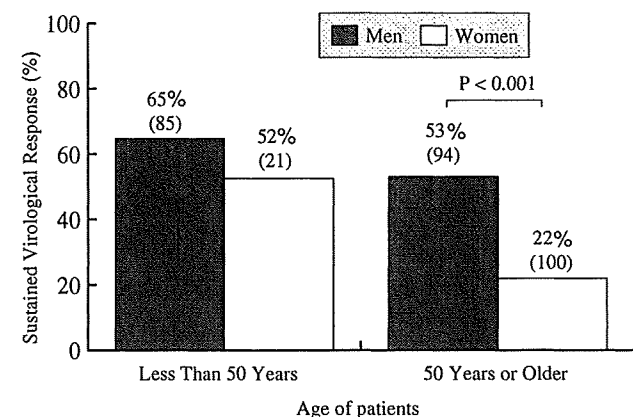
**Fig. 5** Sustained virological response in male and female patients who lost HCV RNA from the serum at various weeks on treatment with PEG-IFN and ribavirin

between on-treatment virological response and SVR was compared among women in different age groups (Fig. 6). In the patients with the earliest virological response at 12 weeks and 24 weeks, SVR was achieved less frequently in women aged  $\geq 50$  years than in those  $< 50$  years, but the difference fell short of being significant due to the small numbers of patients in the comparison.

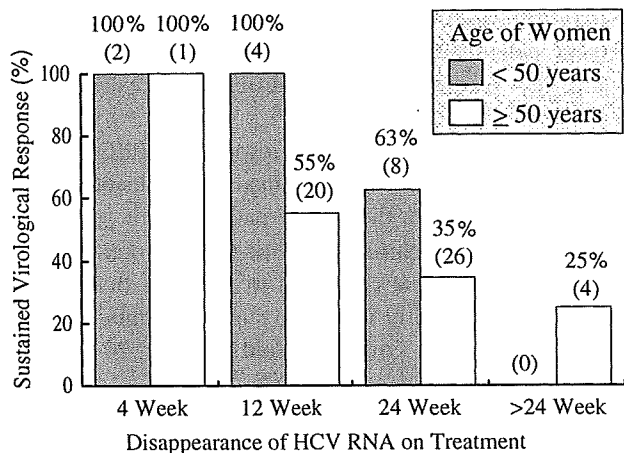
**SVR and Compliance with PEG-IFN Therapy, Ribavirin Therapy, or Both**

Table 2 compares compliance with the combined treatment between men and women. Either or both of PEG-IFN and ribavirin were tolerated to a lesser extent by women than by men. Thus, doses  $\geq 80\%$  were reached less frequently in women than in men for PEG-IFN or ribavirin, or both. The initial dose of ribavirin was no different between men and women.

SVR was achieved less frequently in women than in men who had received  $\geq 80\%$  of the dose of PEG-IFN



**Fig. 4** Sustained virological response to PEG-IFN and ribavirin in male and female patients stratified by age. The number of patients is indicated in parentheses in each column



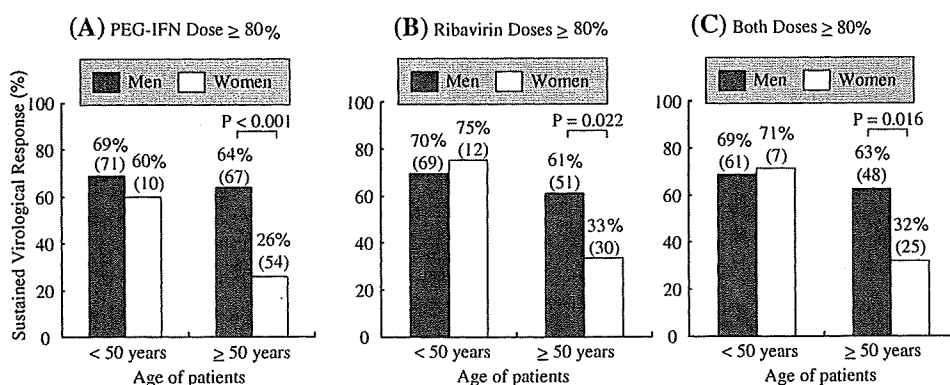
**Fig. 6** Sustained virological response to PEG-IFN and ribavirin in female patients stratified by age who lost HCV RNA from serum at various weeks on treatment

**Table 2** PEG-IFN and ribavirin received by patients with chronic hepatitis with high-titers of HCV-1b RNA

PEG-IFN and ribavirin	Men (n = 179)	Women (n = 121)	Differences P
Initial ribavirin dose (mg/kg body weight)	11.1 (5.0–14.1)	11.2 (3.7–14.3)	0.735
Total dose			
PEG-IFN ≥ 80%	139 (78%)	94 (53%)	<0.001
Ribavirin ≥ 80%	117 (65%)	42 (35%)	<0.001
Both ≥ 80%	110 (61%)	32 (27%)	<0.001
Withdrawn	28 (16%)	28 (23%)	0.131

[20/64 (31%) vs 92/138 (67%),  $P < 0.001$ ], ribavirin [19/42 (45%) vs 79/120 (66%),  $P = 0.027$ ] or both [13/32 (41%) vs 72/109 (66%),  $P = 0.013$ ]. Again, differences were observed only in patients ≥50 years (Fig. 7). In the patients <50 years, in contrast, the rate of SVR was no different between women and men who had received ≥80% of the dose of PEG-IFN, ribavirin, or both.

**Fig. 7** Sustained virological response to PEG-IFN and ribavirin in patients who had received 80% or more of the dose of IFN (a), ribavirin (b) or both of them (c). Results are shown for men and women in two age groups



The influence of age was compared between male and female patients in different age groups. SVR was achieved significantly more frequently in the men aged ≥60 years [88/145 (61%) vs 17/74 (37%),  $P = 0.001$ ] and <60 years [17/34 (50%) vs 6/48 (13%),  $P < 0.001$ ]. Likewise, SVR was more common in male than female patients aged 50–59 years [33/60 (55%) vs 16/53 (30%),  $P = 0.013$ ].

Multivariate Analysis for Factors Accelerating the Response to PEG-IFN and Ribavirin Therapy

In univariate analysis, age, gender, hemoglobin, albumin, ICG<sub>15</sub>, ribavirin dose and compliance with PEG-IFN therapy, ribavirin therapy, or both, influenced SVR. In multivariate analysis, only male gender, ICG<sub>15</sub>, ribavirin dose and compliance with PEG-IFN, as well as both PEG-IFN and ribavirin, accelerated the chance of SVR (Table 3).

Discussion

In a retrospective study, response to PEG-IFN and ribavirin for 48 weeks was compared between 179 men and 121 women with chronic hepatitis C who had been infected with HCV-1b in high viral loads by the Japanese definition (>100 kIU/ml) [14, 15]. Loss of HCV RNA from serum occurred less often in women than in men throughout the 48 weeks of treatment. Both ETR (55% vs 83%,  $P < 0.001$ ) and SVR (27% vs 59%,  $P < 0.001$ ) were achieved significantly less frequently in women than in men. The observed low response to PEG-IFN and ribavirin stands at odds with the better response to antiviral treatments and slow progression of fibrosis in women than in men [9, 16, 17]. There are, however, viral and host factors other than gender that can influence the course of chronic hepatitis C and, by inference, the response to antiviral treatments.

Viral factors such as HCV genotypes and infection load affect the course of chronic hepatitis C. Thus, hepatitis is

**Table 3** Factors promoting the response to PEG-IFN and ribavirin in multivariate analysis

Factors	Odds ratio	95% Confidence interval	<i>P</i>
Male gender	3.50	1.71–7.17	0.001
ICG <sub>15</sub> ≤ 13.5%	2.09	1.07–4.08	0.031
Ribavirin ≥ 11.1 mg/kg per day	2.17	1.11–4.25	0.024
Total PEG-IFN ≥ 80%	6.96	2.26–21.4	0.001
PEG-IFN/ribavirin ≥ 80%	12.66	2.32–71.4	0.003

more severe and less responsive to IFN in patients infected with HCV genotypes 1 and 4 than in those with HCV genotypes 2, 3 and 6 [18–22]. Likewise, high viral loads are associated with rapid progression of liver disease and poor response to IFN [23–25]. In our study, such viral factors were excluded in comparing the response to PEG-IFN and ribavirin between women and men. All the patients were infected with HCV genotype 1b in high viral loads (>100 kIU/ml).

Age influences the severity of chronic hepatitis C [9, 26], and disease progresses faster and response to antiviral therapy is poorer in older patients [23]. There were significant differences in age between female and male patients in our study. The women were older than the men [mean (range) 57 (30–69) years vs 50 (19–66) years,  $P < 0.001$ ], and the proportion of patients ≥60 years was higher in women than in men (39% vs 19%,  $P < 0.001$ ). Hence, the response to PEG-IFN and ribavirin was evaluated in patients aged ≥50 years and <50 years separately. There were no differences in the response between female and male patients <50 years, during and at the end of the 48-week treatment, as well as 24 weeks thereafter. However, ETR (55% vs 84%,  $P < 0.001$ ) and SVR (22% vs 53%,  $P < 0.001$ ) were gained significantly less often in women than men who were aged ≥50 years.

The influence of gender was observed, also, in patients aged ≥60 years and those aged 50–60 years. Hence, women would become less responsive than men to PEG-IFN and ribavirin after they had entered their fifties.

From a therapeutic notion, compliance with treatment can alter the response. Since ribavirin accumulates in erythrocytes and induces hemolysis, it is less tolerated in women who tend to be anemic than men without such an inclination [27]. At the baseline, women had lower levels of hemoglobin and ferritin than men. These would have been responsible for the lower tolerance to PEG-IFN and ribavirin in women than men in our study. In fact, ≥80% of the dose of PEG-IFN, ribavirin, or both, was tolerated less frequently in women than men ( $P < 0.001$  for each). Even in the patients who had received ≥80% of the dose, however, the response to PEG-IFN and ribavirin was gained less frequently in women than in men. Again, the

difference was due to a significantly lower response in female patients than in male patients aged ≥50 years, while the response was no different between those <50 years of age.

Taken altogether, the poorer response to PEG-IFN and ribavirin in women than in men was attributable to impaired response in the female patients aged ≥50 years. Older women with chronic hepatitis C, therefore, would be less responsive to the combined treatment with PEG-IFN and ribavirin currently in use. In support of this view, the response to human lymphoblastoid IFN for 24 weeks is dependent on gender and age [28]. The greatest physiological change precipitated in women by aging is a decreased serum concentration of bioavailable estrogen after they enter the menopause [29]. Estrogen has been shown to have an antifibrotic potential in both experimental and clinical studies. In experimental cirrhosis induced by dimethylnitrosamine in rats, administration of neutralizing antibodies to estradiol and ovariectomy enhanced fibrogenesis in female rats [30]. Hepatocytes have the receptor to estrogen [31], and myofibroblastic transformation in hepatic stellate cells of rats is inhibited in culture supplemented with this hormone [32]. Consequently, hepatic fibrosis progresses faster in menopausal women with chronic hepatitis C, and hormone replacement therapy may be able to prevent it [33]. Furthermore, in women aged ≥50 years, the number of estrogen receptor in hepatocytes decreases to one-half of that in those aged <50 years. This would stand in further support of the notion that the antifibrotic effects of decreased estrogen levels in patients aged ≥50 years with chronic hepatitis C would produce a lesser response to PEG-IFN and ribavirin.

Favorable effects of female sex hormones on hepatitis have long been suggested. Chronic hepatitis C is mild in menstruating women [34]; its activity is suppressed during pregnancy and enhanced after delivery [35]. The velocity of fibrosis progression is extremely low in young women exposed to HCV through mass-administration of immunoglobulin-D. Only two of 184 (1.2%) and four of 1,018 (0.4%) developed cirrhosis over 24 years and 20 years, respectively, in Irish and German studies [36, 37]. It does need to be pointed out, however, that the majority of women in those studies had not been followed beyond the menopause. There is a possibility that chronic hepatitis C may progress at a faster speed during their next few decades. Continued observations of them would be necessary to evaluate the validity of such an assumption.

Although decreased levels of estrogen can explain the enhanced activity of chronic hepatitis C in older women, as well as their concomitant resistance to PEG-IFN and ribavirin, it does not give an account of the better response in men than women who were aged ≥50 years. Feminization represented by gynecomastia is common in men

who have developed cirrhosis, and it can increase even in healthy men with age [38]. Possibly in the background of this phenomenon, circulating levels of free estrogen in men exceed those in women, after they enter their fifties, with margins widening with age [29]. It is tempting to speculate that elevated estrogen levels in men with chronic hepatitis C are responsible for their better response to the combination therapy than women who were aged  $\geq 50$  years. Whether or not such a speculation would hold would have to be evaluated by a comparison of estrogen levels between older men and women with chronic hepatitis C.

Although osteoporosis is an extrahepatic manifestation of chronic hepatitis C [39], hormone replacement therapy has been withheld for fear of potential hepatotoxicity. There is evidence, however, that oral contraceptives inhibit the progression of fibrosis in women [33]. It may lead to the possibility that the response to antiviral treatment in older women with chronic hepatitis C would be improved by substituting estrogen in them. The merit of hormone replacement therapy for them, of course, would need to be balanced against any harmful effects associated with it.

There are limitations in this study. All the patients were infected with genotype 1b in high viral loads. Hence, the results obtained may or may not be extended to patients with chronic hepatitis C who are infected with HCV of other genotypes in low viral loads. The influence of sex hormones needs to be substantiated by their determination in correlation with SVR. These limitations notwithstanding, the results obtained warrant a special caution in the treatment of women older than 50 years due to their lesser responsiveness to PEG-IFN and ribavirin.

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# Correlation Between Serum Hepatitis B Virus Core-Related Antigen and Intrahepatic Covalently Closed Circular DNA in Chronic Hepatitis B Patients

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Nucleos(t)ide analogues are utilized for the treatment of chronic HBV infection, and HBe seroconversion and HBV DNA levels are commonly used as markers of viral status and as primary treatment endpoints. Recently, a new assay was prepared for the detection of serum HBV core-related antigen (HBcrAg), consisting of HBcAg, HBeAg, and p22cr, which is a precore protein from amino acid –28 to at least amino acid 150, by coding the precore/core region. In this study, we examined the correlation between serum HBcrAg concentration and viral status by the analysis of serum HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic covalently closed circular DNA (cccDNA) in 57 chronic hepatitis B patients. Intrahepatic cccDNA was detected in all 57 patients, 42 patients were HBcrAg-positive, and serum HBcrAg concentration level was closely correlated with cccDNA. Additionally, positive HBcrAg concentration level results were observed in 6 out of 13 HBsAg seroclearance patients and 20 out of 31 HBV DNA-negative patients. Moreover, the correlation between HBcrAg and cccDNA in these 31 HBV DNA-negative patients was statistically significant ( $r=0.482$ ,  $P=0.006$ ). These data suggest that serum HBcrAg concentration is well correlated with intrahepatic cccDNA level, and that the measurement of serum HBcrAg may be clinically useful for monitoring intrahepatic HBV viral status, especially in patients under treatment with nucleos(t)ide analogues. *J. Med. Virol.* 81:27–33, 2009. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** HBV DNA; HBcrAg; cccDNA; HBsAg; lamivudine; entecavir

## INTRODUCTION

Hepatitis B virus (HBV) is an important causative agent for liver disease such as chronic hepatitis,

cirrhosis, and hepatocellular carcinoma. Recently, several nucleos(t)ide analogues such as lamivudine [Dienstag et al., 1995], adefovir dipivoxil [Chin et al., 2001], and entecavir [Colonna et al., 2001] have been found to consistently produce rapid and dramatic decreases in viremia [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. For the serological monitoring of chronic hepatitis patients under treatment with nucleos(t)ide analogues, improvement of alanine transaminase level, seroconversion from HBe antigen (HBeAg)-positive to anti-HBe antibody (HBeAb)-positive, and peripheral HBV DNA concentration are used as markers in chronic active hepatitis, and both HBeAg seroconversion and HBV DNA levels below the detection limit and/or of  $10^5$  copies/ml are commonly used as primary treatment endpoints [Lok et al., 2004]. In addition, HBV surface antigen (HBsAg) seroclearance has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolongation of survival [Arase et al., 2006], although spontaneous HBsAg seroclearance and/or remission occurred in only a small proportion of patients during the natural history of chronic HBV infections.

However, a major problem with long-term lamivudine treatment is the potential development of drug-resistance, mainly caused by the mutation of the YMDD motif of reverse transcriptase [Chayama et al., 1998]. We previously reported the efficacy of lamivudine therapy and factors associated with the emergence of resistance in chronic HBV infection in Japan [Suzuki et al., 2003].

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The measurement of the predictive serum markers of residual intrahepatic HBV DNA and/or covalently closed circular DNA (cccDNA), which is intrahepatic HBV replicated intermediate, is more important than the measurement of peripheral HBV DNA for monitoring the viral status of hepatitis patients [Sung et al., 2005]. Additionally, the amount of cccDNA in serum is reported to be higher in patients who develop YMDD mutants than in patients who do not [Yuen et al., 2005]. Recently, it was established that HBV RNA is detectable in serum and the elevation of HBV RNA is a predictor of early occurrence of viral mutation during lamivudine therapy [Rokuhara et al., 2006; Hatakeyama et al., 2007]. However, these HBV DNA, HBV RNA, or cccDNA detection assay methods remain complicated and difficult to perform. Therefore, simple methods of viral status evaluation are required for routine assays rather than for nucleic acid assays.

Recently, a new assay was performed for the detection of hepatitis B core-related antigen (HBcrAg) consisting of HBV core antigen (HBcAg), HBeAg, and 22 kDa precore protein (p22cr) coded with precore/core gene [Kimura et al., 2002, 2005]. p22cr is a precore protein from amino acid -28 to at least amino acid 150, containing an uncleaved signal sequence and lacking the C-terminal arginine-rich domain. p22cr is found in empty and HBV DNA negative virus particles; the production of empty particles is not dependent on the formation of HBV DNA [Kimura et al., 2005]. Several reports indicate that the concentration of serum HBcrAg is closely correlated with peripheral HBV DNA in untreated patients [Rokuhara et al., 2003; Tanaka et al., 2006]. Additionally, HBcrAg is considered as a prospective marker of the appearance of drug-resistant HBV mutants and of the identification of patients with low risk of HBV reactivation after discontinuation of lamivudine administration, while peripheral HBV DNA does not qualify as a prospective marker in these patients [Rokuhara et al., 2005; Shinkai et al., 2006; Tanaka et al., 2006; Matsumoto et al., 2007]. The relationship between HBcrAg and intrahepatic cccDNA levels has not yet been clarified.

In this study, we examined the correlation between HBcrAg and viral status by the analysis of HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic cccDNA in patients with chronic hepatitis B.

## MATERIALS AND METHODS

### Patients and Samples

Serum samples and biopsy specimens were obtained from 57 chronic hepatitis B patients at Toranomon Hospital under informed consent. The median age of patients was 49 (range, 25–71 years). Out of 57 patients, 28 underwent nucleos(t)ide analogue administration (17 patients of lamivudine, 7 patients of both lamivudine and adefovir dipivoxil, 4 patients of entecavir), and 13 were HBsAg-negative/HBs-seroclearance patients with more than 12 months of being HBsAg-positive before HBs-seroclearance.

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## Routine Laboratory Tests

HBsAg, HBeAg, and HBeAb were routinely measured by the commercially available Chemiluminescent Enzyme Immunoassay (CLEIA) (Lumipulse System, Fujirebio, Inc., Tokyo, Japan).

### HBcrAg Test

Serum HBcrAg was measured by CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, Fujirebio, Inc.). Briefly, 150  $\mu$ l of serum was incubated with 150  $\mu$ l of pretreatment solution containing 15% sodium dodecyl sulfate at 60°C for 30 min. After heat treatment, 120  $\mu$ l of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61, and HB114) against denatured HBcAg, HBeAg, and p22cr. After 10 min incubation at 37°C and washing, further incubation was done for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and p22cr. After washing, 200  $\mu$ l of substrate solution [AMPPD: 3-(2'-spiroadamantan)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) was added to the test cartridge which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids: -10 to 183 of precore/core gene product). HBcrAg concentration was expressed as units/ml (U/ml), which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, HBcrAg value was expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analysis, HBcrAg-negative cases were calculated as 3.0 log U/ml.

### HBV DNA Assay

HBV DNA in serum was measured by polymerase chain reaction (PCR) assay kit (Amplicor HBV monitor test, Roche Molecular Systems, Inc., Branchburg, NJ). Values under or over the detection range were calculated as 2.6 log copies/ml or as 7.6 log copies/ml, respectively.

### Measurement of cccDNA

Liver biopsy specimens were taken and stored at -80°C before DNA extraction. HBV DNA was extracted using QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on absorbance at 260 nm. For this study, two oligonucleotide primers, cccF2 (5'-cgtctgtgcccctctcatctga-3', nucleotides 1,424–1,444), cccR4 (5'-gcacagcttgaggcttgaa-3', nucleotides 1,755–1,737), and a probe cccP2 (5'-VIC-accattatgctacag-MGB-3', nucleotides 1,672–1,655), were designed using Primer Express™ software (Applied Biosystems, Foster City, CA) to flank the direct

repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4 oligonucleotide primers spanning the direct repeat region of the HBV genome allows the PCR of native viral DNA in the Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region [Mason et al., 1998]. Twenty-five microliters of the extracted DNA (0.5 µg) were detected with the sequence detector system (ABI 7900HT, Applied Biosystems) in 50 µl of a PCR mixture containing TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer, and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50°C for 2 min, AmpliTaq Gold was activated at 95°C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 90 sec per cycle (SRL, Inc., Tokyo, Japan).

**Statistical Analysis**

The statistical analysis of the correlation data between serum HBcrAg, HBsAg, HBV DNA, and/or cccDNA was performed by SPSS software (version 14.0J, SPSS Japan Inc., Tokyo, Japan), and the statistical significance between the two sides was taken as *P*-value lower than 0.05.

**RESULTS**

**Serological and Genetic Assay Results**

We classified the 57 patients according to assay results of HBsAg, HBeAg, serum HBV DNA, intrahepatic HBV cccDNA, and serum HBcrAg. Positive results were observed with all 57 patients in the cccDNA assay, in 44 patients with the HBsAg test, in 16 patients with the HBeAg test, in 26 patients with the HBV DNA assay, and in 42 patients with the HBcrAg assay (Table I). Among the 13 patients with negative results with HBsAg, HBeAg, and HBV DNA but positive results with cccDNA assay, six patients showed HBcrAg-positive results, although the serum HBcrAg concentration value was low (mean value ± standard deviation: 3.23 ± 0.27 log U/ml) in comparison to that of the group with positive results with HBsAg, HBeAg, and HBV DNA (6.91 ± 1.06 log U/ml) tests. Among the 28 patients with HBsAg-positive but HBeAg-negative results, 20 patients were HBcrAg-positive and 10 out of these 20 patients showed negative HBV DNA assay results.

Next, assay results were analyzed according to presence/absence of nucleos(t)ide analogue treatment, HBsAg-positive/negative results, and HBeAg-positive/negative results by the combination with treated/untreated subgroups (Table II). When patients were classified into two groups, namely 28 patients treated with nucleos(t)ide analogues and 29 untreated patients, no difference was observed in average mean value of HBcrAg, HBV cccDNA, and HBV DNA (data not shown). However, since the 13 HBsAg-negative patients were clinically stable, we further analyzed the 44 HBsAg-positive patients by grouping them according to pres-

TABLE I. Summary of HBcrAg Concentration in Positive and/or Negative Patients by HBsAg, HBeAg, HBV DNA, and HBcrAg Assay

All cases	HBsAg			HBeAg			HBV DNA			HBcrAg		
	Results (number)	HBcrAg (±SD)	Results (number)	Results (number)	HBcrAg (±SD)	Results (number)	Results (number)	HBcrAg (±SD)	Results (number)	Results (number)	HBcrAg (±SD)	
All (N = 57)	4.61 (1.64)	5.05 (1.62)	Positive (N = 44)	6.53 (1.14)	6.91 (1.06)	Positive (N = 12)	Positive (N = 12)	6.91 (1.06)	Positive (N = 12)	Positive (N = 12)	6.91 (1.06)	
						Negative (N = 4)	Negative (N = 4)	5.40 (0.38)	Negative (N = 0)	Negative (N = 0)	5.40 (0.38)	
							Positive (N = 14)	4.29 (1.20)	Positive (N = 10)	Positive (N = 10)	4.81 (1.02)	
							Negative (N = 14)	4.11 (1.20)	Negative (N = 4)	Negative (N = 4)	<3.00	
							Positive Negative (N = 0)		Positive Negative (N = 0)	Positive Negative (N = 0)	4.55 (1.14)	
							Positive (N = 0)		Positive Negative (N = 0)	Positive Negative (N = 0)	<3.00	
							Positive (N = 0)		Positive Negative (N = 6)	Positive Negative (N = 6)	3.23 (0.27)	
							Negative (N = 13)	3.11 (0.21)	Negative (N = 7)	Negative (N = 7)	<3.00	

Mean value: log U/ml ± Standard deviation.