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Original Article

Effectiveness and safety of reduced-dose telaprevir-based triple therapy in chronic hepatitis C patients

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Aim: To compare the early virological effectiveness, sustained virological response and safety of telaprevir 1500 mg/day with telaprevir 2250 mg/day, when combined in triple therapy with pegylated interferon and ribavirin in Japanese patients with high viral loads of genotype 1 hepatitis C virus.

Methods: The telaprevir 2250 mg/day and 1500 mg/day groups each contained 60 patients matched by age, sex and history of previous interferon-based treatment. Serum levels of genotype 1 hepatitis C virus RNA, hemoglobin levels, drug adherence and drug discontinuation rates were monitored during and after triple therapy.

Results: Patients receiving telaprevir 1500 mg/day had significantly lower telaprevir adherence and lower initial ribavirin dose but similar or superior pegylated interferon and ribavirin adherence and a lower rate of telaprevir discontinuation than did those receiving telaprevir 2250 mg/day. The early virological responses and sustained virological response rates were similar in both groups. Hemoglobin levels decreased to a greater extent in patients treated with telaprevir 2250 mg/day.

Conclusion: Compared to triple therapy including telaprevir 2250 mg/day, that including telaprevir at a reduced dose of 1500 mg/day was associated with lower rates of anemia and similar antiviral efficacy. Such a regimen may meaningfully improve sustained virological response rates, especially among female and elderly Japanese patients.

Key words: chronic hepatitis, hepatitis C virus, pegylated interferon, ribavirin, telaprevir

INTRODUCTION

APPROXIMATELY 170 MILLION people are chronically infected with hepatitis C virus (HCV) worldwide,[1] and approximately 30% develop serious liver disease such as decompensated cirrhosis and hepatocellular carcinoma (HCC).[2,3] Currently, interferon (IFN) is the only antiviral drug capable of eliminating HCV infection. The present standard of care (SOC) for patients infected with HCV genotype 1, the most prevalent global genotype, is pegylated interferon (PEG IFN) combined with ribavirin (RBV) for 48 weeks.[4] However, sustained virological response (SVR), defined as the reduction of serum HCV RNA to undetectable levels 24 weeks after the completion of therapy, is achieved in only 42–52% of patients.[5–7] Moreover, response rates are influenced by patient factors such as sex, age and ethnicity,[8–10] as well as virological factors such as genotype and viral load.[11] SVR rates remain unsatisfactorily low (22%) in women aged 50 years or more who are infected with HCV genotype 1 in Japan.[12] Hence, there is a pressing need to improve the efficacy of antiviral treatment in such patients.

Recently, a new class of drugs, with a mechanism based on inhibition of the NS3/NS4 protease of the HCV polyprotein, has been investigated for the treatment of chronic hepatitis C. Of the drugs in this class, telaprevir has been selected as a clinical candidate for further development.[13] Telaprevir combined with PEG IFN and RBV has shown potent antiviral activity in phase II[14,15] and III clinical trials;[16,17] SVR rates of

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approximately 70% have been reported in patients infected with HCV-1. Similarly, in Japan, a phase III study was conducted in patients with HCV-1 to compare the efficacy and safety of the telaprevir regimen with those of the current SOC in treatment-naïve patients,[18] and to assess the efficacy and safety of the telaprevir regimen in relapsers and non-responders after previous IFN-based therapy.[19] However, the high efficacy was offset by treatment-induced anemia: early hemoglobin levels during triple therapy decreased by up to 4 g/dL, whereas decreases with SOC were not higher than 3.0 g/dL.[14,15] Additionally, we have previously reported that the factors associated with decreases in hemoglobin levels during triple therapy included female sex and age of more than 50 years.[20] Japanese patients infected with HCV genotype 1b with high viral loads are, on average, much older than Western patients infected with the same genotype, owing to a widespread HCV infection that occurred in Japan approximately 20 years ago.[21] Therefore, we considered that triple therapy would be highly effective when combined with careful monitoring of hemoglobin levels and prompt modification of RBV dose.

Consequently, in this study, we evaluated the effectiveness and safety of telaprevir-based triple therapy, administrated at an initial telaprevir dose of 2250 or 1500 mg/day, in the retrospective matched control study of 120 Japanese patients with chronic HCV-1 infection with high viral loads.

METHODS

Patients

FROM DECEMBER 2008 to August 2012, 204 patients with chronic hepatitis C were recruited to receive triple therapy with telaprevir, PEG IFN and RBV for 24 weeks at the Department of Hepatology in the Toranomon Hospital in Metropolitan Tokyo. All patients had the following characteristics: (i) positive for HCV RNA genotype 1 and antibody to HCV (anti-HCV), absence of co-infection with HCV of other genotypes; (ii) negative for hepatitis B surface antigen; (iii) HCV RNA levels of 5.0 log IU/mL or more as determined with the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan); (iv) platelet counts of more than $80 \times 10^3/mm^3$ without cirrhosis diagnosed by ultrasonography; (v) not pregnant or lactating; (vi) total previous alcohol intake of less than 500 kg; (vii) absence of HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; and (viii) absence of antiviral or immunosuppressive treatment during the previous 3 months.

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

Study design

Telaprevir (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan) was administrated at the dose of 2250 (750 mg three times daily) or 1500 mg/day (750 mg twice daily). We selected 60 patients per group who were matched by age, sex and history of previous IFN-based treatment from the telaprevir 2250 and 1500 mg/day groups (Table 1), because 204 patients had many differences in baseline characteristics in both groups. PEG IFN-α-2b (PEG-Intron; Schering Plough, Kenilworth, NJ, USA) was injected s.c. at a median dose of 1.5 μg/kg (range, 1.1–1.8) once a week. RBV (Rebetol; Schering Plough) was administrated at 200–1000 mg/day; RBV dose of 600 mg/day (for bodyweight ≤60 kg), 800 mg/day (for bodyweight >60 to ≤80 kg) or 1000 mg/day (for bodyweight >80 kg) in principle. Since November 2011, the initial dose of RBV was reduced by 200 mg in cases of female sex, aged 66 years or older, hemoglobin level of less than 13 g/dL, bodyweight of less than 45 kg or platelet counts of less than $150 \times 10^3/mm^3$ at baseline by the judgment of the physician. All participating patients received these three drugs for the initial 12 weeks, followed by PEG IFN and RBV for an additional 12 weeks. All patients were followed up for at least 24 weeks after the last dose of study drugs to assess SVR.

Doses of telaprevir, PEG IFN and RBV were reduced or their administration discontinued as required, based on the reduction of hemoglobin levels; reduction of white blood cell, neutrophil or platelet counts; or the development of adverse events. Thus, the total dose of each drug administrated during the 12–24 weeks was calculated as the ratio of the actual administrated total dose to the anticipated total dose of each drug; these ratios provided adherence measures for telaprevir, PEG IFN and RBV.

HCV RNA measurements

Blood samples were obtained at weeks 1, 2, 4, 6, 8, 12, 16, 20 and 24 after initiation of treatment and at week 24 after completion of treatment, and routine biochemical

Table 1 Baseline characteristics of the patients infected with genotype 1 HCV who received triple therapy with pegylated interferon, ribavirin and TVR

	TVR 2250 mg/day	TVR 1500 mg/day	*P*-value
n	60	60	
Sex (male/female)	30/30	30/30	Matched
Age (years)	60 (53–63)	62 (56–64)	Matched
Body mass index (kg/m^2)	22.1 (20.4–24.0)	22.7 (20.1–24.8)	0.278
IL28B genotype (rs8099917) TT/TG + GG	40/20	54/6	0.003
ITPA genotype (rs12979860) CC/CA + AA	44/16	36/23	0.175
Hemoglobin (g/dL)	14.3 (13.5–15.2)	14.2 (13.0–14.8)	0.223
Platelets (×104/μL)	17.6 (14.9–21.0)	16.9 (13.8–19.9)	0.227
Albumin (g/dL)	3.8 (3.7–4.0)	3.8 (3.7–4.1)	0.404
Alanine aminotransferase (IU/L)	35 (25–49)	37 (25–58)	0.437
γ-Glutamyltransferase (IU/L)	29 (18–49)	22 (17–39)	0.230
Creatinine (mg/dL)	0.7 (0.6–0.8)	0.6 (0.6–0.7)	0.333
Uric acid (mg/dL)	5.6 (4.9–6.5)	5.5 (4.7–6.3)	0.487
α-Fetoprotein (μg/L)	4 (3–7)	5 (3–8)	0.740
HCV RNA (log10 IU/mL)	6.8 (6.4–7.0)	6.7 (6.3–7.0)	0.551
Core a.a. 70 (wild/mutant)	38/22	45/15	0.235
Core a.a. 91 (wild/mutant)	28/32	36/24	0.200
Previous IFN-based treatment			
Naïve/relapsed/null response	23/25/12	23/25/12	Matched

Values are number with percentage in parentheses or median with interquartile range in parentheses.
a.a., amino acid; HCV, hepatitis C virus; IFN, interferon; TVR, telaprevir.

and hematological tests were performed. The antiviral effects were assessed by measuring plasma HCV RNA levels using the COBAS TaqMan HCV test. The linear dynamic range of the assay was 1.2–7.8 $\log_{10}$ IU/mL; undetectable samples were defined as negative.

Detection of amino acid substitutions in the core of HCV-1b

Amino acid (a.a.) substitutions in the HCV core region were determined using direct sequencing of polymerase chain reaction products after extraction and reverse transcription of HCV RNA. Core a.a. substitutions at positions 70 and 91 (core 70 and 91, respectively) were determined according to the methods of our previous reports.[22,23]

Determination of IL28B and ITPA genotypes

ITPA (rs1127354) and *IL28B* (rs8099917 and rs12979860) were genotyped using the Invader assay, TaqMan assay or direct sequencing, as described.[24,25]

Statistical analyses

Non-parametric tests, including the χ^2-test, Fisher's exact test, Mann–Whitney *U*-test and Kruskal–Wallis tests, were used to analyze differences in the baseline clinical profiles of patients. Kaplan–Meier analysis and the log-rank test were applied to estimate and compare serum HCV RNA elimination rates between the groups. $P < 0.05$ by two-tailed test was considered statistically significant. All analyses were performed using SPSS software version 10.1 (SPSS, Chicago, IL, USA).

RESULTS

Baseline characteristics

THE BASELINE CHARACTERISTICS of the 120 patients are listed in Table 1. There were no significant differences in the baseline characteristics between the telaprevir 2250 mg/day group and 1500 mg/day group, except for *IL28B* genotypes. Patients receiving telaprevir 1500 mg/day had a significantly higher incidence of TT in *IL28B* genotypes than did those receiving 2250 mg/day.

Initial drug doses, drug adherence and discontinuation rate up to 12 weeks

Patients receiving telaprevir 1500 mg/day had a significantly lower initial telaprevir dose and initial RBV dose than those receiving 2250 mg/day (Table 2). Telaprevir adherence was significantly lower in the 1500 mg/day

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.

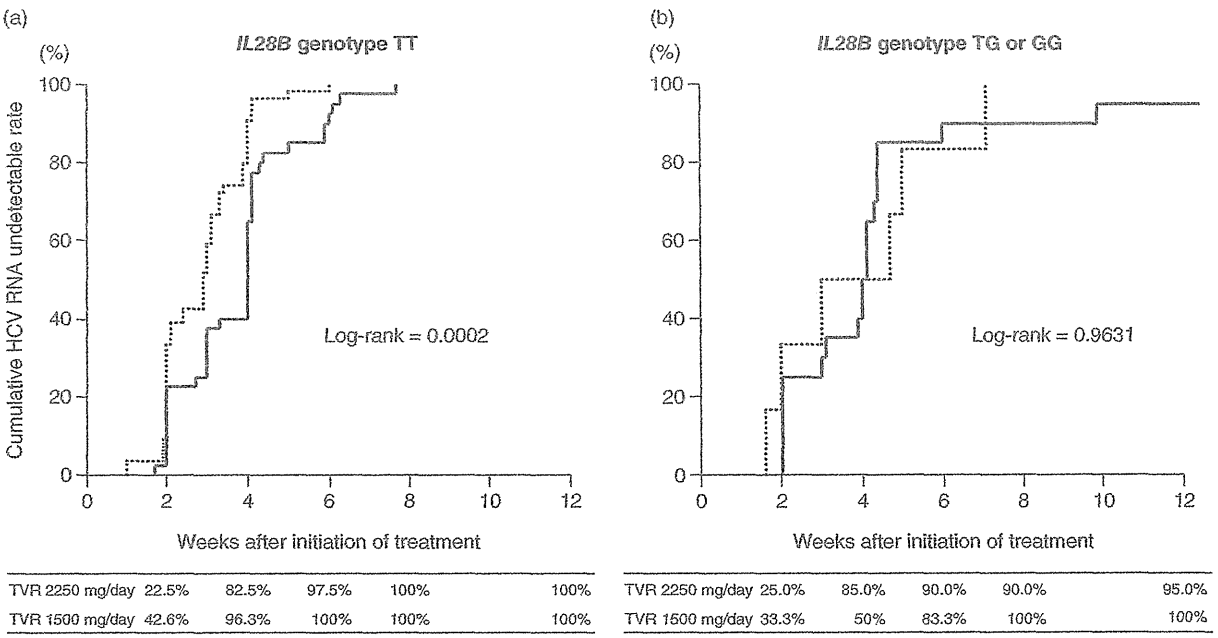

Figure 1 Cumulative rate of undetectable hepatitis C virus (HCV) RNA during triple therapy with pegylated interferon, ribavirin and telaprevir (TVR) at either 2250 mg/day or 1500 mg/day. (a) *IL28B* genotype TT, (b) *IL28B* genotype TG or GG. (———) TVR 2250 mg/day, (·····) TVR 1500 mg/day.

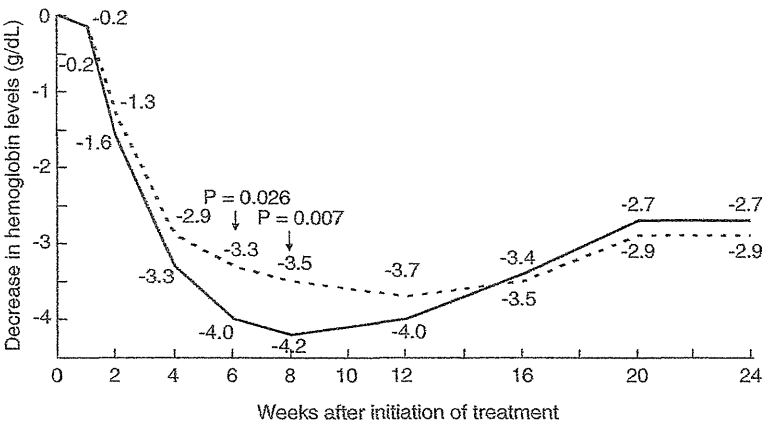

Figure 2 Decreases in hemoglobin levels during triple therapy with pegylated interferon (PEG IFN), ribavirin (RBV) and telaprevir (TVR) at either 2250 mg/day or 1500 mg/day. Each time point in this figure corresponds to median values. Patients evaluated at each time point are indicated below, with the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

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

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).

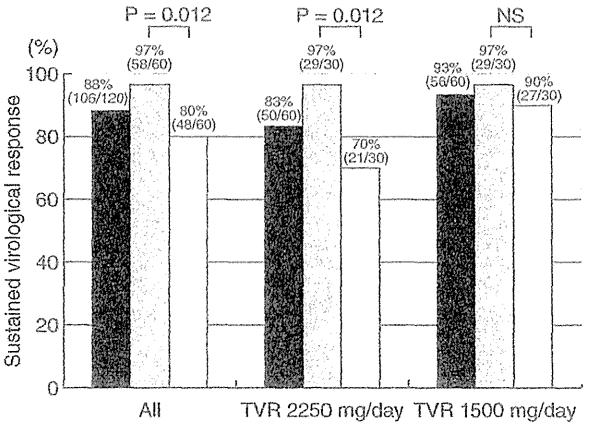

Figure 3 Sustained virological response in patients with chronic hepatitis C to triple therapy with telaprevir (TVR), pegylated interferon and ribavirin for 24 weeks. Sustained virological response was compared among all patients (men and women), TVR 2250 mg/day patients and TVR 1500 mg/day patients,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

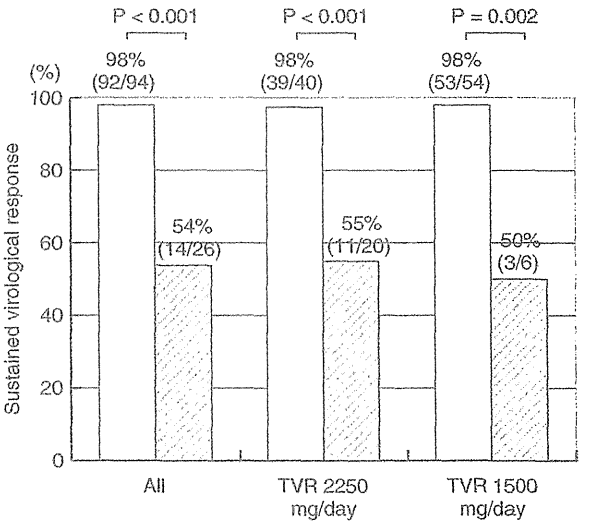

Figure 4 Sustained virological response in patients with chronic hepatitis C to triple therapy with telaprevir (TVR), pegylated interferon and ribavirin for 24 weeks. Sustained virological response was compared between *IL28B* (rs8099917) genotype TT and TG/GG in all patients, TVR 2250 mg/day patients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

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Telaprevir impairs renal function and increases blood ribavirin concentration during telaprevir/pegylated interferon/ribavirin therapy for chronic hepatitis C

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SUMMARY. We aimed to examine the relationship between renal dysfunction and anaemia that may develop during combination therapy involving pegylated interferon, ribavirin and telaprevir (PEG-IFN/RBV/TVR) for the treatment of chronic hepatitis C. Sixty-eight patients with genotype 1b high viral loads were treated with PEG-IFN/RBV/TVR. Peg-IFN and RBV doses were administered according to body weight. TVR was prescribed at 2250 mg/day for 44 patients and at 1500 mg/day for 24 patients who had low haemoglobin level (<12 g/dL). When anaemia had developed, the RBV dose was decreased. The serum TVR concentration at day 8 was measured, and the serum RBV concentration was measured serially. The estimated glomerular filtration rate (eGFR) was estimated to assess renal function. At week 1, serum TVR concentration was not correlated with a decrease in eGFR; however, the TVR dose, on a weight basis (mg/kg), and eGFR were correlated ($r = 0.2691$; $P = 0.0265$). Moreover, there was a negative correlation between eGFR and RBV serum concentration ($r = -0.3694$; $P = 0.0025$), and the serum RBV concentration and decrease in the haemoglobin were significantly correlated from week 1 to week 8. In triple therapy, the TVR dose per weight is correlated with a decline in renal function. Thus, the serum concentration of RBV increases, with a concomitant decrease in haemoglobin. It is important to adjust the doses of TVR and RBV to avoid excessive serum RBV levels and the development of severe anaemia, to achieve a good clinical effect.

Keywords: anaemia, estimated glomerular filtration rate, hepatitis C virus, ribavirin, telaprevir.

INTRODUCTION

An estimated 170 million people are chronically infected with hepatitis C virus (HCV) worldwide [1]. Approximately 30% of the patients with chronic HCV develop life-threatening liver disease, such as decompensated cirrhosis and hepatocellular carcinoma [2,3]. Since Hoofnagle *et al.* [4] first reported the effectiveness of interferon (IFN) in the treatment of the so-called non-A non-B chronic hepatitis, IFN has played a central role in the antiviral therapy for chronic hepatitis C. Until recently, combined therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV), for 48 weeks, has been the standard-of-care for patients infected with HCV genotype 1 (HCV-1), which is the most prevalent genotype worldwide. However, sustained virological response (SVR) is achieved in only 42–52% of the patients treated with this regimen [5–7].

To achieve a better antiviral effect, investigators have developed several direct-acting antivirals, represented by NS3/4A protease inhibitors and NS5B polymerase or NS5A inhibitors [8]. Of these, telaprevir (TVR), an inhibitor of the NS3/4A serine protease, in combination with PEG-IFN and RBV (triple therapy), has been most promising and has been reported to achieve an SVR of up to 70% [9–13]. However, adverse events develop more frequently in patients treated with protease inhibitors than in those treated only with PEG-IFN and RBV. In TVR trials, rash, anaemia, pruritus, nausea and diarrhoea were found to develop more frequently in individuals who received PEG-IFN and RBV along with TVR than in those individuals who received PEG-IFN and RBV only [11]. In addition, a renal functional disorder associated with TVR therapy became evident in Japan after the Ministry of Health, Labour and Welfare approved the use of TVR [14]. RBV is excreted in the urine, and diminished renal function can interfere with its metabolism [15]. In this

Abbreviations: eGFR, estimated glomerular filtration rate; IL28B, interleukin 28B; ITPA, inosine triphosphatase; RBV, ribavirin; Scr, serum creatinine; SNP, single-nucleotide polymorphism; TVR, telaprevir.

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study, we aimed to examine the relationship between renal dysfunction and anaemia in patients undergoing triple therapy for HCV.

PATIENTS AND METHODS

Patients

We enrolled 68 patients with HCV-1 who were treated with PEG-IFN/RBV/TVR at the Sapporo Kosei General Hospital. All participants provided written informed consent according to the process approved by the hospital's ethical committee, and the study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Patients were excluded if they had evidence of autoimmune hepatitis; alcoholic liver disease; congestive liver failure; hepatitis B virus infection; markers for human immunodeficiency virus; hepatocellular carcinoma or other malignancies; or hepatic decompensation, associated with jaundice, ascites, encephalopathy, or gastrointestinal bleeding. The patient characteristics are shown in Table 1.

All patients were treated with PEG-IFN-a-2b, RBV and TVR triple therapy. PEG-IFN-a-2b (MSD, Tokyo, Japan) was injected subcutaneously at a median dose of 1.5 μg/kg per week. TVR and RBV doses were adjusted according to guidelines for the treatment of hepatitis B and C, established in 2012 by the Japanese Ministry of Health, Labour and Welfare [16]. Usually, 750 mg of TVR (Mitsubishi Tanabe Pharma, Tokyo, Japan) was administered orally every 8 h after meals. The dose of RBV (MSD) was adjusted according to the individual's body weight (600 mg for individuals weighing ≤60 kg; 800 mg for individuals weighing <60 to ≤80 kg; and 1000 mg for individuals weighing >80 kg) and was orally administered after breakfast and dinner. If the initial haemoglobin (Hb) level was <14 mg/dL in women, or <13 mg/dL in men, the RBV dose was reduced by 200 mg. Triple therapy with TVR was administered for 12 weeks, followed by an additional 12 weeks of PEG-IFN-a-2b and ribavirin therapy (combination therapy). If severe anaemia was present, the dosage of RBV, followed by that of TVR, was adjusted, as determined by the chief physician.

Hepatitis C virus genotype

Hepatitis C virus genotype was determined by analysis of the sequence in the NS5B region.

Hepatitis C virus RNA levels

Hepatitis C virus RNA levels were determined using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The linear dynamic range of the assay was 1.2–7.8 $\log_{10}$ IU/mL.

Table 1 Characteristics of study patients in each telaprevir (TVR) dose

	Total	1500 mg/day	2250 mg/day	*P*
Number	68	24	44	
Sex (M/F)	34/34	4/20	30/14	<0.0001
Age (years old)	55.8 ± 10.5	59.6 ± 9.4	53.7 ± 10.6	0.0023
Height (cm)	161.4 ± 7.8	157.1 ± 6.8	163.8 ± 7.4	0.0023
Weight (kg)	62.1 ± 10.2	57.9 ± 9.0	64.5 ± 10.2	0.0090
rs12979860 (CC/TC/TT)	43/23/2	16/8/0	27/15/2	0.5186
rs1127354 (CC/CA/AA)	51/16/1	18/5/1	33/11/0	0.7695
WBC (/mm^3)	4663 ± 1271	4107 ± 1101	4934 ± 1288	0.0074
Haemoglobin (g/dL)	13.6 ± 1.2	12.9 ± 1.0	14.0 ± 1.1	0.0005
Platelet (×10^4/mm^3)	16.6 ± 4.6	15.8 ± 4.1	17.1 ± 4.8	0.3521
ALT (IU/L)	57.1 ± 47.4	51.3 ± 39.8	60.3 ± 51.3	0.2905
GGTP (IU/L)	52.1 ± 53.7	44.7 ± 44.9	56.1 ± 58.0	0.1902
estimated glomerular filtration rate (mL/min/1.73 $m^{2)}$	85.6 ± 15.2	81.5 ± 11.7	88.1 ± 16.4	0.1902
Viral genotype (1b/others)	68/0	24/0	44/0	
Virus titre (log IU/mL)	6.5 ± 0.6	6.2 ± 0.7	6.5 ± 0.7	0.2456
Pegylated interferon (μg/kg)	1.52 ± 0.11	1.54 ± 0.13	1.52 ± 0.10	0.5004
TVR (mg/kg)	32.5 ± 6.8	26.5 ± 3.9	35.8 ± 5.7	<0.0001
Ribavirin (RBV) (mg/day) (400/600/800/1000)	19/19/27/3	14/9/1/0	5/10/26/3	<0.0001
RBV (mg/kg)	10.2 ± 2.0	8.5 ± 1.2	11.2 ± 1.7	<0.0001

Data are presented as mean ± SD.

Single-nucleotide polymorphism genotyping

We genotyped each patient for two single-nucleotide polymorphism (SNP)s: rs12979860, an IL28B (interleukin 28B) SNP previously reported to be associated with therapeutic outcome [17], and rs1127354, an inosine triphosphatase (ITPA) SNP reported to be associated with ribavirin-induced anaemia [18]. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, San Diego, CA, USA) or the Invader or TaqMan assay, as described previously [19,20].

Drug concentrations

Blood samples were collected immediately prior to the administration of TVR or RBV in the morning on days 8, 15 and 29. Serum concentrations of TVR were determined at day 8 (week 1) using a high-performance liquid chromatographic (HPLC) apparatus fitted with a mass spectrometer (LC-MS/MS) (Mitsubishi Chemical Medience Corporation, Tokyo, Japan), and the serum concentration of RBV was measured at day 8 (week 1), day 15 (week 2) and day 29 (week 4) by HPLC (SRL, Inc. Tokyo, Japan) using serum stored at −80 °C.

Renal function

To assess the renal function, we used the estimated glomerular filtration rate (eGFR), which was calculated according to the equation [$194 \times (\mathrm{Scr}^{-1.094}) \times (\mathrm{age}^{-0.287}) \times$ (0.739 for women) (mL/min/1.73 m^2) serum creatinine (Scr)], as established by the Japanese Society of Nephrology in 2008 [21].

Statistical analysis

Continuous variables between groups were compared using the Mann–Whitney *U*-test, and categorical variables were compared using the Fisher's exact test. The correlation between the two groups was calculated using Spearman's rank correlation coefficient. Statistical analyses were performed using the statistical software SAS version 9.1 (SAS Institute Inc., Cary, NC, USA); a P value of <0.05 was considered significant.

RESULTS

Telaprevir dose and patient background

The TVR dose was administered according to initial Hb level and gender. The dose per weight of TVR and RBV was significantly higher in the patients receiving the 2250-mg TVR dose; however, the dose per weight of PEG did not differ according to the TVR dose. With regard to background factors, an increased age, higher proportion of women, decreased height and weight, lower white blood cell count and lower levels of Hb and eGFR were noted in the 1500-mg TVR dose group (Table 1).

Telaprevir serum concentration

The TVR serum concentration (trough value) at day 8 (week 1) of triple therapy was measured in 65 of 68 cases. The TVR concentration varied widely (1076–4598 ng/mL). The mean TVR serum concentration of patients receiving the 2250-mg TVR dose was higher than that of patients receiving the 1500-mg TVR dose (2739 ± 833 ng/mL *vs* 2361 ± 838 ng/mL, respectively); however, the difference was not statistically significant ($P = 0.075$). The patients' background factors were used as independent variables in the multiple regression analysis (Table 1), and the TVR serum concentration was estimated using the following formula: TVR concentration (ng/mL) = 56.2 × TVR (mg/kg)− 37.6 × height (cm) + 6878.4 ($R'^2 = 0.3500$, $P < 0.001$) (Fig. 1). In addition, the dose per weight of TVR (mg/kg) and the TVR serum concentration were significantly correlated ($r = 0.4795$, $P < 0.001$) (Fig. 2).

Correlation of telaprevir serum concentration, dose per weight of telaprevir (mg/kg) and decline in renal function

The patients' eGFR declined at an initial stage during triple therapy, and the decline continued for the duration of TVR treatment [baseline: 85.8, week 1: 69.6, week 2: 70.2, week 4: 69.2, week 8: 66.6, week 12: 72.5 (mL/min/1.73 m^2)] (Figure S1). The total TVR serum concentration (trough value) at week 1 of the triple therapy was not correlated with the delta eGFR; however, the dose per body weight of TVR (mg/kg) was significantly correlated with delta eGFR at week 1 and week 4, but not at week 8 (Fig. 3).

Correlation of renal function and ribavirin serum concentration

There was no correlation between the dose per weight of RBV (mg/kg) and eGFR or delta eGFR at week 1 ($P = 0.6422$ and $P = 0.1152$, respectively). However, at week 1, there was a significant negative correlation between eGFR and RBV serum concentration, and a significant positive correlation between delta eGFR and RBV serum concentration ($r = -0.3694$, $P = 0.0025$; $r = 0.3189$, $P = 0.0096$, respectively) (Fig. 4). By multiple regression analysis, the serum concentration of RBV at week 1 was estimated using the formula: RBV concentration (ng/mL) = 413.5 × sex (male = 1, female = 2) + 12.8 × age (years) + 163.5 × RBV (mg/kg)−5.9 × eGFR (1 week)−1291.6 ($R'^2 = 0.4631$, $P < 0.001$) (Fig. 1). Thus, in addition to age, sex and the dose per weight of

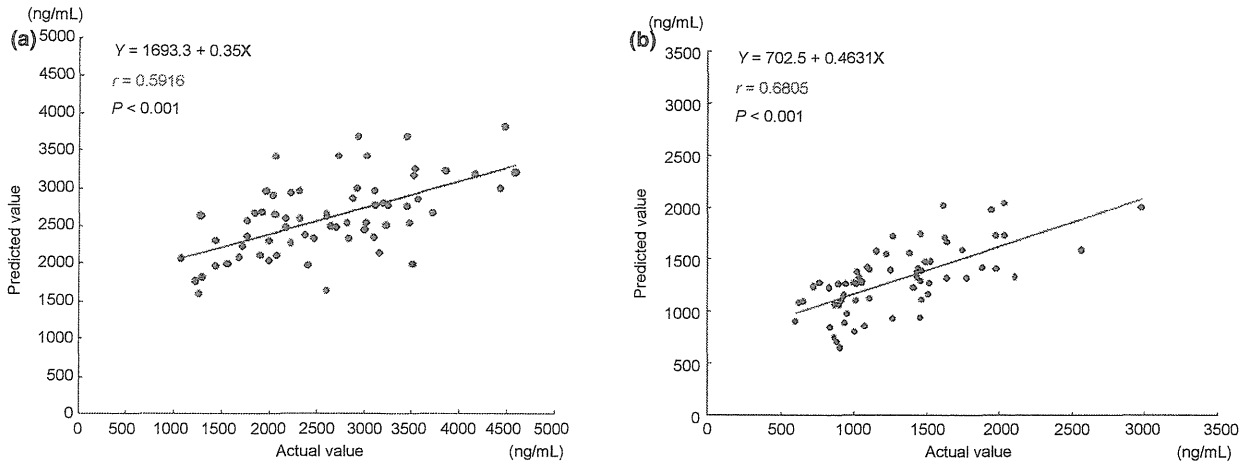

Fig. 1 (a) Correlation of TVR serum concentration actual value and predicted value. The actual TVR serum concentrations and predicted values, calculated by the formula derived from multiple regression analysis, are significantly correlated; (b) correlation of RBV serum concentration actual value and predicted value. The actual values of RBV serum concentration and the predicted values, calculated by the formula derived from multiple regression analysis, are significantly correlated.

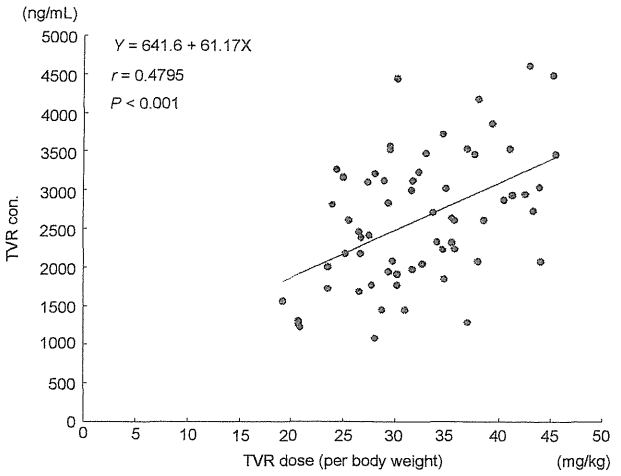

Fig. 2 Correlation of TVR serum concentration and quantity of TVR (mg/kg). The TVR serum concentration and dose per weight (mg/kg) are significantly correlated.

RBV (mg/kg), eGFR was also correlated with the RBV serum concentration.

Correlation of telaprevir, ribavirin serum concentration and haemoglobin level

The TVR serum concentration at week 1 was significantly negatively correlated with Hb levels at week 3, 6, 7 and 8 of treatment and was significantly positively correlated with the delta Hb level only at week 3 of treatment. The RBV serum concentration at week 1 showed a significant negative correlation with the Hb levels from week 2 to week 8 and a significant positive correlation with the delta Hb levels from week 1 to week 8. Moreover, the RBV serum concentration at week 2 and week 4 showed a significant negative and positive correlation with the Hb levels and the delta Hb levels, respectively (Table S1).

DISCUSSION

As RBV is usually eliminated by renal filtration, the development of renal failure would result in the accumulation of RBV, particularly in red blood cells, and may induce haemolytic anaemia. Triple therapy that includes TVR has two well-known serious side effects: anaemia and dermatopathy. In addition to these side effects, a decline in renal function, which was noted in Japan after TVR was made commercially available, is now recognized as a significant problem; this decline in renal function was not noted with PEG-IFN and RBV combination therapy. Renal dysfunction has also recently been associated with boceprevir use [22,23]. The mechanism responsible for the decline in renal function caused by TVR remains unknown; however, the risk factors in Japanese patients include receiving a 2250-mg TVR dose, advanced age and low Hb levels at the start of the therapy.

The serum concentration of TVR reportedly reaches a steady state in 2–7 days of treatment [24,25]. In the present study, the trough serum concentration of TVR at day 8 of triple therapy was estimated through multiple regression analysis using the formula: TVR (ng/mL) = 56.2 × TVR (mg/kg)−37.6 × height (cm) + 6878.4. The dose per weight of TVR (mg/kg) was the strongest determinant of the TVR serum concentration. The renal function impairment in patients treated with the TVR/PEG-IFN/RBV combination therapy was noted at an early stage in the treatment and persisted throughout the TVR treatment duration, but gradually improved after TVR treatment was

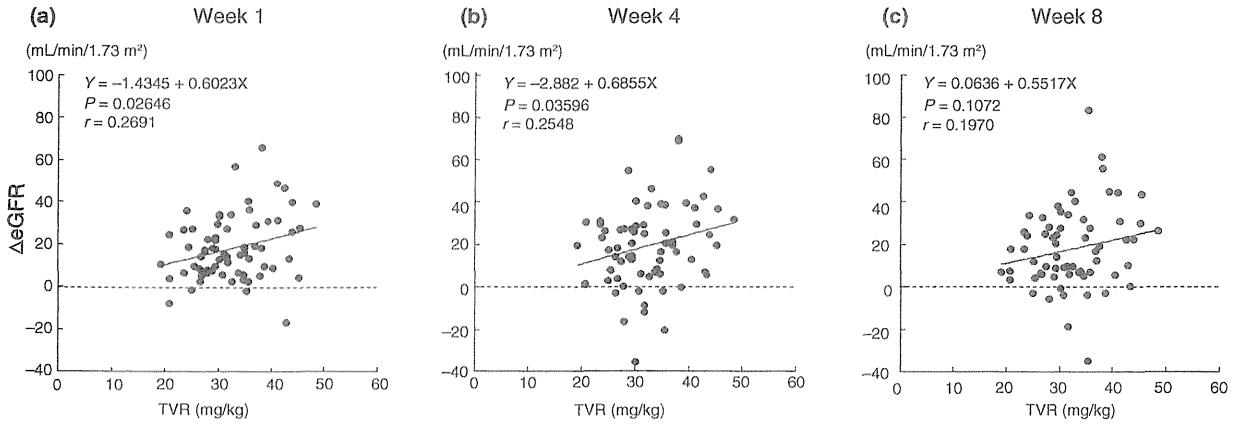

Fig. 3 Correlation between TVR (mg/kg) and delta eGFR. The dose per weight of TVR (mg/kg) is significantly correlated with the delta eGFR, indicating a decline in renal function from the baseline, at week 1 and week 4, but not at week 8 of triple therapy.

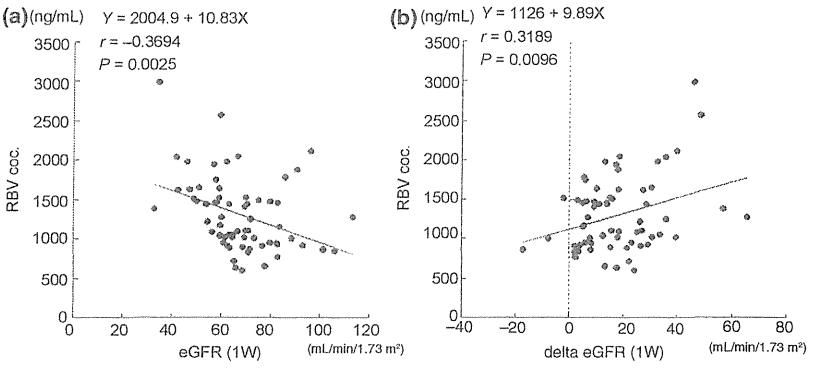

Fig. 4 Correlation between RBV concentration (Week 1) and eGFR (Week 1) and delta eGFR (Week 1). The eGFR at week 1 is negatively correlated with eGFR, whereas the serum RBV concentration at week 1 is positively correlated with delta eGFR.

discontinued. In patients receiving the TVR/PEG-IFN/RBV combination therapy, although the serum TVR concentration at week 1 was not significantly correlated with the decline in renal function during week 1, the dose per weight of TVR (mg/kg) showed a significant correlation with the decline in renal function during week 1. Thus, the degree of renal function impairment worsened with an increase in the dose per weight of TVR (mg/kg). Furthermore, because RBV is eliminated by renal filtration [15], it is expected that a decline in renal function will result in an increase in serum RBV concentration. In the present study, the serum concentration of RBV at week 1 showed a significant negative correlation with eGFR and significant positive correlation with delta eGFR. The RBV serum concentration was estimated through multiple regression analysis using the formula: RBV concentration (ng/mL) = 413.5 × sex (male = 1, female = 2) + 12.8 × age (years) + 163.5 × RBV (mg/kg) − 5.9 × eGFR (week 1) − 1291.6. In addition to sex, age and dose per weight of RBV, the eGFR at week 1 was a regulating factor for RBV serum concentration. After examining the relationships among TVR serum concentration, RBV serum concentration, and Hb level, the TVR serum concentration at week 1 was negatively correlated with the Hb level at week 3, 6, 7 and 8 and was positively correlated with the delta Hb level at week 3. The RBV serum concentration was negatively correlated with the Hb level from week 2 to 8 and positively correlated with the delta Hb level from week 1 to 8. Similarly, the serum RBV concentration at weeks 2 and 4 was correlated with the Hb level and the delta Hb level, respectively.

In patients receiving TVR-containing triple therapy, the development of anaemia can lead to dose reduction or discontinuation of the drug. The administration of TVR or RBV alone can cause anaemia. However, the renal dysfunction caused by TVR can exacerbate this problem by increasing the serum concentration of RBV and thus increase the severity of haemolytic anaemia.

Because TVR was administered at a fixed dose of 2250 mg/day during the third phase of the clinical trial for TVR/PEG-IFN/RBV combination therapy in Japan, the dose per weight of TVR varied widely (25.7–55.3 mg/kg) [13]. Moreover, by multivariate analysis, we noted that the factor that contributed significantly to a decline in the Hb level to less than 8.5 g/dL – which necessitated the discontinuation of TVR/PEG-IFN/RBV combination therapy – was a relatively high dose per weight of TVR [26]. In the present

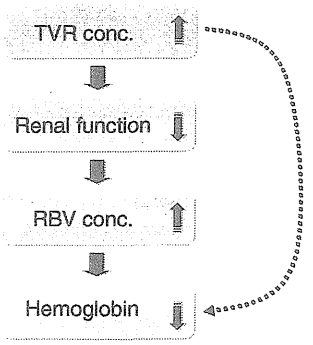

Fig. 5 Cascade reaction of RBV induced anaemia during triple therapy. In the triple therapy, as the TVR concentration increases, renal function (and consequent excretion of RBV) decreases; the RBV serum concentration increases, and the Hb level decreases. In addition, TVR has a mild direct hemolytic effect (interrupted arrow on the right), contributing to the decrease in Hb concentrations.

study, although the TVR dose was reduced to 1500 mg/day in a few female patients and in those with low baseline Hb level, the dose per weight of TVR varied widely (19.2–48.5 mg/kg). A decline in the eGFR at week 1 was related to the dose per weight of TVR, and the RBV serum concentration at week 1 was related to the decline in the eGFR at week 1; moreover, a higher RBV concentration at week 1 was related to a greater decline in Hb levels.

In summary, we noted a certain sequence of events that can occur during anti-HCV treatment that includes TVR: (i) the starting dose of TVR, when not calculated on a per weight basis, may be high, (ii) TVR causes a reduction in renal function, with a reduction in the excretion of RBV, (iii) the blood concentration of RBV rises and (iv) the increased RBV concentration, in addition to the inherent tendency for TVR to lower Hb levels, leads to haemolytic anaemia (Fig. 5). The declining Hb levels may require discontinuation of the regimen or reduction in the anti-HCV drug doses and, consequently, may result in a reduced antiviral effect. For patients requiring TVR/PEG-IFN/RBV combination therapy, the interactions of TVR and RBV should be considered while determining the optimal doses of TVR and RBV.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Correlation between serum concentration of RBV or TVR and Hb, as well as delta Hb levels. The reduction in the eGFR is evident as early as week 1 of therapy and persists for the duration of treatment.

Figure S1. The reduction in the eGFR is evident as early as week 1 of therapy and persists for the duration of treatment.

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Hallmarks of Hepatitis C Virus in Equine Hepacivirus

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ABSTRACT

Equine hepacivirus (EHcV) has been identified as a closely related homologue of hepatitis C virus (HCV) in the United States, the United Kingdom, and Germany, but not in Asian countries. In this study, we genetically and serologically screened 31 serum samples obtained from Japanese-born domestic horses for EHcV infection and subsequently identified 11 PCR-positive and 7 seropositive serum samples. We determined the full sequence of the EHcV genome, including the 3′ untranslated region (UTR), which had previously not been completely revealed. The polyprotein of a Japanese EHcV strain showed approximately 95% homology to those of the reported strains. HCV-like *cis*-acting RNA elements, including the stem-loop structures of the 3′ UTR and kissing-loop interaction were deduced from regions around both UTRs of the EHcV genome. A comparison of the EHcV and HCV core proteins revealed that Ile^{190} and Phe^{191} of the EHcV core protein could be important for cleavage of the core protein by signal peptide peptidase (SPP) and were replaced with Ala and Leu, respectively, which inhibited intramembrane cleavage of the EHcV core protein. The loss-of-function mutant of SPP abrogated intramembrane cleavage of the EHcV core protein and bound EHcV core protein, suggesting that the EHcV core protein may be cleaved by SPP to become a mature form. The wild-type EHcV core protein, but not the SPP-resistant mutant, was localized on lipid droplets and partially on the lipid raft-like membrane in a manner similar to that of the HCV core protein. These results suggest that EHcV may conserve the genetic and biological properties of HCV.

IMPORTANCE

EHcV, which shows the highest amino acid or nucleotide homology to HCV among hepaciviruses, was previously reported to infect horses from Western, but not Asian, countries. We herein report EHcV infection in Japanese-born horses. In this study, HCV-like RNA secondary structures around both UTRs were predicted by determining the whole-genome sequence of EHcV. Our results also suggest that the EHcV core protein is cleaved by SPP to become a mature form and then is localized on lipid droplets and partially on lipid raft-like membranes in a manner similar to that of the HCV core protein. Hence, EHcV was identified as a closely related homologue of HCV based on its genetic structure as well as its biological properties. A clearer understanding of the epidemiology, genetic structure, and infection mechanism of EHcV will assist in elucidating the evolution of hepaciviruses as well as the development of surrogate models for the study of HCV.

The *Flaviviridae* family is composed of four genera: *Flavivirus*, *Pestivirus*, *Pegivirus*, and *Hepacivirus*. *Flaviviridae* family viruses are enveloped and contain a single-stranded, positive-sense RNA genome, which encodes a single large precursor polyprotein composed of approximately 2,800 to 3,000 amino acids. The genus *Hepacivirus* had included only two species, hepatitis C virus (HCV) and GB virus B (GBV-B), until 2010. GBV-B was isolated from serum samples obtained from laboratory tamarins by 11 passages of serum obtained from a human patient with idiopathic hepatitis (1). Although GBV-B experimentally infects tamarins and common marmosets, but not chimpanzees, *in vivo* (2, 3), the natural host of GBV-B has not yet been clarified. Several hepacivirus species were recently detected in dogs, horses, bats, and rodents and tentatively designated nonprimate hepaciviruses (NPHVs). Bat hepaciviruses have been isolated from some species of bats in Kenya (4), while rodent hepaciviruses have been isolated from several species of rodents in Germany, the Netherlands, South Africa, and Namibia (5, 6). GBV-B is phylogenetically more similar to rodent hepacivirus than to HCV (5). Several strains of equine hepacivirus (EHcV) have been isolated from domestic horses in the United States, the United Kingdom, and Germany (5, 7, 8). The canine hepacivirus was isolated from dogs in the United States (9) but has not yet been genetically or serologically detected in any dogs other than those from the first report (5, 7, 8). The polypeptides of canine hepacivirus show approximately 95% amino acid homology to those of the EHcV strains, suggesting that canine hepacivirus may belong to the same species as EHcV and

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that infections may be rare in dogs (5, 7, 8, 10). Recent phylogenetic analyses identified EHcV as the most closely related viral homologue of HCV among the reported NPHV strains; however, epidemiological and virological information on EHcV is limited. The open reading frames of EHcV strains show approximately 95% homology to one another, suggesting that previously reported EHcV strains may be classified into one species. Several genome sequences of rodent hepacivirus have already been completely determined (5). The 3′ untranslated region (UTR) of HCV was found to include three stem-loop (SL) structures, while variable stem-loop structures were found in that of rodent hepacivirus and GBV-B (5). However, the nucleotide sequence of the EHcV 3′ UTR has not yet been determined completely because the adenine-rich [(A)-rich] sequence downstream of the stop codon in the EHcV genome interrupts an ordinary 3′-rapid amplification of cDNA ends (RACE) reaction (8). The RNA secondary structure of the hepacivirus 3′ UTR may indicate species specificity (5).

On the basis of amino acid similarities among the polyproteins of NPHVs and HCV, the N-terminal one-fourth of the NPHV polyprotein has been predicted to be cleaved by signal peptidase into mature structural proteins and a viroporin (core, E1, E2, and p7), while the C-terminal three-fourths has been predicted to be cleaved by viral proteases into maturated nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (6). Core, E1, and E2 have been predicted to form viral particles with host lipids, although it remains unclear whether p7 is incorporated into a viral particle. Signal peptide peptidase (SPP) was shown to further cleave the C-terminal transmembrane region of HCV and GBV-B core protein after signal peptidase-dependent cleavage (11, 12). However, whether SPP cleaves the C-terminal transmembrane region of the NPHV core protein remains unknown.

The mature core proteins of HCV and GBV-B are localized mainly on lipid droplets (LDs) (13, 14). The core proteins of dengue virus are also localized on LDs but are not cleaved by SPP (15), suggesting that localization of the core protein on LDs may be one of the common characteristics of the *Flaviviridae* family. The HCV core protein is known to be partially localized in the detergent-resistant membrane (DRM), which originates from lipid raft-like membranes (16, 17). The DRM is composed of cholesterol and sphingolipids, which are included in the replication compartment known as the membranous web (18, 19). Therefore, LDs and DRM are considered to be the intracellular compartments for the replication and viral assembly of HCV, but it is currently unknown whether NPHV core proteins are localized on LDs and DRM.

Epidemiological information on EHcV is still limited. The results of the present study demonstrated that Japanese-born domestic horses were infected with EHcV, which showed high homology to the reported strains on the basis of its nucleotide and amino acid sequences. We predicted the RNA secondary structures around the 5′ and 3′ UTRs of the EHcV genome and analyzed the biological properties of the EHcV core protein in relation to the HCV core protein.

MATERIALS AND METHODS

Samples. Serum samples 1 to 13 were collected from Japanese-born domestic horses raised on one farm, farm A, located in Hokkaido, Japan, while groups of serum samples numbered 14 to 18 and 19 to 31 were from horses on farms B and C, respectively, located in Tokyo, Japan (Fig. 1). The distance between Hokkaido and Tokyo is about 1,000 km. All sample collections conformed to guidelines for the care and use of laboratory animals (Yamanashi University) and were approved by the Institutional Committee of Laboratory Animal Experimentation (Yamanashi University). All samples were divided into small aliquots and stored at −80°C until nucleic acid extraction.

RT-PCR. Total RNAs were prepared from horse sera using a Qiagen viral RNA extraction kit (Qiagen, Valencia, CA). RNAs were converted to cDNA using a PrimeScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Shiga, Japan) with random primers. The viral gene was amplified by PCR using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ) with three pairs of primers: NPHV-F1 (5′-TGTCACCT ACTATCGGGG-3′) and NPHV-R1 (5′-TCAAGCCTATACAGCAAAG G-3′), NPHV-F2 (5′-ATCATTTGTGATGAGTGCC-3′) and NPHV-R2 (5′-CATAAGGGCGTCCGTGGC-3′), and NPHV-F3 (5′-GTGGTCGCC ACGGATGCC-3′) and NPHV-R3 (5′-ACCCTATGAAGACGCTCTCC-3′). PCR was carried out as follows: one cycle at 92°C for 5 min; 35 repeats of one cycle at 94°C for 0.5 min, 58°C for 0.5 min, and 72°C for 0.5 min, in that order; and one cycle at 72°C for 1 min followed by holding at 4°C. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized using the BioDoc-It imaging system (UVP, Upland, CA).

Determination of the EHcV genomic sequence. The viral genome of EHcV was segmentally amplified by PCR using the primers listed in Table 1. The PCR products were cloned into T vectors prepared from pBluescript II SK(−) (20). The DNA sequences of the PCR products were determined using an ABI Prism BigDye Terminator version 1.1 cycle sequencing kit and an ABI Prism 310 genetic analyzer (Life Technologies, Tokyo, Japan). More than three colonies were picked up among the transformants of *Escherichia coli* with regard to the accuracy of the sequence. The nucleotide sequences of the PCR products were determined in forward and reverse directions. The junction of two adjacent PCR products was confirmed by PCR using primers that overlapped two close regions. The 5′-terminal sequence upstream of the open reading frame was determined with a 5′-RACE core set (TaKaRa) using the 5′ phospholyrated RT primer for the NPHV 5′ UTR (5′-CATCCTATCAGACCG-3′). The 3′-terminal region downstream of the (A)-rich region was determined by the 3′-RACE method (21, 22), modified as follows: Total RNAs were prepared from horse serum using TRIzol LS reagent (Invitrogen, Carlsbad, CA) with 40 μg of glycogen (Nacalai Tesque, Kyoto, Japan). The poly(U) tail was added to the 3′ end of the RNA preparation using *Escherichia coli* poly(U) polymerase (New England BioLabs, Ipswich, MA) and was incubated for 45 min at 37°C. The resulting preparation was reverse transcribed by the SuperScript First-Strand Synthesis system (Life Technologies) using an oligo(dA) adapter primer (5′-TTGCGAGCACAGAATTAATACG ACTCACAAAAAAAAAAAAVN-3′). The sequence of each region was determined by sequencing more than 3 clones. The primers for PCR amplification and the RACE methods are listed in Table 1. The whole sequence of the EHcV strain isolated from serum sample 3 (GenBank accession number AB863589) was determined by the method described above. The EHcV strain was designated JPN3/JAPAN/2013 in this study. The partial NS5B-coding regions and 3′ UTRs were amplified from serum samples 5 and 1. The nucleotide sequences of samples 5 and 1 (GenBank accession numbers AB921150 and AB921151, respectively) were determined by the method described above. The neighbor-joining trees of the nucleotide sequences from the NPHV, HCV, and GBV-B strains were predicted by the method of Saitou et al (23). Trees were constructed by the maximum composite likelihood method calculated by using the program MEGA5 (24) (see Fig. 3). The secondary protein structures were predicted by the method of Garnier et al. (25) (see Fig. 6). Hydrophobicity plots of the EHcV and HCV core proteins were prepared by the method of Kyte and Doolittle (26) and drawn using the software Genetyx (Nihon Genetyx, Tokyo, Japan) (see Fig. 5).

Quantification of viral genomic RNAs in horse sera. Total RNA was prepared from equine serum using a Qiagen viral RNA extraction kit and was then reverse transcribed into cDNA by using a PrimeScript RT-PCR

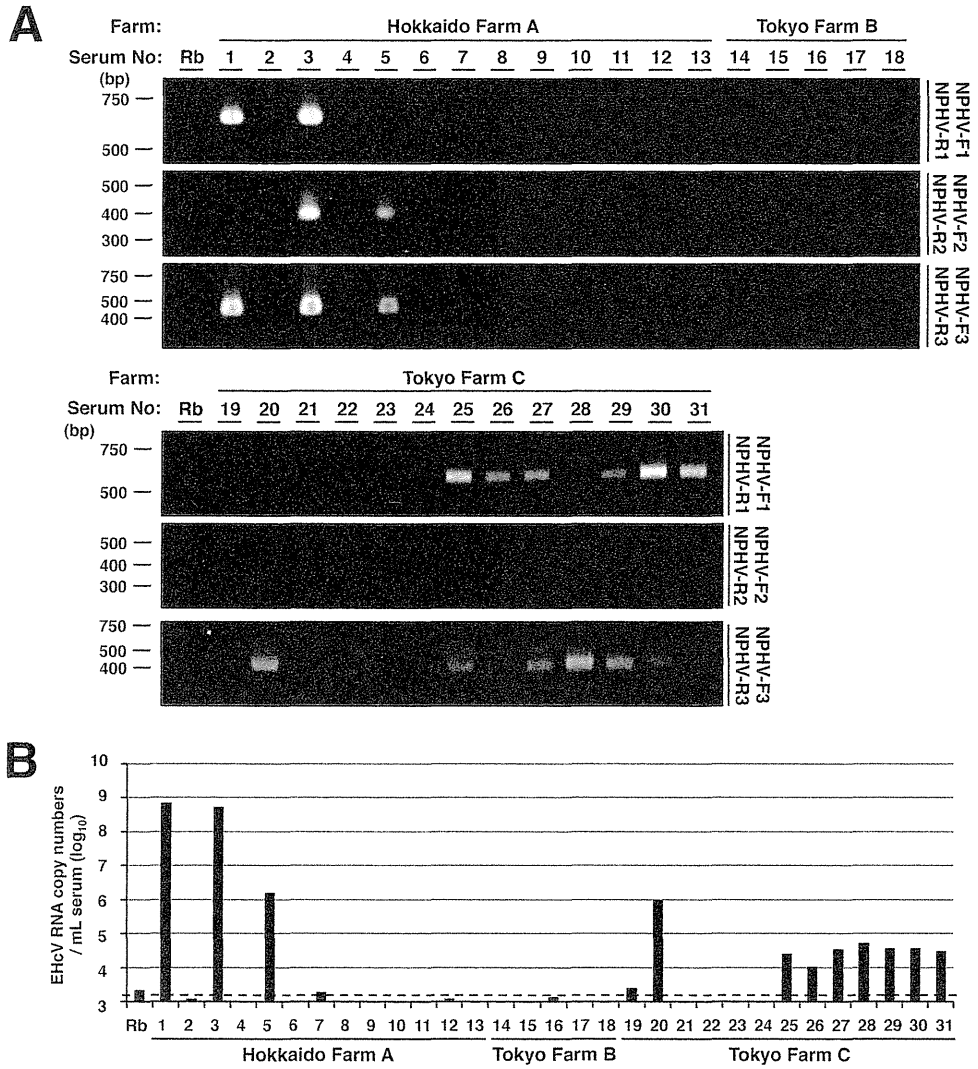

FIG 1 Detection and genetic analyses of NPHV genomic RNA in sera of Japanese domestic horses. (A) Total RNAs extracted from 31 equine sera and normal rabbit serum (Rb) as a negative control were subjected to RT-PCR analysis. Hokkaido Farm A, Tokyo Farm B, and Tokyo Farm C indicate the farms where the individual horses were reared. Three sets of primers, NPHV-F1 and NPHV-R1, NPHV-F2 and NPHV-R2, and NPHV-F3 and NPHV-R3, were used to amplify NPHV-specific gene regions. The PCR products were electrophoresed and stained with ethidium bromide. (B) Total RNAs were isolated from sera, reverse-transcribed, and estimated as a copy number per ml. Normal rabbit serum was used as a negative control. The dashed line indicates the cutoff level.

kit with random primers. The amount of targeted viral RNA was estimated using SYBR GreenER qPCR SuperMix (Life Technologies) and the ABI StepOnePlus real-time PCR system (Life Technologies). The region encoding NS3 was targeted with the primer pair NPHV-F3 (5′-GTGGTC GCCACGGATGCC-3′) and NPHV-R3 (5′-ACCCTATGAAGACGCTC TCC-3′). Total RNAs extracted from conventional rabbit serum were used as a negative control to determine the analytical threshold line. The *in vitro*-transcribed RNA of EHcV was utilized for the standard curve.

Prediction of RNA secondary structures. The 5′-UTR sequences of EHcV strains were aligned with the MUSCLE program and subjected to a manual search for covariant nucleotide substitutions. The RNA folding structure upstream of domain III in the 5′ UTR was predicted using the Mfold web server (27) with conventional phylogenetic conservation analysis due to the lack of sufficient homology to the 5′ UTR sequences of HCV strains. The NS5B-coding regions and 3′ UTRs of EHcV strains were aligned with the program MUSCLE. Conserved secondary structures were predicted as described above. The secondary structures of the 3′ UTR in EHcV were predicted by the Mfold web server without confirming phylogenetic data because of the absence of additional available sequences of the EHcV 3′ UTR and the lack of sufficient homology to the HCV X-tail sequences.

Plasmids. The PCR product encoding the EHcV core protein was amplified from serum sample 3 and was then cloned into the BamHI and XhoI sites of pcDNA3.1-Flag/HA, which encodes the FLAG and hemagglutinin (HA) epitope tags, as reported previously (28). Ala^{204} was replaced with Lys to prevent signal peptidase-dependent cleavage. The translated EHcV core protein was added to the FLAG and HA epitope tags at the N and C termini (EHcVc), respectively. A point mutation was generated using a KOD mutagenesis kit (Toyobo, Osaka, Japan). The PCR products encoding EHcVc or the mutant in which Ile^{190} and Phe^{191} were replaced with Ala and Leu (EHcVc-mt), respectively, were introduced into the AflII and EcoRV sites of pCAGGS using an In-Fusion HD cloning kit (TaKaRa). The introduced fragments of all plasmids were confirmed by sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems). The plasmid encoding the N-terminally FLAG-tagged and C-terminally HA-tagged HCV core protein (HCVc) and the mutant in which

TABLE 1 List of PCR primers used in this study

Primer	Nucleotide position	Genome location	F or R[a]	Sequence (5′→3′)
Primer for cloning of the NPHV genome	92–111	5′ UTR	F	ATGTGTCACTCCCCCTATGG
	367–386	5′ UTR	R	CTATGGTCTACGAGACCGGC
	268–285	5′ UTR	F	AGCCGAAATTTGGGCGTG
	1207–1224	E1	R	AAACAGAAGCCATAGCGG
	1116–1132	E1	F	AGTGCTTGTTGGGTGCC
	1697–1713	E2	R	GTCCTTTGCACTTCGGG
	1605–1623	E2	F	ACTGTTAAGCAGATGTGGG
	2103–2121	E2	R	CACAGAGTTGGTAAGTAGC
	2007–2023	E2	F	AAGCAGTGTGGTGCTCC
	2526–2545	E2	R	AAACAGAACCAGAGAATTGC
	2375–2392	E2	F	CCCTGCCTTCACTACTGG
	2898–2913	NS2	R	CGAGATAGCGCCAAGC
	2847–2867	NS2	F	TTTATGCTAGTAAAGTGGTGG
	3396–3415	NS2	R	GGTGATAAAAGTCTCCATCC
	3318–3334	NS2	F	ATCCTCCATGGCTTGCC
	3819–3835	NS3	R	GGGCCACCTGAACTACC
	3732–3750	NS3	F	ACCAGGACGGGTCAGGTCG
	4254–4270	NS3	R	ATAATGTCATAAGCACC
	4177–4195	NS3	F	CTAGTTGCAAGACAACGGG
	4682–4700	NS3	R	AGTGTTGCAGTCAGTGACG
	4574–4591	NS3	F	TGTCACCTACTATCGGGG
	5199–5218	NS3	R	TCAAGCCTATACAGCAAAGG
	4574–4591	NS3	F	TGTCACCTACTATCGGGG
	5199–5218	NS3	R	TCAAGCCTATACAGCAAAGG
	5134–5152	NS3	F	CTCCCAGCAAAGATGAACG
	5997–6014	NS4B	R	AGCACCCACACCAACAGC
	5919–5934	NS4B	F	AAGATCTTGAGTGGTG
	6651–6632	NS5A	R	GCCGATAACTCTGACAGC
	6547–6564	NS5A	F	ACACCTGGAAAACAGCCG
	7293–7310	NS5A	R	AGATTCCGTGGCCGAAGG
	7235–7252	NS5A	F	AGCTCTCGTTTCCGGGTG
	7573–7590	NS5B	R	TAGCTGACGCTGTTGTGG
	7511–7527	NS5B	F	ACGCCACCCTATAGGCC
	8027–8046	NS5B	R	GTTGACGGGGAGTGTATTGG
	7926–7943	NS5B	F	ATCGTTTACCCCGATTTG
	8528–8545	NS5B	R	CAAGATGTTATCTGCTCC
	8457–8474	NS5B	F	CGTGACTTCACTAATGCC
	9069–9086	NS5B	R	GTCAATCGAGTTTACGCC
Primer for 5′ RACE	235–252	5′ UTR	F	AATCGCGGCTTGAACGTC
	213–230	5′ UTR	R	TGTACTCACGGATTCACG
Primer for 3′ RACE	8979–8999	NS5B	F	CTTAAAGTACGTGGTGGTCGC
Adapter primer			R	GCGAGCACAGAATTAATACGAC

[a] F, forward; R, reverse.

Ile[176] and Phe[177] were replaced with Ala and Leu (HCVc-mt), respectively, were described previously (28). The gene encoding human signal peptide peptidase (SPP) or its mutant was introduced into pcDNA3.1-myc/His C (Invitrogen) instead of the plasmids described previously (28). The resulting plasmids encoded C-terminally myc-$His_{\times 6}$-tagged wild-type SPP (SPP-wt) or the mutant protein in which Asp[219] was replaced with Ala (SPP-D219A).

Cell culture and transfection. The human embryonic kidney cell line 293FT and the human hepatoma cell line Huh7OK1 (29) were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids (Sigma, St. Louis, MO), sodium pyruvate (Sigma), and 10% fetal bovine serum (FBS) and were then cultured at 37°C under the conditions of a humidified atmosphere and 5% CO_2. Plasmids were transfected into cell lines using XtremeGene 8 (Roche) according to the manufacturer's protocol.

Western blot analysis. 293FT cells were cultured in 6-well plates and transfected with the appropriate plasmids. The transfected cells were harvested at 18 h posttransfection, washed with cold phosphate-buffered saline (PBS), and suspended in 50 μl of the lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 135 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail (Merck Bioscience, Calbiochem, San Diego, CA). The lysates were centrifuged at 19,000 × *g* for 5 min at 4°C. The supernatants were mixed with 16 μl of 4× SDS sample buffer and then boiled at 60°C for 20 min. The resulting mixtures were subjected to SDS-PAGE. The proteins in a gel were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with mouse anti-FLAG antibodies (Sigma), mouse anti-HA antibodies (Covance, Princeton, NJ), mouse