Table 2 Initial drug doses, drug adherence up to 24 weeks and discontinuation rates up to 12 weeks

	TVR 2250 mg/day	TVR 1500 mg/day	*P*-value
n	60	60	
Initial TVR dose (mg/kg per day)	38.1 (33.6–45.1)	25.6 (22.5–29.6)	<0.001
TVR adherence up to 12 weeks (%)	100 (75–100)	67 (65–67)	<0.001
Discontinuation of TVR	15 (25.0%)	6 (10.0%)	0.053
Discontinuation of TVR due to anemia	12 (20%)	3 (5%)	0.025
Initial PEG IFN dose (μg/kg per week)	1.5 (1.4–1.6)	1.5 (1.4–1.6)	0.706
PEG IFN adherence up to 24 weeks (%)	100 (85–100)	100 (89–100)	0.062
Initial RBV dose (mg/kg per day)	11.6 (10.6–12.8)	9.9 (7.9–11.3)	<0.001
RBV adherence up to 24 weeks (%)	51 (41–61)	59 (46–68)	0.090
Discontinuation of all drugs up to 12 weeks	5 (8.3%)	1 (1.7%)	0.207

Values are number with percentage in parentheses or median with interquartile range in parentheses.
PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir.

group than in the 2250 mg/day group, while there were no differences in adherence for the other two drugs. Although there were no significant differences between the groups in the rates of discontinuation of telaprevir or all drugs up to 12 weeks, the rates of discontinuation of telaprevir due to anemia in the 1500 mg/day group were significantly lower than in 2250 mg/day group.

Loss of serum HCV RNA according to *IL28B* genotypes

Figure 1 compares the on-treatment virological response over the first 12 weeks for the telaprevir 2250 and 1500 mg/day groups according to *IL28B* genotypes, respectively, because there were significant differences in distribution of *IL28B* genotypes between both groups.

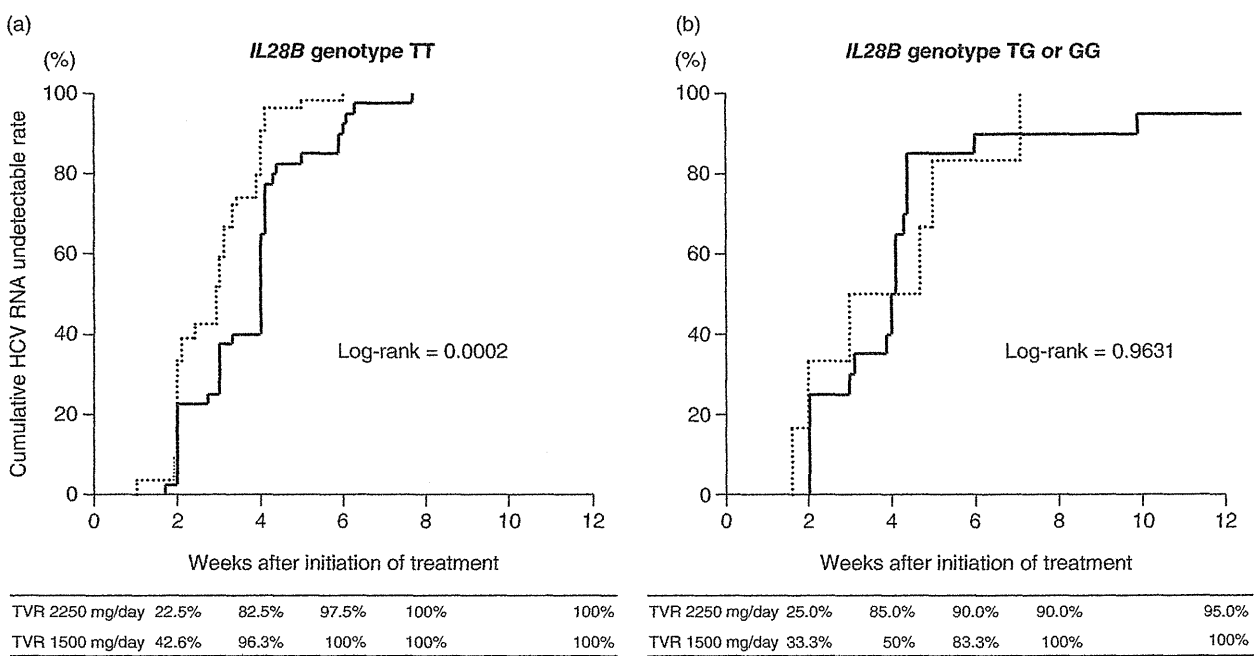
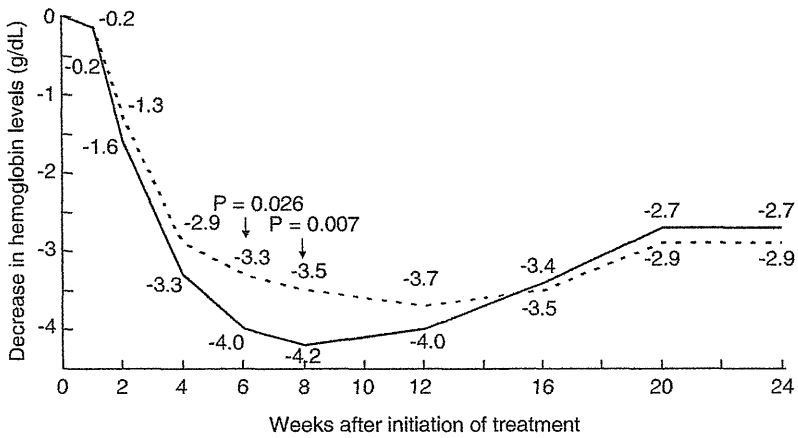


Figure 1 Cumulative rate of undetectable hepatitis C virus (HCV) RNA during triple therapy with pegylated interferon, ribavirin and telaprevir (TVR) at either 2250 mg/day or 1500 mg/day. (a) *IL28B* genotype TT, (b) *IL28B* genotype TG or GG. (———) TVR 2250 mg/day, (·····) TVR 1500 mg/day.



Number of patients (TVR withdrawn)

2250 mg/day	60	60	60 (1)	59 (4)	55 (10)	55	55	55
1500 mg/day	60	60	60 (1)	59 (2)	59 (3)	59	59	59

Figure 2 Decreases in hemoglobin levels during triple therapy with pegylated interferon (PEG IFN), ribavirin (RBV) and telaprevir (TVR) at either 2250 mg/day or 1500 mg/day. Each time point in this figure corresponds to median values. Patients evaluated at each time point are indicated below, with the number of patients who discontinued TVR (continued PEG IFN and RBV) in parentheses. (———) TVR 2250 mg/day, (·····) TVR 1500 mg/day.

Triple therapy suppressed HCV RNA levels quickly and effectively in both groups. In the 2250 and 1500 mg/day groups of *IL28B* genotype TT, HCV RNA became undetectable in 22.5% and 42.6% of patients at 2 weeks, 82.5% and 96.3% at 4 weeks, and 100% and 100% at 8 weeks, respectively (Fig. 1a). The early virological response of the telaprevir 1500 mg/day group was significantly higher than that of the 2250 mg/day group in *IL28B* genotype TT (log-rank test = 0.0002).

In the subgroups of *IL28B* genotype non-TT patients receiving telaprevir 2250 and 1500 mg/day, HCV RNA became undetectable in 25.0% and 33.3% of patients at 2 weeks, 85.0% and 50% at 4 weeks, 90.0% and 100% at 8 weeks, and 95.0% and 100% at 12 weeks, respectively. The virological responses during the first 12 weeks in this subgroup of patients did not significantly differ between the telaprevir 2250 and 1500 mg/day groups (log-rank test = 0.9631, Fig. 1b).

Safety

Figure 2 shows the decreases in hemoglobin levels in telaprevir 2250 and 1500 mg/day recipients. Data from six patients were omitted (five receiving telaprevir 2250 mg/day and one receiving 1500 mg/day) because treatment was withdrawn between 8 and 12 weeks after initiation. Telaprevir was discontinued in 15 of the 60 (25.0%) patients receiving telaprevir 2250 mg/day (one at week 6, four at week 8 and 10 at week 12) and six of the 60 (10.0%) receiving 1500 mg/day (one at week 6, two at week 8 and three at week 12). Hemoglobin decreased to a greater extent in patients receiving telaprevir 2250 mg/day than in those receiving 1500 mg/day at week 6 (−4.0 [−6.7 to −1.2] vs −3.3 [−5.2 to 0.2] g/dL, $P = 0.026$) and week 8 (−4.2 [−7.7 to −1.3] vs −3.5 [−6.9 to −1.3] g/dL, $P = 0.007$).

Skin disorder frequency was comparable between the telaprevir 2250 mg/day group and 1500 mg/day group (81.7% and 75%, respectively). However, skin disorders of grades 2–3 occurred more frequently in the telaprevir 2250 mg/day group than in the 1500 mg/day group (55% vs 35%, $P = 0.043$).

With respect to renal dysfunction, increases in serum creatinine (sCR) levels during therapy were not significantly different between both groups. However, blood uric acid levels increased to a greater extent in patients receiving telaprevir 2250 mg/day than in those receiving 1500 mg/day at week 1 (1.3 [−1.6 to 4.8] vs 0.9 [−2.1 to 4.3] g/dL, $P = 0.015$), week 2 (1.2 [−2.3 to 4.1] vs 0.5 [−2.3 to 2.7] g/dL, $P = 0.004$), week 4 (1.6 [−1.1 to 5.5] vs 0.7 [−2.4 to 3.8] g/dL, $P < 0.001$), week 6 (1.6 [−1.7 to 4.8] vs 0.5 [−3.5 to 3.6] g/dL, $P < 0.001$) and week 8 (1.1 [−3.6 to −4.9] vs 0.7 [−1.6 to 3.7] g/dL, $P = 0.029$).

Predictive factors associated with SVR

The overall SVR rate was 83% (169/204) in our hospital. SVR was accomplished in 106 (88%) of 120 patients selected for this study, including 50 of 60 (83%) patients in the telaprevir 2250 mg/day and 56 of 60 (93%) patients in telaprevir 1500 mg/day groups (Fig. 3).

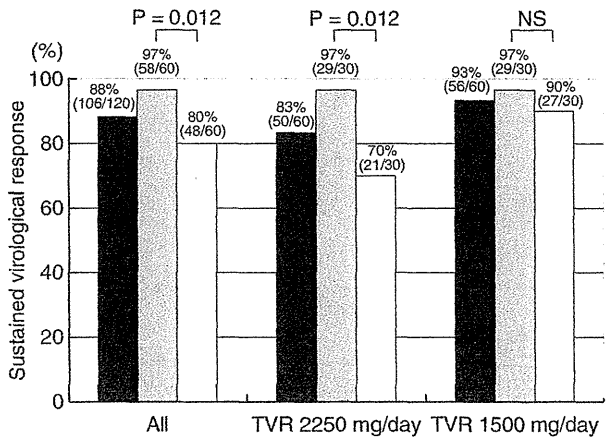


Figure 3 Sustained virological response in patients with chronic hepatitis C to triple therapy with telaprevir (TVR), pegylated interferon and ribavirin for 24 weeks. Sustained virological response was compared among all patients (men and women), TVR 2250 mg/day patients and TVR 1500 mg/day patients, respectively. (■) Total, (□) male, (□) female.

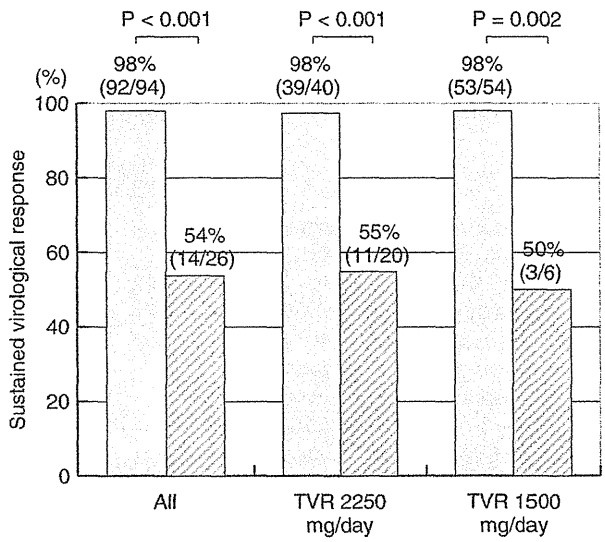


Figure 4 Sustained virological response in patients with chronic hepatitis C to triple therapy with telaprevir (TVR), pegylated interferon and ribavirin for 24 weeks. Sustained virological response was compared between *IL28B* (rs8099917) genotype TT and TG/GG in all patients, TVR 2250 mg/day patients and TVR 1500 mg/day patients, respectively. (□) TT, (□) TG or GG.

Significant univariate predictors for SVR included male sex, *IL28B* genotype TT, and HCV core a.a. 70 wild type, except for null response to prior treatment, initial telaprevir dose of 37.5 mg/kg per day or more, telaprevir dosing period of 10 weeks or more, 100% PEG IFN adherence up to 24 weeks, PEG IFN adherence up to 12 weeks of 80% or more, RBV adherence up to 12 weeks of 50% of more, γ-glutamyltransferase of 35 IU/mL or less, and sCr of 0.6 mg/dL or more ($P < 0.05$). Of these, male sex (odds ratio [OR] = 13.7; $P = 0.028$) and *IL28B* genotype TT (OR = 44.4; $P = 4.47 \times 10^{-5}$) were identified as significant independent predictors for SVR (Table 3).

Therefore, we assessed the SVR rate of triple therapy according to sex and *IL28B* genotype. SVR was much less frequent in women than in men (48/60 [80%] vs 58/60 [97%], $P = 0.0012$, Fig. 3). Especially, in the telaprevir 2250 mg/day group, there were significant differences between men and women (29/30 [97%] vs 21/30 [70%], $P = 0.0012$). However, there were no differences between men and women in the telaprevir 1500 mg/day group (29/30 [97%] and 27/30 [90%], respectively).

Patients with *IL28B* genotype TT were significantly more likely to achieve SVR (92/94 [98%] vs 14/26 [54%], $P < 0.001$, Fig. 4), compared with patients with TG or GG genotypes. There were significant differences between *IL28B* genotype TT and non-TT in both the telaprevir 2250 and 1500 mg/day groups (39/40 [98%] vs 11/20 [55%], $P < 0.001$ and 53/54 [98%] vs 3/6 [50%], $P = 0.002$, respectively).

Table 3 Multivariate analysis of factors associated with sustained virological response of TVR, pegylated interferon and ribavirin triple therapy in Japanese patients infected with HCV

Factor	Category	Odds ratio (95% CI)	*P*-value
Sex	1; female	1	
	2; male	13.7 (1.33–141.2)	0.028
IL28B genotype (rs8099917)	1; TG or GG	1	
	2; TT	44.4 (7.18–274.2)	4.47×10^{-5}

CI, confidence interval; HCV, hepatitis C virus; TVR, telaprevir.

DISCUSSION

IN JAPANESE PATIENTS, virological response to triple therapy with telaprevir, PEG IFN and RBV was excellent. We have previously reported that in 20 patients with chronic HCV-1b infection with high viral load who received triple therapy for 12 weeks, HCV RNA became undetectable in 50% at 2 weeks, 79% at 4 weeks, 88% at 6 weeks, 94% at 8 weeks and 100% at 12 weeks.[26] This previous study was a randomized open-label study in which telaprevir was administrated at doses of 2250 or 1500 mg/day. Early virological response at 7 and 14 days was similar for both telaprevir doses, suggesting that virological response to triple therapy is not affected by lowering the telaprevir dose. Therefore, to expand the dataset, we retrospectively evaluated HCV RNA response and safety during 12 weeks of triple therapy including the two different telaprevir doses followed by PEG IFN and RBV for an additional 12 weeks: we analyzed 204 cases in total. However, because of the non-random nature of treatment allocation, there was a preponderance of women, elderly and anemic patients in the group receiving telaprevir 1500 mg/day. Because there were many differences in baseline characteristics between telaprevir 2250 and 1500 mg/day groups, we selected 60 patients per group who were matched by age, sex and history of previous IFN-based treatment. Therefore, there were no differences in baseline characteristics between both groups in this analysis, except for *IL28B* genotype. Although we tried to match the distribution of *IL28B* genotypes between both groups, this was not possible because of the small number of cases. Therefore, we matched the groups by the history of previous IFN-based treatment, which we considered a similarly strong predictive factor of triple therapy. Moreover, there was a significant difference in the initial dose of RBV between both groups. A significant number of patients underwent RBV dose reductions at the beginning of treatment in the telaprevir 1500 mg/day group because we considered that such patients were likely to experience hemoglobin decrements during triple therapy, but before November 2011, we could not reduce the initial dose of telaprevir and RBV. Nine patients (15.0%) receiving telaprevir 2250 mg/day and 32 cases (53.3%) receiving 1500 mg/day underwent RBV dose reduction at the beginning of treatment. In other words, the group receiving telaprevir 1500 mg/day had a significantly lower initial dose of telaprevir and RBV dose than did the group receiving 2250 mg/day (Table 2).

However, in the present study, HCV RNA became undetectable during the 12 weeks of treatment at similar or higher rates in the telaprevir 1500 mg/day group than in the 2250 mg/day group (Fig. 1). In the *IL28B* TT genotype, the early virological response of the telaprevir 1500 mg/day group was significantly higher than that of the 2250 mg/day group. Although we assessed baseline factors, drug adherence and drug discontinuation rates only in the *IL28B* TT genotype, there were no significant differences between both groups, except for lower telaprevir adherence up to 12 weeks and a greater number of cases of PEG IFN and RBV dose reductions at the beginning of treatment in the telaprevir 1500 mg/day group. Therefore, the reason for significant differences in the early virological response between both groups is unclear. However, we considered that these results did not affect the SVR rate because HCV RNA became undetectable in all patients in both groups at 8 weeks after the start of triple therapy. In all cases, *IL28B* TT cases and non-TT cases, there were no significant differences in SVR rates after triple therapy between those receiving telaprevir 2250 and 1500 mg/day (Figs 3,4). By examining the detailed course of drug administration from 12–24 weeks (Table 2), we found that the group receiving telaprevir 1500 mg/day had a lower discontinuation rate of telaprevir and higher adherence to RBV and PEG IFN up to 24 weeks in spite of the low initial RBV dose. Furthermore, hemoglobin levels showed greater reductions during triple therapy with telaprevir 2250 mg/day than with telaprevir 1500 mg/day, and the group receiving telaprevir 2250 mg/day had a significantly higher discontinuation rate of telaprevir due to anemia than did the group receiving telaprevir 1500 mg/day (Fig. 2). Therefore, telaprevir 1500 mg/day may be a safe option as part of triple therapy, while maintaining PEG IFN and RBV adherence.

Viral breakthrough or relapse can occur during telaprevir monotherapy or telaprevir plus PEG IFN dual therapy (without RBV) because of the development of mutations that confer resistance to telaprevir.[14,27–29] Furthermore, in a Japanese phase III trial of triple therapy in relapsers and non-responders who had not achieved SVR to a previously administrated IFN-based regimen, SVR rates increased as RBV adherence increased, particularly in previous non-responders.[19] In triple therapy with telaprevir, PEG IFN and RBV, we consider that telaprevir could be important for early virological response, but it could also be important for maintaining high adherence to PEG IFN and RBV, which is a key factor for achieving SVR. We speculate that triple therapy including telaprevir at the reduced dose of 1500 mg/day could maintain high levels of adherence

to PEG IFN and RBV, and consequently achieve high SVR rates.

In this study, we investigated the independent predictors for SVR in the multivariate analysis (Table 3). As reported in previous studies, *IL28B* genotype remained the strongest predictor of SVR.[30,31] The next strongest predictive factor was sex: women had significantly lower SVR rates than did men (Fig. 3). However, when we investigated the SVR rates of the telaprevir 2250 mg/day group and 1500 mg/day group, we found that there were significant differences in SVR rates between men and women in the telaprevir 2250 mg/day group but no differences in the telaprevir 1500 mg/day group. In the previous study, we reported that female sex was one of the factors influencing decreases in hemoglobin levels during triple therapy administrated 2250 mg/day of initial telaprevir dose.[20] In the present study, the discontinuation rates of telaprevir due to anemia were significantly higher in women in the telaprevir 2250 mg/day group as compared with men (36.7% vs 3.3%, $P = 0.002$, data not shown), but there were no differences in the discontinuation rates of telaprevir due to anemia between men and women in the telaprevir 1500 mg/day group (0% vs 10%, $P = 0.237$, data not shown). Therefore, we speculate that there were significant differences in SVR rates between men and women because of high telaprevir discontinuation rates owing to anemia in women.

In conclusion, after the completion of 24 weeks of therapy, triple therapy including telaprevir at a reduced dose of 1500 mg/day was as effective as triple therapy including telaprevir 2250 mg/day at suppressing HCV RNA to undetectable levels and achieving SVR. Of note, we found that telaprevir 1500 mg/day was associated with lower levels of anemia and discontinuation of telaprevir owing to anemia, and higher PEG IFN and RBV adherence during triple therapy. These results suggest that the telaprevir 1500 mg/day regimen is an effective and safe alternative for the treatment of elderly and female Japanese patients. This study is a retrospective study. Prospective randomized controlled studies with longer follow-up periods are required to fully assess the efficacy and safety of an initial telaprevir dose of 1500 mg/day.

ACKNOWLEDGMENT

THIS STUDY WAS supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

1 Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41–52.
2 Alberti A, Chemello L, Benvegnu L. Natural history of hepatitis C. *J Hepatol* 1999; **31** (Suppl 1): 17–24.
3 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**: S35–46.
4 Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335–74.
5 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis c virus infection. *N Engl J Med* 2002; **347**: 975–82.
6 Hadziyannis SJ, Sette H, Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346–55.
7 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.
8 Kanwal F, Hoang T, Spiegel BM *et al.* Predictors of treatment in patients with chronic hepatitis C infection – role of patient versus nonpatient factors. *Hepatology* 2007; **46**: 1741–9.
9 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The obsvirc, metavir, clinivir, and dosvirc groups. *Lancet* 1997; **349**: 825–32.
10 Conjeevaram HS, Fried MW, Jeffers LJ *et al.* Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. *Gastroenterology* 2006; **131**: 470–7.
11 Tsubota A, Chayama K, Ikeda K *et al.* Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology* 1994; **19**: 1088–94.
12 Sezaki H, Suzuki F, Kawamura Y *et al.* Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig Dis Sci* 2009; **54**: 1317–24.
13 Lin C, Kwong AD, Perni RB. Discovery and development of vx-950, a novel, covalent, and reversible inhibitor of hepatitis C virus ns3.4a serine protease. *Infect Disord Drug Targets* 2006; **6**: 3–16.
14 Hezode C, Forestier N, Dusheiko G *et al.* Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009; **360**: 1839–50.
15 McHutchison JG, Everson GT, Gordon SC *et al.* Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009; **360**: 1827–38.
16 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405–16.

17 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417–28.
18 Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012; **56**: 78–84.
19 Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. *J Viral Hepat* 2012; **19**: e134–142.
20 Suzuki F, Suzuki Y, Akuta N *et al.* Influence of itpa polymorphisms on decreases of hemoglobin during treatment with pegylated interferon, ribavirin, and telaprevir. *Hepatology* 2011; **53**: 415–21.
21 Yoshizawa H, Tanaka J, Miyakawa Y. National prevention of hepatocellular carcinoma in Japan based on epidemiology of hepatitis C virus infection in the general population. *Intervirology* 2006; **49**: 7–17.
22 Akuta N, Suzuki F, Kawamura Y *et al.* 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* 2007; **46**: 403–10.
23 Akuta N, Suzuki F, Sezaki H *et al.* 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* 2006; **78**: 83–90.
24 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput snp typing system for genome-wide association studies. *J Hum Genet* 2001; **46**: 471–7.
25 Suzuki A, Yamada R, Chang X *et al.* Functional haplotypes of padi4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003; **34**: 395–402.
26 Suzuki F, Akuta N, Suzuki Y *et al.* Rapid loss of hepatitis C virus genotype 1b from serum in patients receiving a triple treatment with telaprevir (mp-424), pegylated interferon and ribavirin for 12 weeks. *Hepatol Res* 2009; **39**: 1056–63.
27 Reesink HW, Zeuzem S, Weegink CJ *et al.* Rapid decline of viral RNA in hepatitis C patients treated with VX-950: A phase 1b, placebo-controlled, randomized study. *Gastroenterology* 2006; **131**: 997–1002.
28 Sarrazin C, Kieffer TL, Bartels D *et al.* Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007; **132**: 1767–77.
29 Lawitz E, Rodriguez-Torres M, Muir AJ *et al.* Antiviral effects and safety of telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in hepatitis C patients. *J Hepatol* 2008; **49**: 163–9.
30 Chayama K, Hayes CN, Abe H *et al.* IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. *J Infect Dis* 2011; **204**: 84–93.
31 Akuta N, Suzuki F, Hirakawa H *et al.* Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology* 2010; **57**: 421–9.

Hepatology Research

Hepatology Research 2013; 43: 557–562 doi: 10.1111/j.1872-034X.2012.01091.x

Short Communication

Prediction of a favorable clinical course in hepatitis C virus carriers with persistently normal serum alanine aminotransferase levels: A long-term follow-up study

Takeshi Nishimura,[1] Kanji Yamaguchi,[1] Hideki Fujii,[1] Yorihisa Okada,[1] Chihiro Yokomizo,[1] Toshihisa Niimi,[1] Yoshio Sumida,[1] Kohichiroh Yasui,[1] Hironori Mitsuyoshi,[1] Masahito Minami,[1] Atsushi Umemura,[1] Toshihide Shima,[2] Takeshi Okanoue[2] and Yoshito Itoh[1]

[1]*Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, and* [2]*Hepatology Center, Saiseikai Suita Hospital, Osaka, Japan*

Aim: This study examined serum alanine aminotransferase (ALT) levels at first visit and their relationship with long-term normal serum ALT levels in hepatitis C virus (HCV) carriers with persistently normal ALT (PNALT).

Methods: HCV carriers with PNALT were identified as those patients with positivity of serum HCV RNA, ALT levels of 30 IU/L or less over a 12-month period on at least three different occasions, platelet count of more than 15×10^4 μl/mL and body mass index of 30 kg/m^2 or less. Outcome was retrospectively studied in 49 HCV carriers with PNALT, who were followed up for more than 10 years.

Results: During the mean follow-up period of 14.7 ± 2.5 years, ALT levels of 30 IU/L or less were preserved in only eight patients (8/49; 16.3%). Among the 17 patients with initial ALT levels of 19 IU/L or less, nine patients remained with ALT levels of 30 IU/L or less after 10 years (9/17; 52.9%). The probability of ALT levels in PNALT being maintained at 30 IU/L or less was significantly higher ($P = 0.001$) in these patients than in those with initial ALT levels of 20 IU/L or more ($n = 32$). Abnormal ALT levels were more common in female PNALT patients aged 45–55 years, which is usually the time of menopause onset.

Conclusion: Because antiviral therapy in the treatment of chronic hepatitis C is rapidly advancing, waiting for more effective and safer treatments may be an option. The results of this study provide an important insight into this issue.

Key words: alanine aminotransferase threshold, hepatitis C virus carriers with persistently normal alanine aminotransferase, long-term follow up

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major public health concern worldwide. Antiviral therapy to eradicate HCV has progressed.[1,2] Currently, peginterferon (PEG IFN) and ribavirin (RBV) combination therapy is widely used to treat chronic hepatitis C, and triple therapy with a protease inhibitor, telaprevir, is also available.[3–5] However, some physicians are reluctant to treat patients using IFN-based therapy because of the development of new therapies, some of which may be more effective and safer.[6]

Compared with fibrosis progression in patients with elevated transaminase levels, HCV carriers with persistently normal alanine aminotransferase (PNALT) and mild liver fibrosis are unlikely to develop severe fibrosis,[7–10] whereas only some reports presented dissimilar results.[11,12] A report at the consensus meeting of the Japan Society of Hepatology held in 2009 concluded that the progression of hepatic fibrosis in HCV carriers with PNALT is generally slow.[2]

Sustained viral response (SVR) rates of HCV carriers with PNALT are similar to those of patients with elevated transaminase levels.[13,14] The decision to utilize IFN-based therapies should be determined not by ALT

Correspondence: Dr Yoshito Itoh, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kawaramachi-Hirokouji, Kamigyou-ku, Kyoto 602-8566, Japan. Email: yitoh@koto.kpu-m.ac.jp
Received 24 April 2012; revision 15 August 2012; accepted 19 August 2012.

values but by the patient's physical condition, probability of successful therapy or prolonged survival, and likelihood of serious adverse effects.[1,2]

Prediction of ALT abnormality in patients with PNALT may be helpful in determining treatment timing, namely, immediately or 2–3 years later, taking into account the probability of hepatocellular carcinoma (HCC) occurrence.[15] The present retrospective study addressed this issue by evaluating outcome in HCV carriers with PNALT who were followed up for more than 10 years.

METHODS

Patients and follow-up study

WE HAVE REPORTED a follow-up study (>5 years) of 69 patients among the 129 HCV carriers with PNALT.[10] In the present study, 49 HCV carriers with PNALT, in whom follow up was possible every 3–6 months, in principle, at our outpatient clinic for more than 10 years, were retrospectively studied. All 49 patients belonged to the previous study[10] and 16 patients who showed ALT levels of 30 IU/L or more before 10 years follow up were treated with PEG IFN-α-2b and RBV (Shering-Plough, Kenilworth, NJ, USA). Other patients with ALT levels of 30 IU/L or more were followed or treated with ursodeoxycholic acid. The other 80 patients in the previous study[10] were excluded from this study because they were lost to follow up before 10 years or received IFN-based therapy while the ALT levels were 30 IU/L or less. The end-points of follow up in this study are ALT elevation of 30 IU/L or more or last visit to our hospital (≥10 years from the first visit).

Hepatitis C virus carriers with PNALT were identified as those patients with positivity of serum HCV RNA, serum ALT levels of 30 IU/L or less over a 12-month period on at least three different occasions, platelet counts of more than 15×10^4 μl/mL, body mass index (BMI) of 30 kg/m^2 or less, and no evidence of oral contraceptive, co-infection with HIV or known liver disease other than hepatitis C.

Liver biopsy was performed using a Menghini needle guided by ultrasound. Liver biopsy specimens were fixed in 10% formalin and stained with hematoxylin–eosin and Masson-trichrome. Histopathological diagnosis was based on the scoring of the New Inuyama Classification.[16] Evaluation was performed by two expert hepatologists who were blinded to the clinical data of the patients.

This study was a retrospective sub-analysis of the study entitled "Analysis of the pathophysiology of HCV carriers with persistent normal ALT levels", which was approved by the ethics committee of the university and conformed to the provisions of the Declaration of Helsinki.

Statistical analysis

All data analyses were performed using SPSS statistical software (ver. 17.0; SPSS, Chicago, IL, USA). Individual characteristics were presented as means ± standard deviations and compared by Mann–Whitney *U*-test or Pearson's χ^2-test. Receiver–operator curve (ROC) analysis was performed, followed by proper categorization of the data. Probability of PNALT maintenance was determined using the Kaplan–Meier method and analyzed using the log–rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of PNALT

CLINICAL CHARACTERISTICS OF the HCV carriers with PNALT are summarized in Table 1. We inves-

Table 1 Clinical characteristics of the 49 HCV carriers with PNALT at first visit

Follow-up period (years)	14.7 ± 2.5
Age (years)	
Male ($n = 4$)	34.8 ± 5.9
Female ($n = 45$)	48.0 ± 11.2
ALT (IU/L)	
Male ($n = 4$)	16.8 ± 4.7
Female ($n = 45$)	21.9 ± 5.3
PLT ($\times 10^4/\mu L$)	
Male ($n = 4$)	20.3 ± 4.7
Female ($n = 45$)	21.5 ± 4.7
BMI (kg/m^2)	
Male ($n = 4$)	20.3 ± 1.5
Female ($n = 45$)	21.3 ± 2.5
Genotype (G1/G2/ND)	25/16/8
Liver histology	
Male (F0/F1/F2/F3/F4)	3/1/0/0/0
(A0/A1/A/2/A3)	1/3/0/0
Female (F0/F1/F2/F3/F4)	11/32/2/0/0
(A0/A1/A2/A3)	2/39/4/0

Data are presented as means ± standard deviations.
Liver histology was classified based on New Inuyama Classification.[16]
ALT, alanine aminotransferase; BMI, body mass index; G1, genotype 1; G2, genotype 2; HCV, hepatitis C virus; ND, not determined; PLT, platelets; PNALT, persistently normal alanine aminotransferase.

tigated whether or not the patients who maintained normal ALT levels (≤30 IU/L) for 10 years or more ($n = 8$) are significantly different from those who did not ($n = 41$) in clinical characteristics. We revealed no significant differences in age ($P = 0.109$), platelet count ($P = 0.371$), BMI ($P = 0.989$), hemoglobin concentration ($P = 0.549$), HCV load ($P = 0.712$), HCV genotype (1 or 2; $P = 0.495$), serum ferritin ($P = 0.710$), hepatic fibrosis score (F0/1,2) ($P = 0.588$), hepatic activity score (A0/1,2) ($P = 0.421$) or iron deposition (positive or negative; $P = 0.251$, $n = 20$). Only the initial ALT levels were significantly lower in patients who maintained normal ALT levels (≤30 IU/L) for 10 years or more ($P = 0.003$).

Initial ALT values and clinical outcome of patients with PNALT

To estimate a cut-off initial ALT level predicting the maintenance of ALT of 30 IU/L or less, the ROC analysis was performed (Fig. 1). The result revealed that 19.5 IU/L was an optimal ALT level predicting the maintenance of ALT of 30 IU/L or less, because it achieved the highest sensitivity (0.756%) and specificity (0.875%), yielding an area under the curve of 0.83 and *P*-value of 0.003.

Among the 17 patients with initial ALT levels of 19 IU/L or less, nine patients remained at ALT levels of 30 IU/L or less after 10 years (52.9%) (Fig. 2). The probability of ALT levels being maintained at 30 IU/L or less was significantly higher ($P = 0.001$) in these patients than in those with initial ALT levels of 20 IU/L or more ($n = 32$).

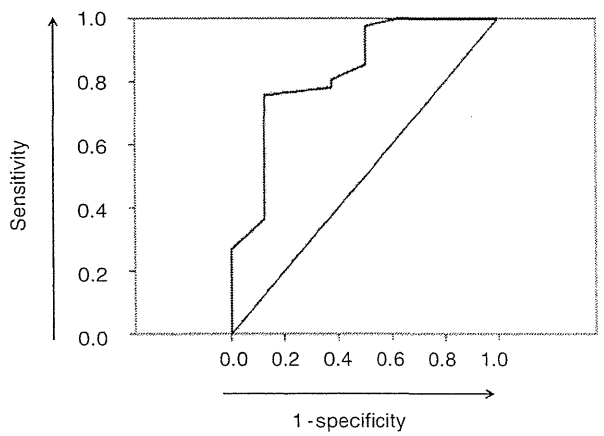


Figure 1 Receiver–operator curve analysis of the relationship between initial alanine aminotransferase (ALT) values and maintenance of normal ALT.

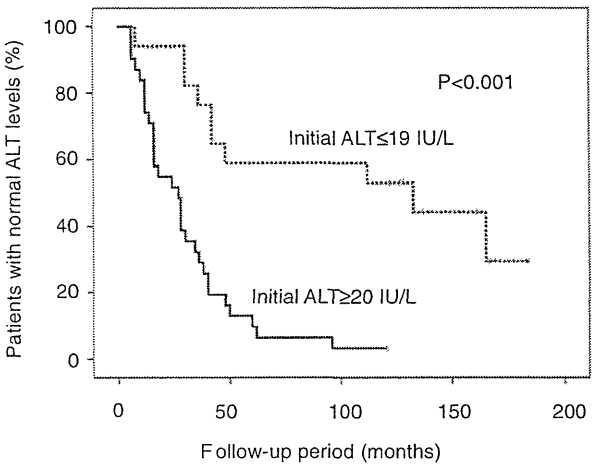


Figure 2 Maintenance of normal alanine aminotransferase (ALT) values (≤30 IU/L) during the follow up. Seventeen patients had initial ALT levels of ≤19 IU/L and 32 of ≥20 IU/L.

Relationship between menopause and ALT elevation

The ages of female PNALT patients at which abnormal ALT first occurred are presented in Figure 3(a). Abnormal ALT levels were most frequently recorded in female patients aged 45–55 years, which is usually the time of menopause onset. We sent a questionnaire to 45 female patients to investigate the relationship between ALT elevation and menopause, but only 16 patients responded. Of the respondents, age of menopause onset varied between 48 and 56 years, except for one patient who underwent hysterectomy at 37 years old and experienced menopause before consulting our hospital. ALT levels were found to be elevated within 3 years of their awareness of menopause in 10 patients (Fig. 3b), but before 3 years of menopause in three patients (Fig. 3c). The remaining three patients experienced menopause before consultation to our outpatient clinic (data not shown).

DISCUSSION

THE COURSE OF illness in HCV carriers with PNALT is not well known. The general consensus in Japan is that most HCV carriers with PNALT exhibit mild liver damage and/or fibrosis.[10] During the follow-up period of 10 years, interestingly, ALT levels remained stable at 30 IU/L or less in 52.9% (9/17) of patients with initial ALT levels of 19 IU/L or less. The probability of PNALT being maintained was significantly higher in patients

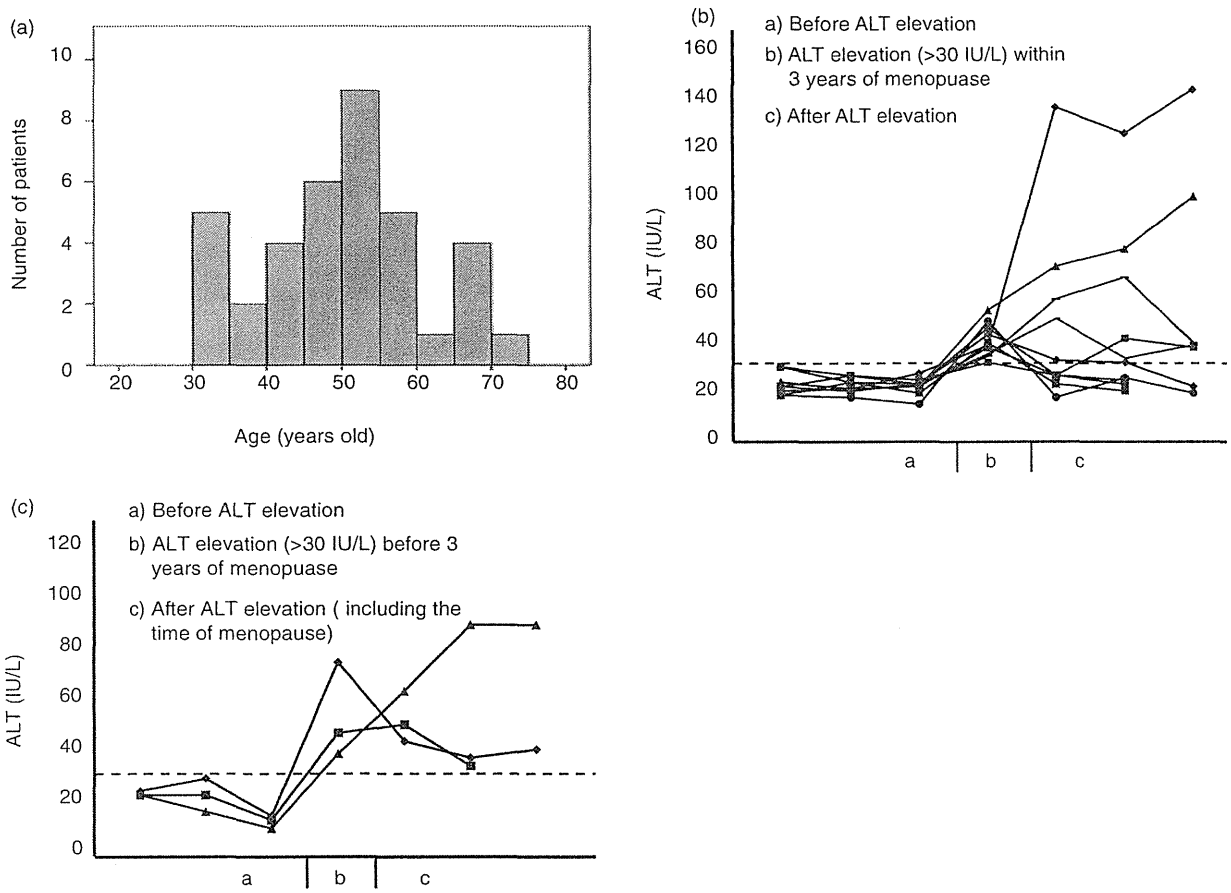


Figure 3 (a) Age of female persistently normal alanine aminotransferase (PNALT) patients at which abnormal ALT first occurred ($n = 37$). (b) PNALT with first ALT levels of >30 IU/L within 3 years of menopause. (c) PNALT with first ALT levels of >30 IU/L before 3 years of menopause.

with initial ALT levels of 19 IU/L or less than in those with initial ALT levels of 20 IU/L or more (Fig. 2, $P < 0.001$). Although the progression of hepatic fibrosis could not be evaluated by repeated liver biopsy during the observation period, this result suggests a benign course in a subgroup of HCV carriers with PNALT, whose ALT levels were 19 IU/L or less at the first visit.

Interestingly, a report from a hyperendemic area in Japan revealed that a basal ALT level of 20 IU/L or more was an important predictive factor of ALT flare-up in HCV carriers with PNALT.[17] This result accords with the favorable ALT levels documented in our study (Fig. 2). Furthermore, 19 IU/L is the updated upper limit of the healthy range for serum ALT level in female patients with chronic HCV infection or non-alcoholic fatty liver disease, as advocated by Prati *et al.*[18]

Concerning the possibility of HCC, one Japanese report demonstrated that HCV carriers with PNALT and ALT levels of more than 20 IU/L were, to some extent, at risk of both hepatocarcinogenesis and ALT elevation.[19] These results reinforce the finding in this study that patients with initial ALT levels of 20 IU/L or more and 30 IU/L or less were at a high risk for ALT elevation during the follow-up period (Fig. 2).

The relationship between menopause and the first abnormal ALT level in female patients was also examined. As shown in Figure 3(a), first abnormal ALT levels in female PNALT patients were frequently observed at 45–55 years of age, which is usually the time of menopause onset.

Although only 16 patients responded to the questionnaire, ALT levels were found to be elevated within

3 years of their awareness of menopause in 10 patients. This finding is interesting because previous studies have reported an association between menopause and progression of hepatic fibrosis[20,21] or resistance to antiviral therapy.[22] Recently, production of the HCV particle has been reported to be inhibited by 17-β-estradiol *in vitro*.[23] Further study in this field will clarify this issue.

Although the mechanism of abnormal ALT is uncertain, we speculate that one of the plausible causes of abnormal ALT levels might be enhanced immunological response against HCV. Recently, Itose *et al.*[24] demonstrated that the frequency of regulatory T cells is higher in PNALT patients and that depletion of $CD25^+$ cells enhanced HCV-specific T-cell response. So, we speculate that some immunological activation may underlie the cause of ALT elevation. Increased BMI during the observation may be another cause of abnormal ALT, although we do not have precise data on that point.

In conclusion, because antiviral therapy for chronic hepatitis C is making rapid and encouraging progress, waiting for more effective and safer treatments may be an option. The results of this study provide an important insight into this issue.

REFERENCES

1 Ghany MG, Strader DB, Thomas DL, Seeff LB. AASLD practice guidelines Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; 49: 1335–74.
2 Namiki I, Nishiguchi S, Hino K *et al.* Management of Hepatitis C: report of the consensus meeting at the 45th annual meeting of the Japan society of Hepatology 2009. *Hepatol Res* 2010; 40: 347–68.
3 Ghany MG, Nelson SR, Strader DB *et al.* An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practical guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; 54: 1433–44.
4 Kumada H, Toyoda J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naïve patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012; 56: 78–84.
5 Hayashi N, Okanoue T, Tsubouchi H, Toyoda J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis. *J Viral Hepat* 2012; 19: 134–42.
6 Chayama K, Takahashi S, Toyota J *et al.* Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012; 55: 742–8.
7 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet* 1997; 349: 825–32.
8 Persico M, Persico E, Suzzo R *et al.* Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. *Gastroenterology* 2000; 118: 760–4.
9 Hui C-K, Belaye T, Montegrande K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. *J Hepatol* 2003; 38: 511–7.
10 Okanoue T, Makiyama A, Nakayama M *et al.* A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. *J Hepatol* 2005; 43: 599–605.
11 Shiffman ML, Diago M, Tran A *et al.* Chronic hepatitis C in patients with persistently normal transaminase levels. *Clin Gastroenterol Hepatol* 2006; 4: 645–52.
12 Lowson A. Hepatitis C virus-infected patients with a persistently normal alanine aminotransferase: do they exist and is this really a group with mild disease? *J Viral Hepat* 2010; 17: 51–8.
13 Hui CK, Monto A, Belaye T, Lau E, Wright TL. Outcome of interferon alpha and ribavirin treatment for chronic hepatitis C in patients with normal serum aminotransferase. *Gut* 2003; 52: 1644–8.
14 Zeusem S, Diago M, Gene E *et al.* Peginterferon alfa-2a(40 Kilodalton) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* 2004; 127: 1724–32.
15 Okanoue T, Itoh Y, Minami M *et al.* Guideline for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet count. *Hepatol Res* 2008; 28: 27–36.
16 Ichida F, Tsuji T, Omata M *et al.* New Inuyama Classification; new criteria for histological assessment of chronic hepatitis. *Int Hepatol Commun* 1996; 6: 112–9.
17 Uto H, Kurogi L, Takahama Y *et al.* Aiamine aminotoransferase levels in a hyperendemic area of Janan. *J Gastroenterol* 2007; 42: 673–80.
18 Prati D, Taioli E, Zanella A *et al.* Update definitions of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; 137: 1–9.
19 Kumada T, Toyoda J, Kiriyama S *et al.* Long-term follow up of patients with hepatitis C with a normal alanine aminotransferase. *J Med Virol* 2009; 81: 446–51.
20 Di Martino V, Lebray P, Myers RP *et al.* Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. *Hepatology* 2004; 40: 1426–33.
21 Codes L, Asselah T, Cazala- Hatem D *et al.* Liver fibrosis in women with chronic hepatitis C: evidence for the negative role of the menopause and steatosis and the potential benefit of hormone replacement therapy. *Gut* 2007; 56: 390–5.

22 Villa E, Karampatou A, Camma C *et al.* Early menopause is associated with lack of response to antiviral therapy in women with chronic hepatitis C. *Gastroenterology* 2011; **140**: 818–29.
23 Hayashida K, Shoji I, Deng L, Jand DP, Hotta H. 17beta-estradiol inhibits the production of infectious particles of hepatitis C virus. *Microbiol Immunol* 2010; 54: 684–90.
24 Itose I, Kanto T, Kakita N *et al.* Enhanced ability of regulatory T cells in chronic hepatitis Cpatients with persistently normal alanine aminotransferase levels than those with active hepatitis. *J Viral Hepat* 2009; **16**: 844–52.

Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2013, Article ID 686420, 8 pages
http://dx.doi.org/10.1155/2013/686420

Hindawi

Research Article

Pathogenic Role of Iron Deposition in Reticuloendothelial Cells during the Development of Chronic Hepatitis C

Hironori Mitsuyoshi,[1] Kohichiroh Yasui,[1] Kanji Yamaguchi,[1] Masahito Minami,[1] Takeshi Okanoue,[2] and Yoshito Itoh[1]

[1] *Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi Hirokouji, Kamigyo-ku, Kyoto 602-8566, Osaka 564-0013, Japan*
[2] *Saiseikai Suita Hospital, Suita, Japan*

Correspondence should be addressed to Hironori Mitsuyoshi; hmitsu@koto.kpu-m.ac.jp

Received 26 November 2012; Revised 28 February 2013; Accepted 15 March 2013

Academic Editor: Kazuhiko Koike

Aim. Chronic hepatitis C (CHepC) is frequently associated with hepatic iron overload, yet mechanisms underlying iron-induced liver injury have not been elucidated. We examined the significance of iron deposition in hepatocytes (HC) and reticuloendothelial cells (REC) in CHepC. *Methods.* Stainable hepatic iron was scored according to the iron deposition pattern in 373 patients. The levels of serum soluble TNF-α receptor (sTNFR2) and hepatic hepcidin mRNA and the efficacy of phlebotomy were compared among patients with different iron deposition patterns. *Results.* Serum transaminase levels and hepatic scores of stage, grade, and steatosis were higher in patients with REC iron staining than in those without. REC iron scores were independently associated with advanced stage. Serum sTNFR2 levels were significantly higher in patients with REC iron than in those without. REC iron scores were independently correlated with sTNFR2 levels. Compared with patients without stainable iron, those with iron overload had decreased ratios of hepcidin mRNA to serum ferritin. The efficacy of phlebotomy was greater in patients with REC iron than in those without REC iron. *Conclusions.* The present results show the importance of REC iron for the development of CHepC and the therapeutic effect of phlebotomy in CHepC.

1. Introduction

Chronic hepatitis C (CHepC) is frequently associated with hepatic iron overload [1–3]. Elevation of serum iron indices or stainable hepatic iron has been shown in 40 to 70% of patients with CHepC [1–3]. From these observations, iron-induced oxidative stress has been considered to be an underlying mechanism of liver injury and of development of hepatocellular carcinoma [4–6].

The mechanisms of hepatic iron overload in CHepC have not yet been elucidated. However, hepcidin has attracted much attention as an important factor in the disease process. Hepcidin is exclusively produced in the liver and regulates body iron stores [7, 8]. Hepcidin causes internalization and degradation of iron-transporter ferroportin on duodenal enterocytes and macrophages, thereby blocking iron absorption and iron recycling, respectively [9]. In hereditary hemochromatosis (HH), defective hepcidin synthesis results in a subsequent increase in body iron stores [10]. In CHepC, hepatic iron overload has been attributed to the mutation of the hemochromatosis protein (HFE) gene [11], since several reports have found an association between HFE genotypes and iron overload in patients with CHepC [12–14]. Another possible mechanism is the direct effect of the hepatitis C virus (HCV) on hepcidin synthesis [15]. Transgenic mice expressing HCV polyprotein have been shown to have decreased hepatic expression of hepcidin due to HCV-induced oxidative stress [15].

When hepatic iron overload develops, stainable iron can be seen either in hepatocytes (HC), reticuloendothelial cells (REC), or both cell types [16]. Recently, patterns of hepatic iron distribution have attracted a considerable attention in chronic liver diseases, since the patterns would predict the histological progressions. In particular, nonparenchymal iron deposition has been associated with advanced stages of alcoholic liver disease (ALD) and nonalcoholic steatohepatitis (NASH) [17, 18]. In CHepC, Di Bisceglie et al. initially reported the presence of hepatic iron deposition both in HC and REC [19]. Hézode et al. reported the positive relationship between histological activity and iron deposition either in REC or mixed HC/REC in patients with CHepC [2].

The mechanism and pathogenicity underlying hepatic iron distribution still remain unclear. However, hepcidin is one of the candidates that could potentially resolve these issues. Hepcidin is synthesized by HC in response to iron overload [7] and can sequestrate iron in Kupffer cells and macrophages through the downregulation of ferroportin [9]. Thus, hepcidin can modify outcomes of patients with CHepC by determining iron deposition patterns. In the present study, relationships between iron deposition patterns and histological scores in CHepC were examined. Then, levels of TNF-α and hepatic hepcidin mRNA and the effect of phlebotomy on liver function tests were compared among patients with different iron deposition patterns. The present study examines the significance of nonparenchymal iron deposition and discusses the mechanisms and pathogenicity underlying iron deposition patterns.

2. Patients and Methods

2.1. Patients. Patients with CHepC who underwent liver biopsies at our institutes between January 2007 and April 2012 were retrospectively reviewed. Patients were selected according to the following criteria: positive anti-HCV antibody; positive serum HCV-RNA confirmed by reverse transcription-polymerase chain reaction (RT-PCR); no history of antiviral therapy; no excessive alcohol intake (intake less than 40 g/week); negative for hepatitis B surface antigen or antibodies to human immunodeficiency virus; and absence of other forms of chronic liver disease, including autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. Informed written consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

2.2. Laboratory Determination. After a 12 h overnight fast, venous blood samples were drawn to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting plasma glucose (FPG), immunoreactive insulin (IRI), total cholesterol, triglycerides, and ferritin levels. The index of insulin resistance was calculated only in patients without overt diabetes (FPG >126 mg/dL), according to the homeostasis model assessment (HOMA). The formula used for insulin resistance was as follows: HOMA-R = FPG (mg/dL) × IRI (μU/mL)/405.

HCV-RNA levels were determined by RT-PCR. HCV genotypes were determined by PCR of the core region with genotype-specific PCR primers [20]. HCV serogroups 1 and 2 were determined by a serologic genotyping assay [21].

Serum TNF-α concentrations were evaluated by the soluble TNF-α receptor type 2 (sTNFR2) levels, since sTNFR2 levels can be easily and stably measured and have been shown to be associated with the serum level of TNF-α [22]. For measurement of sTNFR2 concentrations, serum was stored at −80°C until use. The serum sTNFR2 levels were then measured in 148 patients using a commercial, sensitive enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA).

2.3. Histological Evaluation. Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin-eosin, Masson's trichrome, and Berlin blue. Histopathological diagnosis was based on the scoring of the New Inuyama Classification [23]. Briefly, degree of hepatic fibrosis (stage) was scored as follows: 0 = none, 1 = portal expansion, 2 = bridging fibrosis, 3 = bridging fibrosis with lobular distortion, and 4 = cirrhosis. Degree of inflammation (grade) was scored as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = severe. Steatosis was assessed according to the percentage of hepatocytes containing fat droplets: 0 = less than 5%, 1 = 5–9%, 2 = 10–29%, and 3 = more than 29%. HC iron deposition was scored from 0 to 4 as described previously [24]. REC iron deposition was scored from 0 to 2: 0 = scarcely seen, 1 = sporadically seen in the acinar and/or the portal tract, and 3 = frequently seen in the acinar and/or the portal tract. We considered cellular iron deposition only when granular iron deposition was observed.

2.4. Quantification of Hepatic mRNA Levels of Hepcidin. Hepatic mRNA levels of hepcidin were measured in 84 patients whose biopsy specimens were available. Total RNA was isolated using the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The PCR mixture contained first-strand cDNA and specific primers for human hepcidin: sense, 5′-ACCAGAGCAAGCTCAAGACC-3′ and antisense, 5′-AAACAGAGCCACTGGTCAGG-3′. Real-time PCR was performed to quantify mRNA levels of the target genes using the StepOnePlus Real-Time PCR system (Life Technologies), and mRNA levels of hepcidin were normalized to those of β-actin: sense, 5′-CTGGAACGGTGAAGGTGACA-3′ and antisense, 5′-AAGGGACTTCCTGTAACAATGCA-3′.

2.5. Phlebotomy. Phlebotomy was received in 48 patients after the liver biopsy. All patients showed elevated serum ferritin levels and/or persistent abnormal ALT levels, and none showed anemia (hemoglobin <11.0 g/dL). They underwent phlebotomy (200–400 mL) either biweekly or monthly until serum ferritin levels were <20 ng/mL. However, treatments were terminated irrespective of serum ferritin levels when blood hemoglobin concentrations decreased to less than 10 g/dL.

2.6. Statistical Analysis. Differences and correlations between quantitative variables were analyzed using the Student's t-test and the Pearson product-moment correlation coefficient, respectively. Distributions of qualitative variables were compared using the Chi-squared test. When differences between variables were considered among more than two groups, post hoc comparisons (*Bonferroni test*) were employed after the analysis of variance (ANOVA). Logistic regression model was used to analyze independent variables associated with advanced fibrosis. Multiple-regression model was used to analyze independent variables associated with sTNFR2 levels. A P value of less than 0.05 was considered significant.

3. Results

3.1. General Characteristics of Patients. Three hundred and seventy-three patients met the eligibility criteria. Overall, 208 patients (56%) had HC iron deposition and 125 patients (34%) had REC iron deposition, comprising of no stainable iron in 141 patients (None group), HC deposition alone in 107 (HC group), mixed HC/REC deposition in 101 (Mix group), and REC deposition alone in 24 patients (REC group). Receiver operating characteristic analysis showed that the cut-off values of ferritin for iron deposition in HC and REC were 104.5 ng/mL (area under the curve: AUC = 0.832, $P < 0.0000001$) and 224.5 ng/mL (AUC = 0.827, $P < 0.0000001$), respectively. The differences among the 4 groups were significant with regards to gender, body mass index (BMI), and serum levels of AST, ALT, and ferritin (Table 1). There were a greater number of male patients in the Mix and REC groups than in the other groups. Serum levels of AST and ALT were significantly higher in the Mix and REC groups than in the other groups. Ferritin levels were significantly higher in the Mix group than in the other groups and were significantly higher in the HC and REC groups than in the None group (Table 1).

3.2. Results of Liver Biopsies. The results of liver biopsies are summarized in Table 1. Iron deposition patterns were significantly associated with stage, grade, and steatosis. HC iron scores were significantly higher in the Mix group than in the HC group ($P < 0.0005$, Chi-squared test). Patients in the Mix and REC groups had higher scores of stage, grade, and steatosis than those of the other groups. In contrast, patients in the HC group had similar scores of stage, grade, and steatosis compared to patients in the None group.

3.3. Association between REC Iron Deposition and Fibrosis. In order to examine the variables associated with the fibrosis, patients with an early fibrosis stage were compared with those with an advanced fibrosis stage (Table 2). Age, BMI, levels of AST, ALT, FPG, IRI, and ferritin, HOMA-R, and hepatic scores of grade, steatosis, and REC iron were significantly higher in patients with advanced stage than in those with early stage. The presence of diabetes was significantly associated with advanced stage. On logistic regression analysis, serum levels of AST, ALT, and ferritin and hepatic scores of grade and REC iron were independently associated with advanced stage.

3.4. Association between REC Iron Scores and Serum sTNFR2 Levels. Serum sTNFR2 levels were measured in 148 patients. Figure 1 represents the distributions of sTNFR2 levels among patients with different iron deposition patterns. Serum sTNFR2 levels were significantly higher in the REC group than in the other groups and were significantly higher in the Mix group than in the HC group. Serum sTNFR2 levels were significantly correlated with age, serum levels of AST, ALT, and ferritin, and hepatic scores of stage, grade, steatosis, and REC iron (Table 3). On regression analysis, age and hepatic scores of grade and REC iron were independently correlated with sTNFR2 levels (Table 3).

3.5. Hepcidin mRNA Levels. Hepcidin mRNA levels were quantified in 84 patients. Overall, hepatic hepcidin mRNA levels were higher in patients with stainable iron than in those without stainable iron and the difference was achieved significance between the None and HC groups (Figure 2(a)). However, this significance disappeared after normalization relative to ferritin concentrations (Figure 2(b)). These corrected values tended to be lower in the Mix and REC groups than in the None group (Figure 2(b)).

3.6. Efficacy of Phlebotomy on ALT Levels. Clinical and histological characteristics of the 48 patients who underwent phlebotomy are summarized in Table 4. Figure 3(a) represents the change in ALT levels after phlebotomy. ALT levels were significantly decreased in the HC and Mix groups. The decrease in ALT levels in the REC group did not achieve statistical significance due to the small number of patients. The effects of phlebotomy on ALT levels tended to be greater in the Mix and REC groups than in the HC group ($P = 0.082$, ANOVA) (Figure 3(b)).

4. Discussion

The current study showed high frequency of stainable hepatic iron in patients with CHepC, as previously reported [1–3]. Overall, 61% of the patients had stainable iron either in HC, REC, or both cell types. HC iron scores were mild except in the 11% of patients who had severe HC iron scores. Although it was not examined whether the patients were genetically predisposed to iron overload, the previously reported prevalence of the mutations of the HFE gene in Japanese population is less than 1% [25].

First, relationships between hepatic iron distribution and biochemical and histological findings of CHepC were examined. It was found that REC iron depositions were significantly associated with the severities of liver function tests, stage, grade, and steatosis and were independently associated with advanced fibrosis. In contrast, HC iron itself seemed less significant than REC iron, because the liver function tests and scores of stage and grade were almost identical between patients with HC iron deposition alone and

Table 1: Clinical characteristics of patients.

Group	None (n = 141)	HC (n = 107)	Mix (n = 101)	REC (n = 24)	ANOVA
Age	54.5 ± 12.3	55.9 ± 11.0	56.7 ± 11.0	59.1 ± 12.1	0.216
Gender (male/female)	37/104	52/55	68/33	14/10	<0.0000001*
BMI (kg/m^2)	22.7 ± 4.0	22.8 ± 3.5	23.9 ± 3.4	23.7 ± 3.6	0.037
Diabetes (yes/no)	10/131	10/97	9/92	2/22	0.925*
Genotype (1a/1b/2a/2b/3a)	2/61/32/11/1	1/67/11/4/0	2/50/11/11/0	0/12/3/2/0	0.185*
Serogroup (G1/G2)	25/9	18/6	21/6	5/2	0.985*
HCV-RNA (logIU/mL)	5.9 ± 0.8	6.1 ± 0.8	6.0 ± 0.8	6.1 ± 0.8	0.057
AST (IU/L)	51.3 ± 36.5	43.9 ± 28.9	72.6 ± 39.1[cg]	81.9 ± 62.0[bf]	<0.0000001
ALT (IU/L)	59.6 ± 53.0	53.7 ± 35.2	101.2 ± 66.9[dg]	94.7 ± 75.5[ae]	<0.0000001
Triglyceride (mg/dL)	103.2 ± 86.8	100.9 ± 56.6	99.2 ± 42.0	103.5 ± 34.4	0.984
IRI (μU/mL)	10.7 ± 11.7	8.3 ± 3.7	11.2 ± 5.7	11.5 ± 6.0	0.341
FPG (mg/dL)	99.9 ± 25.6	98.7 ± 13.4	102.6 ± 17.7	104.2 ± 26.0	0.468
HOMA-R	3.4 ± 6.3	2.1 ± 1.1	2.8 ± 1.4	3.0 ± 1.8	0.424
Ferritin (ng/mL)	93.9 ± 85.1	191.5 ± 109.1[b]	425.1 ± 300.9[dgh]	215.4 ± 155.6[a]	<0.0000001
Stage (0/1/2/3/4)	1/82/42/15/1	2/60/36/9/0	1/29/41/21/9	0/3/11/5/5	<0.0000001
Grade (0/1/2/3)	4/79/53/5	2/67/31/7	0/31/55/15	0/2/15/7	<0.0000001
HC iron score (1/2/3/4)	—	63/32/10/2	30/42/21/8	—	—
REC iron score (1/2)	—	—	2.2	3.8	—
Steatosis (0/1/2/3)	91/28/19/3	67/26/13/1	39/36/19/7	7/10/4/3	<0.0005

[a]$P < 0.05$, [b]$P < 0.005$, [c]$P < 0.0001$, [d]$P < 0.0000001$ versus None; [e]$P < 0.01$, [f]$P < 0.0001$, [g]$P < 0.0000001$ versus HC; and [h]$P < 0.00005$ versus REC (Bonferroni test). *Chi-squared test.

None: no stainable iron, HC: hepatocytes, Mix: mixed hepatocytes/reticuloendothelial cells, REC: reticuloendothelial cells, BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, IRI: immunoreactive insulin, FPG: fasting plasma glucose, and HOMA-R: homeostasis model assessment ratio.

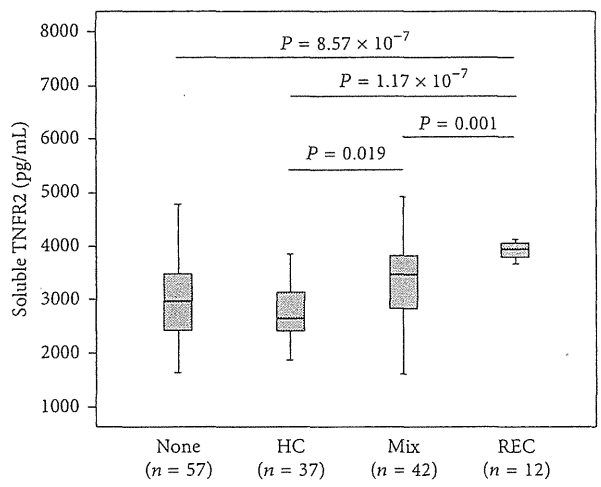


Figure 1: Distributions of serum soluble TNF-α (sTNFR2) levels among patients with different iron deposition patterns are seen. None: no stainable iron, HC: iron deposition in hepatocytes alone, Mix: iron deposition in mixed hepatocytes/reticuloendothelial cells, and REC: iron deposition in reticuloendothelial cells alone. Differences between the groups were analyzed by post hoc comparisons (Bonferroni test).

those without stainable iron. These findings expand Hézode's report that showed the association between liver cirrhosis and the presence of macrophage iron accumulation in CHepC [2]. The present study was unique for its examination of the levels of TNF-α and hepcidin mRNA. The cut-off value of ferritin for REC iron deposition was higher than that for HC iron deposition. Therefore, it can be assumed that iron deposition initially develops in HC followed by REC iron deposition in the development of CHepC.

The association between nonparenchymal iron deposition and disease severity has also been shown in patients with NASH and ALD [17, 18]. Taken together with the current study, it is likely that nonparenchymal iron deposition is a common feature of progressing chronic liver diseases.

Second, the pathogenesis of CHepC was examined in terms of TNF-α production. TNF-α has been implicated as an important pathogenic mediator in a variety of liver diseases [26]. Serum sTNFR2 levels, which have been shown to reflect disease progression in CHepC [27], were significantly higher in patients with REC iron than in those without REC iron. Moreover, REC iron scores were independently correlated with sTNFR2 levels. Thus, the increase in TNF-α production suggests that disease progression is closely associated with iron deposition in REC.

The progression of hepatic fibrosis is driven by activated hepatic stellate cells (HSC). Our findings indicated that iron-loading in nonparenchymal, not parenchymal, cells was correlated with progressive fibrosis in CHepC. Although oxidative stress has been shown to activate HSC [28], the effect of iron-induced oxidative stress on HSC may depend on the localized environment of iron-filled cells. Using a rodent model of secondary iron overload, iron deposition

Table 2: Logistic regression analysis of factors associated with advanced stage.

	Stage 0–2	Stage 3–4	Univariate	Multivariate
Age	54.2 ± 12.1	59.3 ± 9.9	0.019	0.968
Gender (male/female)	136/172	35/30	0.099*	—
BMI (kg/m^2)	22.4 ± 3.1	26.6 ± 5.0	<0.000005	0.450
Diabetes (yes/no)	20/288	11/54	0.009*	0.072
Genotype (1a/1b/2a/2b/3a)	3/159/46/26/1	2/31/11/2/0	0.350*	—
Serogroup (G1/G2)	56/17	13/6	0.469*	—
HCV-RNA (logIU/mL)	6.0 ± 0.8	6.3 ± 0.7	0.536	—
AST (IU/L)	48.4 ± 33.7	80.9 ± 40.9	<0.0000001	<0.05
ALT (IU/L)	64.9 ± 59.5	90.2 ± 52.7	<0.0000001	<0.05
IRI (μU/mL)	9.0 ± 6.4	14.9 ± 10.6	0.008	0.151
FPG (mg/dL)	100.2 ± 26.4	110.2 ± 31.8	<0.0001	0.675
HOMA-R	2.5 ± 3.9	4.7 ± 6.4	0.040	0.324
Ferritin (ng/mL)	214.1 ± 235.3	221.2 ± 185.8	0.034	<0.05
Grade (0/1/2/3)	6/177/115/10	0/2/39/24	<0.0000001*	<0.005
Steatosis (0/1/2/3)	188/78/37/5	16/22/18/9	<0.0000001*	0.811
HC iron (0/1/2/3/4)	139/76/57/28/8	26/17/17/3/2	0.511*	—
REC iron (0/1/2)	223/60/25	25/28/12	<0.0000005*	<0.05

BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, IRI: immunoreactive insulin, FPG: fasting plasma glucose, HOMA-R: homeostasis model assessment ratio, HC: hepatocytes, and REC: reticuloendothelial cells.
Univariate: Student's t-test, Multivariate: logistic regression, and *Chi-squared test.

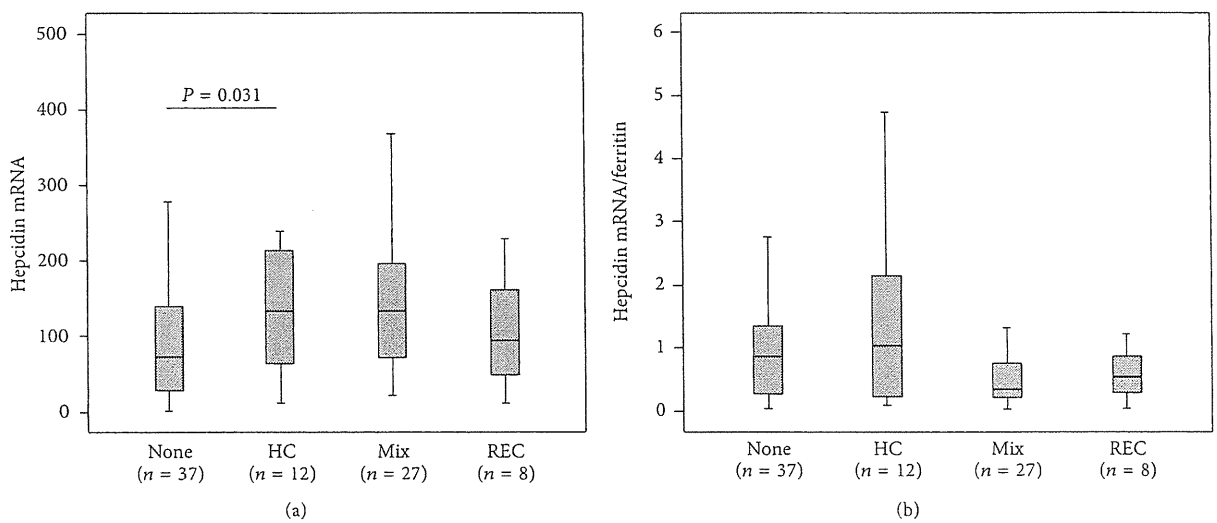


Figure 2: Distributions of absolute hepatic hepcidin mRNA levels (a) and hepcidin mRNA levels normalized to serum ferritin concentrations (b) among patients with different iron deposition patterns. mRNA levels of hepcidin were normalized to those of β-actin. None: no stainable iron, HC: iron deposition in hepatocytes alone, Mix: iron deposition in mixed hepatocytes/reticuloendothelial cells, and REC: iron deposition in reticuloendothelial cells alone. Differences between the None and HC groups were analyzed by post hoc comparisons (Bonferroni test).

in nonparenchymal Kuppfer cells was shown to induce HSC proliferation and activation, leading to liver cirrhosis [29]. These phenomena, however, were ameliorated by treatment with antioxidant [29]. Thus, the redox-active properties of localized iron deposition may be greater in REC than in HC. Alternatively, iron loading in REC may alter their redox status affecting cytokine production by these cells [30].

Third, the mechanisms involved in iron deposition in HC and REC were examined. It has been reported that HCV infection causes hepatic iron overload by the downregulation of hepcidin synthesis [15]. The current study showed that hepatic hepcidin mRNA levels were significantly increased in patients with HC iron than in those without stainable iron. When normalized relative to serum ferritin concentrations, however, the difference in hepcidin mRNA levels between these two groups was not significant. Moreover, normalized hepcidin mRNA levels tended to be lower in the Mix and REC groups than in the None group. The current study cannot

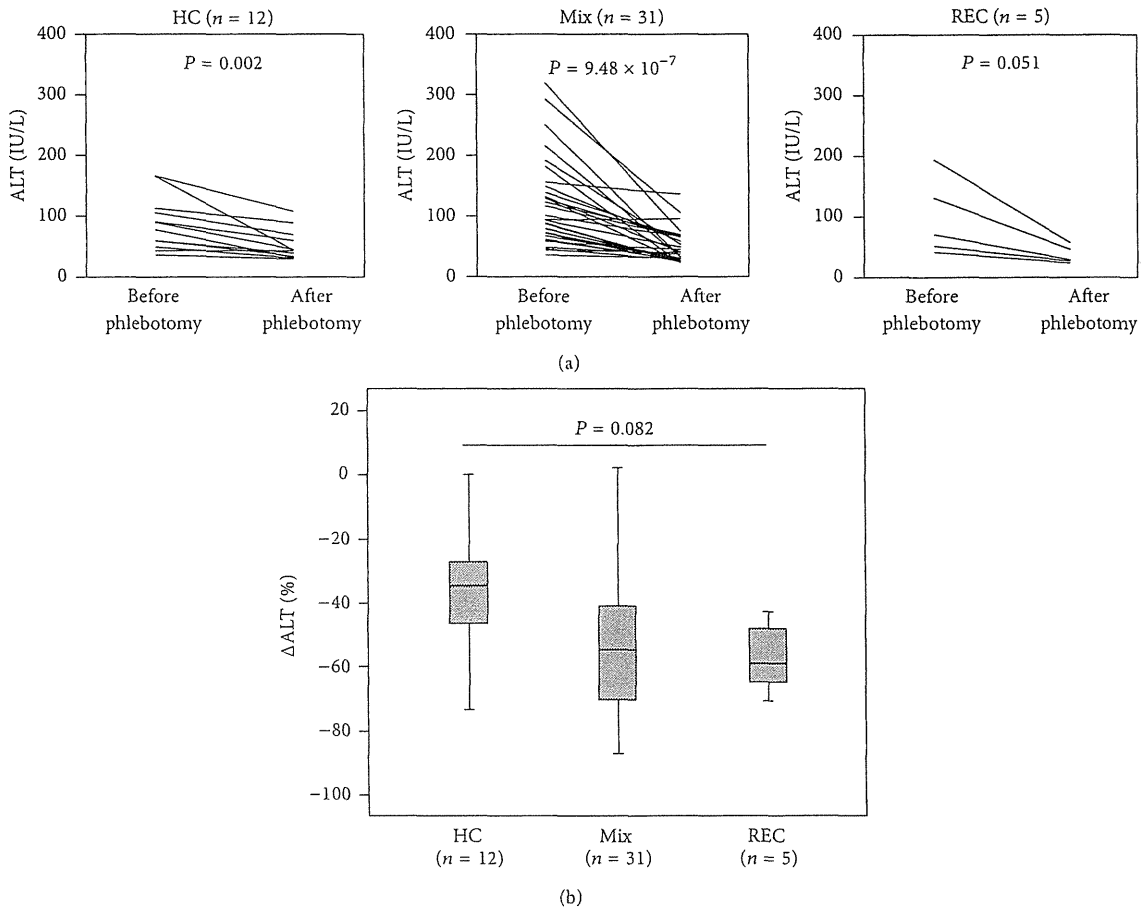


FIGURE 3: Line graphs (a) show the alanine aminotransferase (ALT) levels before and after phlebotomy. Differences in ALT levels were analyzed by the Wilcoxon signed-rank test. Box graph (b) shows the percentage change in ALT levels after phlebotomy. Differences among the three groups were analyzed by ANOVA (P = 0.082). None: no stainable iron, HC: iron deposition in hepatocytes alone, Mix: iron deposition in mixed hepatocytes/reticuloendothelial cells, and REC: iron deposition in reticuloendothelial cells alone.

TABLE 3: Variables associated with sTNFR2 levels.

	Coefficient	Univariate	Regression
Age	0.271	<0.001	<0.01
AST	0.387	<0.000005	0.363
ALT	0.367	<0.000005	0.628
Ferritin	0.302	<0.0005	0.929
Stage	0.292	<0.0005	0.394
Grade	0.389	<0.000005	<0.05
Steatosis	0.169	<0.05	0.434
REC iron score	0.401	<0.0000005	<0.005

Univariate: Pearson's correlation coefficient, and Regression: regression analysis.
AST: aspartate aminotransferase, ALT: alanine aminotransferase, and REC: reticuloendothelial cells.

TABLE 4: Forty-eight patients who underwent phlebotomy.

Age	57.2 ± 10.6
Gender (male/female)	31/17
Ferritin (ng/mL)	438.3 ± 322.1
ALT (IU/L)	109.8 ± 65.8
Stage (0/1/2/3/4)	0/10/27/9/2
Grade (0/1/2/3)	0/17/26/5
Steatosis (0/1/2/3)	14/18/5/11
Hepatocyte iron score (0/1/2/3/4)	5/18/10/13/2
REC iron score (0/1/2)	12/28/8
Pattern	
HC alone	12
Mixed HC/REC	31
REC alone	5

ALT: alanine aminotransferase, HC: hepatocytes, and REC: reticuloendothelial cells.

verify the appropriateness of hepcidin production against iron overload because of the lack of data from the patients without HCV infection. With regard to the response to iron overload of hepcidin synthesis, Fujita et al. also reported that relative hepatic hepcidin mRNA levels to serum ferritin levels were low in CHepC compared to the other chronic

liver diseases [31]. Thus, alterations in hepcidin synthesis may have facilitated hepatic iron deposition, especially in the Mix and REC groups. Since oxidative stress can affect hepcidin synthesis in hepatocytes [15], exacerbated oxidative stress resulting from iron deposition in REC may have affected hepcidin synthesis in hepatocytes, resulting in further hepatic iron overload.

Hepatic macrophages and Kupffer cells can take in iron exclusively through phagocytosis of senescent erythrocytes and/or damaged hepatocytes. Then, iron can be recycled to the blood through the iron-exporter ferroportin [8]. Therefore, ferroportin levels can affect iron sequestration within hepatic macrophages and Kupffer cells. However, the differences in hepcidin mRNA levels among the groups of patients with stainable iron did not reach statistical significance, making it difficult to determine whether hepcidin alone could affect iron deposition patterns. Mechanisms other than hepcidin may therefore be responsible for iron deposition in hepatic macrophages and Kupffer cells.

Finally, the effects of phlebotomy on ALT levels were examined. To the best of our knowledge, the current study is the first to compare the efficacy of phlebotomy among patients with different iron deposition patterns. Interestingly, the effects of phlebotomy on ALT levels tended to be greater in patients with REC iron deposition. These findings indicate the importance of iron reduction in nonparenchymal cells for inhibition of disease progression.

5. Conclusion

In summary, REC iron deposition in CHepC was associated with disease severities and enhanced production of TNF-α. Although inappropriate hepatic synthesis of hepcidin can promote hepatic iron deposition, additional mechanisms should be considered to explain how iron deposition patterns develop. Phlebotomy should be especially considered for patients with nonparenchymal hepatic iron deposition.

References

[1] Y. Ikura, H. Morimoto, H. Johmura, M. Fukui, and M. Sakurai, "Relationship between hepatic iron deposits and response to interferon in chronic hepatitis C," *American Journal of Gastroenterology*, vol. 91, no. 7, pp. 1367–1373, 1996.

[2] C. Hézode, C. Cazeneuve, O. Coué et al., "Liver iron accumulation in patients with chronic active hepatitis C: prevalence and role of hemochromatosis gene mutations and relationship with hepatic histological lesions," *Journal of Hepatology*, vol. 3, pp. 979–984, 1999.

[3] M. Pirisi, C. A. Scott, C. Avellini et al., "Iron deposition and progression of disease in chronic hepatitis C: role of interface hepatitis, portal inflammation, and HFE missense mutations," *American Journal of Clinical Pathology*, vol. 113, no. 4, pp. 546–554, 2000.

[4] J. Choi and J. H. J. Ou, "Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus," *American Journal of Physiology*, vol. 290, no. 5, pp. G847–G851, 2006.

[5] S. Mueller, N. H. Afdhal, and D. Schuppan, "Iron, HCV, and liver cancer: hard metal setting the pace?" *Gastroenterology*, vol. 130, no. 7, pp. 2229–2234, 2006.

[6] J. Kato, K. Miyanishi, M. Kobune et al., "Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C," *Journal of Gastroenterology*, vol. 42, no. 10, pp. 830–836, 2007.

[7] C. H. Park, E. V. Valore, A. J. Waring, and T. Ganz, "Hepcidin, a urinary antimicrobial peptide synthesized in the liver," *Journal of Biological Chemistry*, vol. 276, no. 11, pp. 7806–7810, 2001.

[8] M. W. Hentze, M. U. Muckenthaler, and N. C. Andrews, "Balancing acts: molecular control of mammalian iron metabolism," *Cell*, vol. 117, no. 3, pp. 285–297, 2004.

[9] E. Nemeth, G. C. Preza, C. L. Jung, J. Kaplan, A. J. Waring, and T. Ganz, "The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study," *Blood*, vol. 107, no. 1, pp. 328–333, 2006.

[10] T. Ganz, "Iron homeostasis: fitting the puzzle pieces together," *Cell Metabolism*, vol. 7, no. 4, pp. 288–290, 2008.

[11] A. Pietrangelo, "Hemochromatosis gene modifies course of hepatitis C viral infection," *Gastroenterology*, vol. 124, no. 5, pp. 1509–1523, 2003.

[12] L. Kazemi-Shirazi, C. Datz, T. Maier-Dobersberger et al., "The relation of iron status and hemochromatosis gene mutations in patients with chronic hepatitis C," *Gastroenterology*, vol. 116, no. 1, pp. 127–134, 1999.

[13] B. Y. Tung, M. J. Emond, M. P. Bronner, S. D. Raaka, S. J. Cotler, and K. V. Kowdley, "Hepatitis C, iron status, and disease severity: relationship with HFE mutations," *Gastroenterology*, vol. 124, no. 2, pp. 318–326, 2003.

[14] H. L. Bonkovsky, N. Troy, K. McNeal et al., "Iron and HFE or TfR1 mutations as comorbid factors for development and progression of chronic hepatitis C," *Journal of Hepatology*, vol. 37, no. 6, pp. 848–854, 2002.

[15] S. Nishina, K. Hino, M. Korenaga et al., "Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription," *Gastroenterology*, vol. 134, no. 1, pp. 226–238, 2008.

[16] E. M. Brunt, "Pathology of hepatic iron overload," *Seminars in Liver Disease*, vol. 25, no. 4, pp. 392–401, 2005.

[17] Y. Kohgo, T. Ohtake, K. Ikuta et al., "Iron accumulation in alcoholic liver diseases," *Alcoholism*, vol. 29, no. 11, pp. 189S–193S, 2005.

[18] J. E. Nelson, L. Wilson, E. M. Brunt et al., "Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease," *Hepatology*, vol. 53, no. 2, pp. 448–457, 2011.

[19] A. M. Di Bisceglie, C. A. Axiotis, J. H. Hoofnagle, and B. R. Bacon, "Measurements of iron status in patients with chronic hepatitis," *Gastroenterology*, vol. 102, no. 6, pp. 2108–2113, 1992.

[20] T. Ohno, M. Mizokami, R. R. Wu et al., "New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a," *Journal of Clinical Microbiology*, vol. 35, no. 1, pp. 201–207, 1997.

[21] K. Tsukiyama-Kohara, K. Yamaguchi, N. Maki et al., "Antigenicities of group I and II hepatitis C virus polypeptides-molecular basis of diagnosis," *Virology*, vol. 192, no. 2, pp. 430–437, 1993.

[22] G. A. Spinas, U. Keller, and M. Brockhaus, "Release of soluble receptors for tumor necrosis factor (TNF) in relation to circulating TNF during experimental endotoxinemia," *Journal of Clinical Investigation*, vol. 90, no. 2, pp. 533–536, 1992.

[23] F. Ichida, T. Tsuji, M. Omata et al., "New Inuyama Classification, new criteria for histological assessment of chronic hepatitis,"

International Hepatology Communications, vol. 6, no. 2, pp. 112–119, 1996.

[24] B. Turlin and Y. Deugnier, "Evaluation and interpretation of iron in the liver," *Seminars in Diagnostic Pathology*, vol. 15, no. 4, pp. 237–245, 1998.

[25] T. Sohda, J. Yanai, H. Soejima, and K. Tamura, "Frequencies in the Japanese population of HFE gene mutations," *Biochemical Genetics*, vol. 37, no. 1-2, pp. 63–68, 1999.

[26] C. A. Bradham, J. Plümpe, M. P. Manns, D. A. Brenner, and C. Trautwein, "Mechanisms of hepatic toxicity: I. TNF-induced liver injury," *American Journal of Physiology*, vol. 275, no. 3, pp. G387–G392, 1998.

[27] Y. Itoh and T. Okanoue, "Serum levels of soluble tumor necrosis factor receptors and effects of interferon therapy in patients with chronic hepatitis C virus infection," *American Journal of Gastroenterology*, vol. 94, no. 5, pp. 1332–1340, 1999.

[28] N. Nieto, S. L. Friedman, P. Greenwel, and A. I. Cederbaum, "CYP2E1-mediated oxidative stress induces collagen type I expression in rat hepatic stellate cells," *Hepatology*, vol. 30, no. 4, pp. 987–996, 1999.

[29] A. Pietrangelo, R. Gualdi, G. Casalgrandi, G. Montosi, and E. Ventura, "Molecular and cellular aspects of iron-induced hepatic cirrhosis in rodents," *Journal of Clinical Investigation*, vol. 95, no. 4, pp. 1824–1831, 1995.

[30] S. Recalcati, M. Locati, E. Gammella, P. Invernizzi, and G. Cairo, "Iron levels in polarized macrophages: regulation of immunity and autoimmunity," *Autoimmunity Reviews*, vol. 11, no. 12, pp. 883–889, 2012.

[31] N. Fujita, R. Sugimoto, M. Takeo et al., "Hepcidin expression in the liver: relatively low level in patients with chronic hepatitis C," *Molecular Medicine*, vol. 13, no. 1-2, pp. 97–104, 2007.