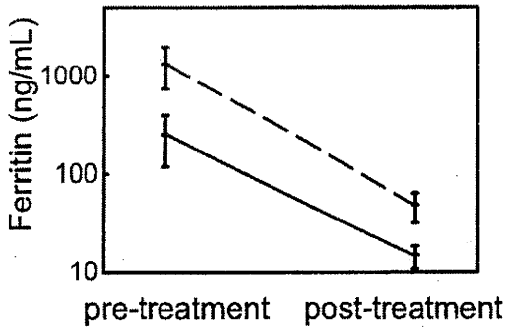

Figure 2. Reduction of serum ferritin levels in the 2 patient groups by venesection. Note that the endpoints were set differently: 20 ng/mL for chronic hepatitis C (a straight line), and 50 ng/mL for HFE-hemochromatosis (a dotted line). High starting points of 1347±620 ng/mL in hemochromatosis patients indicate severe iron overload in the genetic disorder as compared to those of 250±132 ng/mL in chronic hepatitis C patients. Vertical bars indicate mean ± SD.

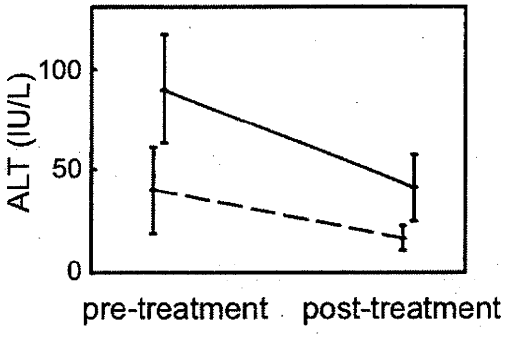

Figure 3. Reduction of serum ALT levels in the 2 patient groups by venesection. The parameter of biochemical liver disease was normalized in HFE-hemochromatosis (dotted line), but remained slightly elevated in chronic hepatitis C (straight line), suggesting that viral insult persisted after iron hepatotoxicity was removed. ALT; alanine aminotransferase. Vertical bars indicate mean ± SD.

with HFE-hemochromatosis might have caused a false negative result.

There were no side effects of venesection requiring discontinuation of therapy. Hemoglobin concentration remained normal during venesection in hemochromatosis patients but decreased slightly in CHC patients. The serum ferritin concentrations used for monitoring body iron stores decreased linearly from 250 ± 32 ng/mL to levels of <20 ng/mL in CHC, and from 1,347 ± 620 ng/mL to levels <50 ng/mL in hemochromatosis (Fig. 2). The treatment effectively reduced ALT to only slightly increased levels in CHC, and normalized these levels in hemochromatosis (Fig. 3). One exceptional patient with hemochromatosis showed an increase in the liver enzyme level from 15 to 23 U/L. Serum hepcidin was significantly decreased in both CHC and hemochromatosis reaching comparable levels after iron depletion (2.1 ± 2.3 ng/mL in hemochromatosis vs. 2.0 ± 1.4 ng/mL in

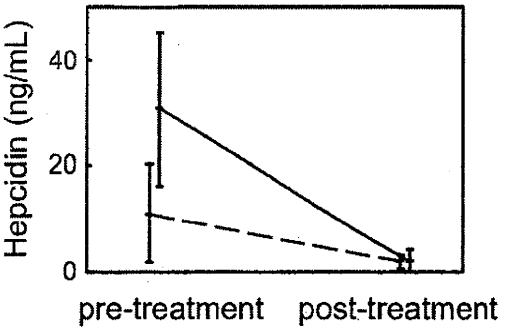

Figure 4. Reduction of serum hepcidin levels in the 2 patient groups by venesection. Serum hepcidin 25 levels of chronic hepatitis C (straight line) were similar to those of controls at the pre-treatment stage, while those of HFE-hemochromatosis (dotted line) were low at 11.1±9.2 ng/mL. In both patient groups, hepcidin levels were reduced to quite low levels after venesection. It is clear that the hepcidin system is set at lower levels in HFE-hemochromatosis than in chronic hepatitis C. Genetic setting with low hepcidin regulation caused a large amount of iron absorption in the intestine over 50 years. Vertical bars indicate mean ± SD.

CHC) (Fig. 4). One exceptional patient with hemochromatosis showed an increase in serum hepcidin from 3.5 to 7.7 ng/mL.

The regimens and effects of venesection for patients with CHC and hemochromatosis are summarized in Table 2. Estimated body iron stores of 730 ± 560 mg were removed from hepatitis patients over 7±3 months, while 5,960 ± 2,750 mg were removed from hemochromatosis patients over 15 ± 8 months. There were differences in the body iron stores and treatment periods between the 2 patient groups. In CHC patients, reduction of ALT activity by venesection was larger than that in hemochromatosis patients (49 ± 30 vs. 22 ± 20 U/L). There were correlations between the reduction in ALT activity and pre-treatment ALT activity in both patient groups (Fig. 5, 6). IHI was quite low in hemochromatosis with a significant difference between the 2 patient groups (0.097 ± 0.083 in CHC vs. 0.0032 ± 0.0046 U/L/mg in hemochromatosis).

Discussion

In the present paper, we compared the behavior of serum iron indices including hepcidin-25 and liver enzymes in CHC and HFE-hemochromatosis during the first stage of venesection to remove body iron stores. Since, in addition to genetic background, dietary customs between Italians and Japanese, and the venesection protocols used for CHC and hemochromatosis are different, this comparative study may involve confounding factors. However, most of the findings observed are potentially significant. Considering that both conditions responded to venesection, iron hepatotoxicity is involved in their pathogenesis, but these iron disorders substantially differ with regard to mechanisms and amounts of

Table 2. Regimens and Effects of Venesection Treatment in Chronic Hepatitis C and HFE-Hemochromatosis

	CHC	Hemochromatosis	Statistical Analysis
Treatment Periods (months)	7 ± 3	15 ± 8	0.03
Blood Volume Removed (mL)	2,010 ± 1,190	12,000 ± 5,560	<0.01
Iron Removed by Venesection. (mg)	940 ± 550	5,960 ± 2,750	<0.01
Iron in Reduced Hb (mg)	210 ± 150	negligible	
Body Iron Stores (mg)	730 ± 560	5,960 ± 2,750	<0.01
ALT Reduction (U/L)	49 ± 30	22 ± 20	0.02
Iron Hepatotoxicity Index	0.097 ± 0.083	0.0032 ± 0.0046	<0.01

CHC; chronic hepatitis C, ALT; alanine aminotransferase.

Treatment methods for CHC and hemochromatosis are different so that statistical analysis is not fully reliable. Body iron stores were calculated from the blood volume removed according to the formula described in the text. Iron hepatotoxicity index (IHI) was calculated by dividing reduction in ALT by body iron stores. Provided that iron-induced hepatotoxicity is represented by ALT reduction during iron removal treatment, IHI might be a sensitivity to iron hepatotoxicity. A high IHI in CHC suggests not only a high sensitivity to iron hepatotoxicity, but also a good benefit of iron removal treatment. In contrast, liver cells with HFE-mutation are highly tolerant of iron hepatotoxicity. Treatment regimens for hemochromatosis were stronger but effects were smaller than those for CHC.

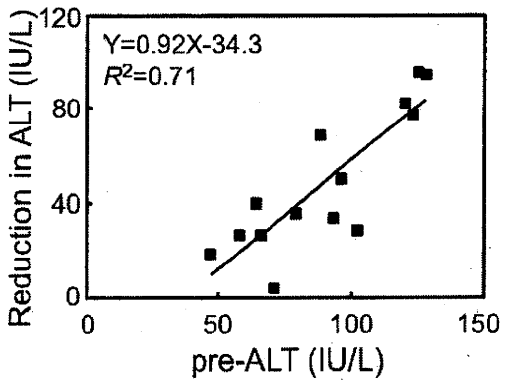

Figure 5. A good correlation between the reduction of ALT and pre-treatment ALT in chronic hepatitis C. Pre-treatment ALT level is predictive of efficacy by venesection. It may be important that other parameters including ferritin concentration are not predictive of treatment effects. In other words, the higher ALT is, the greater the treatment effect will be. ALT: alanine aminotransferase.

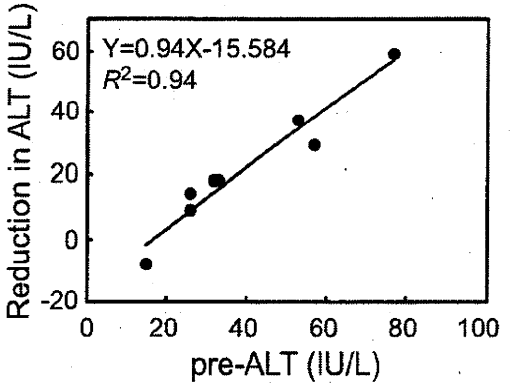

Figure 6. A good correlation between the reduction of ALT and pre-treatment ALT in HFE-hemochromatosis. Seven of 8 patients treated showed decreased serum ALT activities, while one of them had increased enzyme activity from 15 to 23 U/L after iron removal. It may be important that even though the levels are low, the effect of iron removal is ALT-dependent and not ferritin-dependent even in patients with HFE-hemochromatosis. ALT reduction may be the primary effect, and suppression of hepatic fibrosis might be a secondary event of venesection in both chronic hepatitis C and HFE-hemochromatosis. ALT: alanine aminotransferase.

iron overload. In HFE-hemochromatosis, iron accumulates due to HFE-dependent derangement of hepcidin production (10, 11). Serum hepcidin-25 levels in our Italian HFE-hemochromatosis patients were low at 11.1 ± 9.2 ng/mL. The current observations of circulating hepcidin 25 confirmed the findings previously observed in urine from patients with HFE-hemochromatosis (12). Low levels of serum hepcidin 25 were also found in Japanese patients with non-HFE hemochromatosis (18).

In CHC patients, the mechanism of hepatic iron accumulation appears to be more complex and is still not completely understood. Iron regulation by hepcidin might be partially impaired in CHC. Serum hepcidin-25 levels in our Japanese patients were relatively high at 30.7 ± 14.5 ng/mL compared to those in Italian HFE-hemochromatosis patients. Our findings, including the reduced hepcidin/ferritin ratio and correlation between hepcidin and ferritin concentrations fit well with reports showing that hepcidin induction was

relatively impaired in CHC patients, but also that its regulation by iron stores was maintained (13, 14). These findings are also in agreement with recent studies in animal and cellular models suggesting that HCV suppresses hepcidin production and may contribute to the development of iron overload in CHC (19, 20).

Previous study reported that none of the iron indices were predictive of the venesection effect, but rather the pretreatment ALT activities were predictive in CHC (21). The present study confirmed that this is also likely in HFE-hemochromatosis. The pretreatment ALT was higher, the reduction in ALT was larger in both CHC and HFE-hemochromatosis. Active liver cell damage could be removed by venesection regardless of the amount of stored iron. HCV co-infection in hemochromatosis patients markedly increases the risk of cirrhosis in the presence of a relatively low amount of iron (22). Therefore, with or without HCV infection, venesection should be recommended for HFE-hemochromatosis patients not only to remove excess iron, but also to suppress biochemical liver damage as in CHC patients. Provided that iron-induced hepatotoxicity is represented by ALT reduction during venesection, IHI obtained by [dividing ALT reduction by body iron stores] might be representative of sensitivity to iron hepatotoxicity under disease conditions. The IHI suggests not only sensitivity to iron hepatotoxicity, but also beneficial effects of venesection. CHC patients with a high IHI may be more sensitive to iron-induced liver damage than patients with HFE-hemochromatosis with low IHI. Therefore, liver cells with HFE-mutation are more tolerant to iron hepatotoxicity than liver cells infected by HCV.

In HFE-hemochromatosis patients, iron accumulates slowly and redox active iron emerges only when the storage capacity for ferritin and hemosiderin is overwhelmed. In other words, because of the high tolerance to iron hepatotoxicity, clinical manifestation might be delayed in HFE-hemochromatosis. The mean amount of iron removed from HFE hemochromatosis was reported to be 4.98 g (3.9-6.1 g in the range) (12), while that of CHC was 0.61 g (0-1.6 g in the range) (17). The results of the current study did not differ from those in the literature. It is also important that the amount of body iron in CHC patients is considered a non-toxic level in healthy subjects because sensitivity to iron hepatotoxicity disappeared in complete responders to IFN without iron removal (13). However, the combination of even a slightly increased iron level with HCV-related insult may act synergistically to increase iron hepatotoxicity and the risk of progressive liver damage so that iron removal can be beneficial in this setting (3, 4).

Patients with HFE-hemochromatosis showed normalized serum ALT activity after venesection, while all CHC patients showed significantly reduced but not normalized activity on biochemical liver function test. Iron hepatotoxicity was totally removed from HFE-hemochromatosis at 50 ng/mL of serum ferritin, while it remained in CHC around 20 ng/mL serum ferritin. The modified endpoint of 20 from 10 ng/mL for CHC still induced a reduction of Hb concentration in the posttreatment period (1.6 ± 1.1 g/dL). This incomplete effect of venesection in CHC indicates at least 2 compartments of hetatotoxicity of iron-induced oxidative stress and HCV-dependent insult. Hb concentration did not change in HFE-hemochromatosis with a high serum ferritin endpoint of 50 ng/mL. The high endpoint for HFE-hemochromatosis may account for the good tolerance to venesection to remove large amounts of body iron stores. Thus, threshold and sensitivity for iron hepatotoxicity were apparently different under the 2 conditions.

The remarkable reduction in serum hepcidin levels after venesection for iron overload conditions indicates suppressed internalization of ferroportin to enhance iron absorption in the gut. Thus, patients will rapidly recover iron stores if receiving a normal diet. Based on those findings, low-iron diets may be recommended to reduce active iron absorption in the intestine during the second stage of venesection in order to maintain free from iron-induced hepatotoxicity in Japanese patients with CHC (4, 23). In HFE-hemochromatosis with good tolerance to venesection, the effect of dietary iron restriction might be negligible.

To summarize an iron-induced hepatotoxicity under these 2 disease conditions, CHC patients were loaded with a minimum iron burden, but were more sensitive to iron-induced hepatotoxicity and less tolerant of venesection. In contrast, HFE-hemochromatosis patients were loaded with excessive iron due to genetic dysregulation of the hepcidin system and were more tolerant to both iron-induced hepatotoxicity and venesection to remove large amounts of body iron stores.

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Original Article

Impact of early elevation of serum bilirubin during treatment with pegylated interferon and ribavirin in patients with chronic hepatitis C

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Aim: Hemolytic anemia is a well-known adverse effect of interferon and ribavirin combination treatment. Herein, we analyzed the impact of early elevation of serum bilirubin level as a marker for predicting severe anemia during treatment.

Methods: We studied 245 chronic hepatitis C patients who received pegylated interferon and ribavirin combination treatment, and divided them using two different threshold levels: (i) elevation of total bilirubin of 0.5 mg/dL or more within 1 week of starting treatment; and (ii) drop of hemoglobin (Hb) by 3 g/dL or more within 4 weeks of starting treatment. We compared the dynamics in each group and then investigated independent factors for predicting a severe Hb drop (≥3 g/dL) at 4 weeks after beginning treatment and dose reduction of ribavirin.

Results: Total bilirubin levels at 1 week were significantly higher in patients with a Hb drop of 3 g/dL or more as compared to those with a drop of less than 3 g/dL ($P < 0.0001$). Hb levels at 4 weeks were significantly lower in the group of 0.5 mg/dL or more increase of total bilirubin levels than in the group with a less than 0.5 mg/dL increase ($P < 0.0001$). Therefore, elevation of total bilirubin after 1 week of treatment was shown to be an independent factor for predicting severe Hb drop (≥3 g/dL) at 4 weeks ($P < 0.0001$), and dose reduction of ribavirin during treatment ($P = 0.0321$).

Conclusion: Early elevation of serum bilirubin level was found to be a possible predictive marker of both a severe drop of Hb in the early phase of treatment and dose reduction of ribavirin.

Key words: bilirubin, hemolytic anemia, hepatitis C virus, pegylated interferon and ribavirin.

INTRODUCTION

ANEMIA IS ONE of the most common and important adverse effects of interferon (IFN) and ribavirin combination treatment that occurs in chronic hepatitis C patients.[1] Two different mechanisms of anemia are induced by such combination treatment: (i) suppression of bone marrow hematopoiesis caused by IFN;[2] and (ii) dose-dependent hemolytic anemia caused by ribavirin.[3] However, clinically, the contribution of ribavirin to anemia during this combination treatment overshadows the effect of IFN on bone marrow.[1]

Hemolytic anemia associated with ribavirin has been reported in recent studies. A median maximum decrease in hemoglobin (Hb) of 3.7 g/dL was found in patients who received pegylated interferon (PEG IFN)-α-2a plus ribavirin.[4] Another study found that 50% of patients experienced a decrease in Hb of 3 g/dL or more,[3] while a mean maximal decrease in Hb of 4.0 g/dL was reported in patients who received PEG IFN-α-2b plus ribavirin treatment.[5] Consequently, ribavirin dose modification was needed in approximately 25% of patients.[3–6]

Hemolytic anemia can induce cardiovascular morbidity, as well as substantial negative effects on cerebral function and quality of life (QOL).[1] On the other hand, a reduction in the dose of ribavirin can lower sustained viral response (SVR) rate in patients with hepatitis C, as well as both the starting dose of ribavirin and duration of treatment; thus, the cumulative dose of ribavirin is critical to achieve an optimal SVR by lowering the

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relapse rate after treatment, especially in genotype 1 infected patients.[1,7,8] So, prediction of severe anemia following ribavirin treatment is critical not only for patients' safety, but also for forming a proper dosing strategy.

It has been noted that the majority (80–85%) of bilirubin, the end product of haem, comes from Hb with only a small fraction derived from other haem-containing proteins such as cytochrome P450.[9] This report also noted that bilirubin elevation can be classified into three types: (i) pre-hepatic; (ii) hepatic; and (iii) cholestatic. Thus, it is caused by not only liver dysfunction, but also for a pre-hepatic reason: hemolysis.[9]

In this study, we found some patients with a steep elevation of serum total bilirubin level after starting PEG IFN and ribavirin combination treatment for chronic hepatitis C. The ability of early elevation of serum bilirubin to predict both a severe Hb drop in the early phase after starting combination treatment and dose reduction of ribavirin during treatment was analyzed. Furthermore, we discuss whether this marker is useful to prevent severe anemia which is one of the main causes of cessation of the treatment.

METHODS

Patients

BETWEEN 2002 AND 2009, we treated 245 patients (140 men and 105 women, mean age 52.2 ± 12.2 years) with PEG IFN-α-2b and ribavirin combination treatment at our institute. All were included and retrospectively analyzed in this study.

Patients' age and sex, initial dose of ribavirin and liver biopsy findings if available (205 patients), as well as Hb levels and serum bilirubin levels at 0, 1, 2, 4, 6, 8, 12 and 24 weeks after induction of combination treatment were analyzed.

Treatment protocol

Patients who were diagnosed with hepatitis C by both abnormalities of liver function test results and hepatitis C virus (HCV) RNA-positive findings by Cobas Amplicor HCV Monitor V2.0 test (Roche Diagnositics, Branchburg, NJ, USA) until 2007, or Cobas Taqman HCV test (Roche Diagnositics) from 2008. In some cases, liver biopsy findings based on the French Metavir score were included in this study. All patients in this study were treated with a combination of PEG IFN-α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough). The initial dose was determined by bodyweight (PEG IFN, 1.5 μg/kg; ribavirin, 400 mg/day for patients weighing <40 kg, 600 mg/day for those weighing 40–60 kg, 800 mg/day for those weighing 60–80 kg, 1000 mg/day for those weighing >80 kg). Dose reduction of ribavirin was done when the Hb level became less than 10 g/dL, and treatment was stopped when that became less than 8.5 g/dL.

Definition of severe Hb decline and classification of patients based on elevation of serum bilirubin

A Hb drop of 3 g/dL or more from baseline at 4 weeks after the start of combination treatment was defined as severe Hb decline in the early phase of treatment. Thus, our analyses were performed between groups divided by that threshold level. In addition, we performed analyses after dividing the patients based on a total bilirubin elevation of 0.5 mg/dL or more from the baseline after 1 week.

Dose reduction and treatment cessation by hemolytic anemia during combination treatment

In this study, 94 of 245 (38.4%) patients required a dose reduction of ribavirin, and four patients (1.6%) stopped treatment because of hemolytic anemia that developed during treatment. We performed analyses to find factors associated with dose reduction of ribavirin; however, we could not analyze factors associated with treatment cessation because of the limited number of patients.

Statistical analysis

Two-way repeated-measures ANOVA was used to compare the change of serum bilirubin level between groups divided by the extent of Hb decline as well as the change of Hb level between groups divided by the extent of serum bilirubin increase. The Mann–Whitney *U*-test was used in the comparison of values obtained at each time point. Univariate analysis was used to analyze predictive factors, with the Mann–Whitney *U*-test applied for the continuous values, and the χ^2-test applied for the categorical values. In multivariate analysis, a multiple logistic regression test was applied. $P < 0.05$ was considered to indicate statistical significance. All analyses were performed with Stat View ver. 5.0.

Ethical consideration

All data were retrospectively obtained from patients' records and the study was performed according to the Declaration of Helsinki.

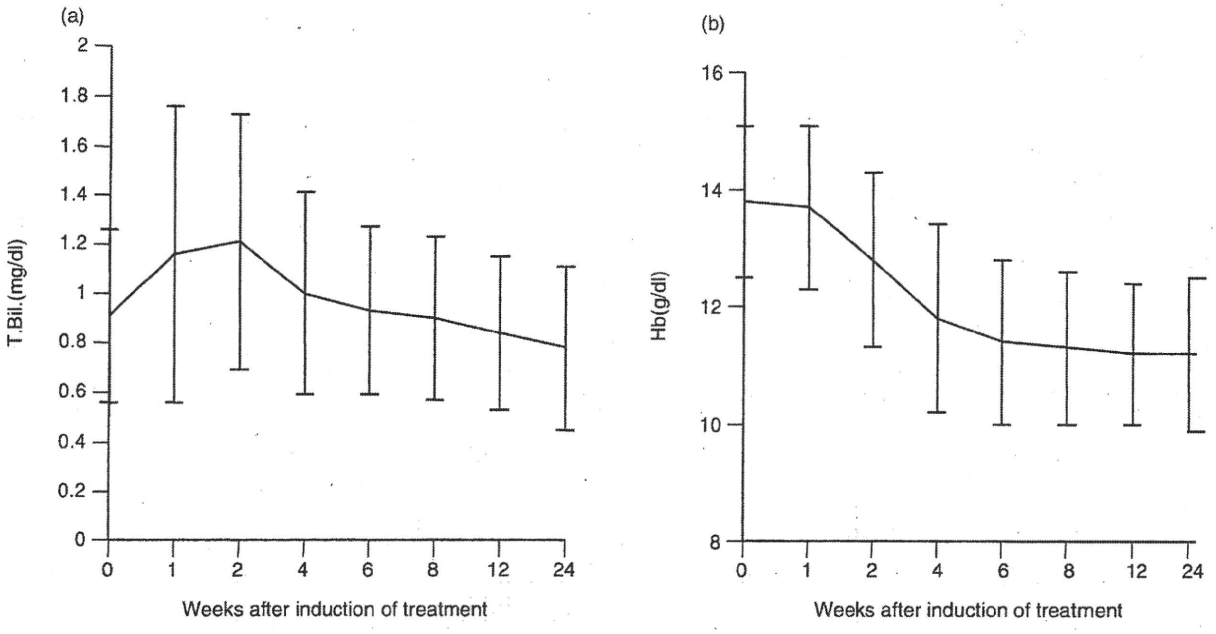

Figure 1 (a) Dynamics of serum total bilirubin (T.Bil) levels after beginning combination treatment. (b) Dynamics of hemoglobin (Hb) levels after beginning combination treatment. Each data indicate the mean values and error bars indicate the standard deviation.

RESULTS

Dynamics of Hb levels and serum bilirubin levels after induction of PEG IFN and ribavirin combination treatment

FIGURE 1(A) SHOWS the dynamics of serum total bilirubin after starting combination therapy in all of the study patients. Total bilirubin gradually increased from the baseline level until 2 weeks after starting treatment, then returned to the baseline level. Figure 1(b) shows the dynamics of Hb levels after starting treatment in all patients included in this study. Hb levels gradually declined and reached a plateau at 4–8 weeks after starting treatment.

Different dynamics of serum total bilirubin levels between patients with and without severe hemolytic anemia in the early phase of treatment

We found a steep increase of serum total bilirubin level (≥0.5 mg/dL) at 1 week after starting treatment in 62 of the 245 patients (25.3%). Furthermore, 65 (26.5%) of the patients had a severe decline in Hb (≥3 g/dL) after 4 weeks of treatment. Figure 2(a) shows the difference of dynamics of serum bilirubin levels between patients with and without severe declines of Hb. In those with a severe Hb decline, serum total bilirubin levels were increased at 1 week after beginning treatment, which was earlier than the total study population (Fig. 2a). In contrast, in patients without a steep Hb decline after 4 weeks, the change in total bilirubin level was quite similar compared to that of the total population (Fig. 2a). The difference between these groups was shown to be statistically significant by ANOVA ($P = 0.0095$). In addition, total bilirubin levels at 1 ($P < 0.0001$), 2 ($P < 0.0001$) and 4 weeks ($P = 0.0306$) after the start of treatment in the patients with a severe Hb decline were significantly higher as compared to those without a severe Hb decline.

Different dynamics of Hb levels between patients classified by the extent of elevation of serum total bilirubin

Next, we compared dynamics of Hb levels between the patients with a steep increase of serum total bilirubin (≥0.5 mg/dL) and those without steep increase (<0.5 mg/dL) at 1 week after starting treatment. Figure 2(b) shows the comparison of dynamics by ANOVA, which were not statistically significant ($P = 0.1539$).

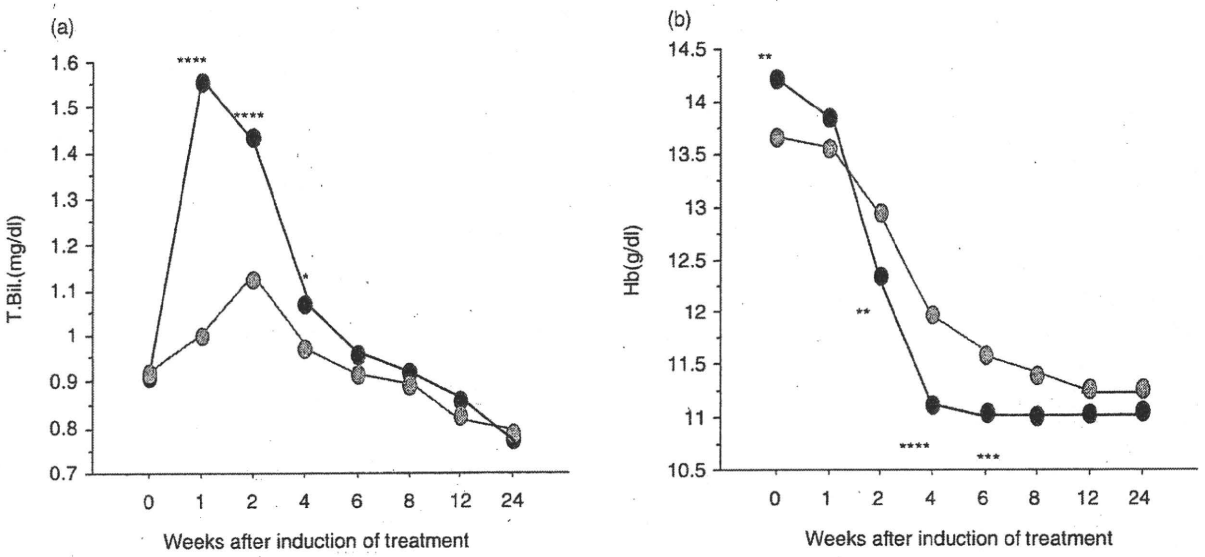

Figure 2 (a) Comparison of the dynamics of serum bilirubin levels between patients with severe hemolytic anemia (Hb decline ≥3 g/dL) to those without severe hemolytic anemia at 4 weeks after start of treatment. Two-way repeated-measures ANOVA showed a significant difference of dynamics between these two groups ($P = 0.0095$). Black lines indicate serum bilirubin levels in the group with severe hemolytic anemia, and gray lines indicate those levels in the group without severe hemolytic anemia. $^{*}P < 0.05$, $^{****}P < 0.0001$ for comparisons of each values at each time point. (b) Comparison of the dynamics of Hb levels between patients group divided by the extent of elevation of serum total bilirubin (≥0.5 vs <0.5 mg/dL at 1 week after starting treatment).Two-way repeated-measures ANOVA did not reveal a statistically significant difference between the two groups ($P = 0.1539$). Black lines indicate Hb levels in the group with elevation of serum bilirubin of ≥0.5 mg/dL, and gray lines indicate these levels in the group with elevation of serum bilirubin of <0.5 mg/dL. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ for comparison of each values at each time point. Hb, hemoglobin; T.Bil, total bilirubin.

As for why it did not reach statistical significance, baseline Hb levels were higher in the group with a steep increase of serum total bilirubin ($P = 0.0043$), though that trend was reversed at 2 weeks after the start of treatment ($P = 0.0030$). Furthermore, the extent of Hb decline was higher in the group with that steep increase.

Factors associated with severe Hb drop in the early phase

Next, we investigated the predictive factors associated with a severe Hb decline after beginning treatment. By univariate analysis, male sex ($P = 0.0201$), higher initial dose of ribavirin ($P = 0.0029$), higher baseline Hb level ($P < 0.0001$) and higher elevation of serum total bilirubin after 1 week ($P < 0.0001$) were shown to be significant predictive factors (Table 1). By multivariate analysis, though higher patient age (odds ratio [OR] = 1.055, 95% confidence interval [CI] = 1.020–1.090, $P = 0.0090$), higher initial dose of ribavirin (OR = 1.266, 95% CI = 1.015–1.580, $P = 0.0361$) and higher baseline Hb level (OR = 1.765, 95% CI = 1.202–2.592, $P = 0.0038$) were selected as the significant independent factors associated with a steep decline in Hb after treatment, though a higher elevation of serum bilirubin at 1 week after starting treatment was found to be the strongest factor than the other three (OR = 9.448, 95% CI = 3.727–23.965, $P < 0.0001$) (Table 2).

Factors associated with dose reduction of ribavirin during combination treatment

To evaluate the clinical relevance of a steep increase of serum bilirubin level, we investigated the factors associated with reduction of ribavirin dose during the combination treatment. By univariate analysis, higher patient age ($P < 0.0001$), female sex ($P = 0.0207$), higher initial dose of ribavirin ($P = 0.0003$), more advanced fibrosis ($P < 0.0001$) and lower baseline Hb levels ($P = 0.0007$) were considered as significant factors associated with dose reduction of ribavirin during treatment. In contrast, a higher elevation of serum bilirubin after 1 week was not considered a statistically significant factor though it showed a tendency for significance

Table 1 Univariate analysis of factors associated with severe hemoglobin decline (Hb decline ≥3 g/dL in 4 weeks after starting treatment) after induction of combination treatment in this study

	Yes ($n = 65$)	No ($n = 180$)	*P*-value
Age, range (median), years	29–70 (56)	18–74 (53)	0.1200
Sex, *n* (M/F)	45/20	95/85	0.0201*
Initial dose of ribavirin, range (median), mg/kg bodyweight	5.4–15.8 (12.2)	2.4–16.2 (11.1)	0.0029**
Histological grading, *n*, A1/2/3	26/28/2	84/63/2	0.3114
Histological staging, *n*, F0/1/2/3/4	10/22/15/8/1	34/60/38/16/1	0.8340
Baseline Hb, range (median), g/dL	12.2–18.1 (14.6)	11.0–16.0 (13.6)	<0.0001****
Baseline Hb, range (median), g/dL	0.4–2.0 (0.9)	0.3–2.5 (0.8)	0.2711
Elevation of total bilirubin at 1 week from baseline, range (median), mg/dL	−0.6–2.7 (0.6)	−1.0–2.2 (0.1)	<0.0001****

*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

($P = 0.0684$) (Table 3). By multivariate analysis, higher elevation of serum bilirubin at 1 week was a significant independent factor ($P = 0.0321$), as were higher age ($P = 0.0007$), higher initial dose of ribavirin ($P < 0.0001$) and lower baseline Hb level ($P = 0.0162$) (Table 4).

DISCUSSION

HEMOLYTIC ANEMIA THAT occurs after beginning PEG IFN and ribavirin combination treatment for patients with chronic hepatitis C is a critical adverse effect that causes dose modification in 20–25% of treated patients,[1,4,5] of whom more than half must discontinue treatment.[5]

In this report, we first evaluated the extent of hemolytic anemia after beginning combination treatment in chronic hepatitis C patients. The mean Hb decrease from baseline for all patients was approximately 2.5 g/dL and reached the bottom and plateau at 4 weeks after treatment, which was similar to previous reports. We also focused on the dynamics of total bilirubin, and found that the mean elevation was 0.3 mg/dL at 2 weeks after beginning treatment (Fig 1a,b).

Table 2 Multivariate analysis of factors associated with severe hemoglobin decline after induction of combination treatment in this study

Factors	Odds ratio (95% confidence interval)	*P*-value
Age (reference)	1	
1 year old higher	1.055 (1.020–1.090)	0.0090**
Sex (reference)	1	
Male sex	1.093 (0.398–2.999)	0.8629
Initial dose of ribavirin (reference)	1	
1 mg/kg bodyweight higher	1.266 (1.015–1.580)	0.0361*
Histological grading (reference)	1	
1 point higher	1.091 (0.438–2.715)	0.8515
Histological staging (reference)	1	
1 point higher	1.088 (0.655–1.806)	0.7449
Baseline Hb (reference)	1	
1 g/dL higher	1.765 (1.202–2.592)	0.0038**
Baseline total bilirubin (reference)	1	
1 mg/dL higher	2.245 (0.701–7.189)	0.1731
Elevation of total bilirubin at 1 week from baseline (reference)	1	
1 mg/dL higher	9.448 (3.727–23.965)	<0.0001****

*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

Table 3 Univariate analysis of factors associated with dose reduction of ribavirin after induction of combination treatment in this study

Dose reduction of Ribavirin	Yes ($n = 94$)	No ($n = 151$)	*P*-value
Age, range (median), years	22–71 (59)	18–74 (51)	<0.0001****
Sex, *n* (M/F)	45/49	95/56	0.0207*
Initial dose of ribavirin, range (median) mg/kg bodyweight	7.0–16.2 (12.0)	2.4–16.2 (11.1)	0.0003***
Histological grading, *n*, A1/2/3	38/42/1	72/49/1	0.2052
Histological staging, *n*, F0/1/2/3/4	13/14/30/14/0	31/58/23/10/2	<0.0001****
Baseline Hb, range (median), g/dL	11.1–16.1 (13.3)	11.0–18.1 (14.2)	0.0007***
Baseline total bilirubin, range (median), mg/dL	0.4–1.8 (0.8)	0.3–2.5 (0.8)	0.9497
Elevation of total bilirubin at 1 week from baseline, range (median), mg/dL	−0.8–2.7 (0.3)	−1.0–2.2 (0.0)	0.0684†

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; †$P < 0.1$.
Hb, hemoglobin.

In addition, serum bilirubin levels showed a steep and rapid elevation (≥0.5 mg/dL) at 1 week after the start of treatment in 25.3% of our patients, and seemed to occur more frequently in those with severe hemolytic anemia. This was the clue that motivated us to begin this study.

Various studies have been conducted regarding serum bilirubin levels after starting PEG IFN and ribavirin combination treatment. In one report, serum bilirubin levels were significantly increased in HCV/HIV co-infected patients who were treated with atazanavir, a protease inhibitor of HIV following the start of PEG IFN and ribavirin combination treatment.[10] However, to the best of our knowledge, there are no studies focused on serum bilirubin elevation after beginning combination treatment in standard chronic hepatitis C patients.

The main pre-hepatic cause of serum bilirubin elevation is hemolysis. Thus, we speculated that the extent of serum bilirubin elevation is correlated with hemolytic anemia following the start of PEG IFN and ribavirin combination treatment. As expected, the elevation of serum bilirubin at 1 week after starting treatment in patients with a severe Hb decline at 4 weeks after starting treatment was greater than that in patients without a severe Hb decline after 4 weeks. Hepatic dysfunction

Table 4 Multivariate analysis of factors associated with dose reduction of ribavirin after induction of combination treatment

Factors	Odds ratio (95% confidence interval)	*P*-value
Age (reference)	1	
1 year old higher	1.056 (1.023–1.090)	0.0007***
Sex (reference)	1	
Male	1.144 (0.484–2.704)	0.7598
Initial dose of ribavirin (reference)	1	
1 mg/kg bodyweight higher	1.539 (1.249–1.897)	<0.0001****
Histological grading (reference)	1	
1 point higher	0.808 (0.373–1.750)	0.5886
Histological staging (reference)	1	
1 point higher	1.395 (0.904–2.153)	0.1324
Baseline Hb (reference)	1	
1 g/dL higher	0.667 (0.480–0.928)	0.0162*
Baseline total bilirubin (reference)	1	
1 mg/dL higher	1.975 (0.732–5.327)	0.1786
Elevation of total bilirubin at 1 week from baseline (reference)	1	
1 mg/dL higher)	2.143 (1.061–3.806)	0.0321*

*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.
Hb, hemoglobin.

might be another cause of serum bilirubin elevation, but there were no changes in liver functions in patients in this study (data not shown).

We also found that the extent of elevation of serum bilirubin could be a predictive marker for severe hemolytic anemia after beginning PEG IFN and ribavirin combination treatment, and that after 1 week of treatment was the strongest independent factor for predicting a severe Hb drop in multivariate analysis. In recent reports, an early decrease of Hb (≥2 g/dL) at 2 weeks after beginning treatment was useful for predicting the treatment discontinuation,[11,12] as were a decrease in Hb to less than 10 g/dL,[13] and ribavirin apparent clearance (CL/F), which reflects ribavirin concentration at 4 weeks after the start of combination therapy.[14–16]

Among these markers, elevation of serum bilirubin after 1 week of treatment is new and at least as useful as the others; it is easier to employ than CL/F because a calculation formula is not needed, and can predict severe hemolytic anemia earlier than Hb decline at 2 weeks after beginning treatment.

Another interesting finding is that higher baseline Hb level was another independent factor of severe decline in Hb after starting treatment as were initial ribavirin dose and age, both of which are not surprising and have been shown in several reports.[3,5,13] Similar studies found that patients with a higher baseline Hb and who were maintained on the initial dose of ribavirin showed a trend toward larger Hb decline, while they also noted that the endogenous erythropoietin response is blunted in this population.[17,18]

A severe Hb decline by ribavirin administration due to hemolysis can cause anemia, which has a negative impact not only on the QOL of the patients, but also on cardiovascular comorbidity and cerebral function; thus, dose modification is needed in some cases.[1,19] However, dose modification has been shown to reduce the efficacy of PEG IFN and ribavirin combination treatment.[19] To investigate the factors associated with dose reduction of ribavirin during such combination treatment, the extent of serum bilirubin elevation was again found to be a significant independent factor. Thus, the extent of early elevation of serum bilirubin may be an important marker to predict dose reduction of ribavirin early after the start of treatment, which would be useful not only for patients' safety but also for predicting treatment response.

In summary, significant proportions of patients have been shown to have a steep elevation of serum bilirubin level at 1 week after beginning PEG IFN and ribavirin combination treatment for chronic hepatitis C. We found that this steep elevation was significantly correlated with both a severe decline in Hb and dose reduction of ribavirin, and consider that it may be an easier and earlier predictive marker compared with those previously reported, including CL/F and Hb decline at 2 weeks after starting treatment

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Association between HCV amino acid substitutions and outcome of peginterferon and ribavirin combination therapy in HCV genotype 1b and high viral load

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Key words
amino acid substitution, chronic hepatitis C, hepatitis C virus, peginterferon and ribavirin therapy, resistance to interferon.

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Abstract

Background and Aim: We prospectively compared the sensitivity to interferon (IFN) and the efficacy of antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin for chronic hepatitis C virus (HCV) genotype 1b infection according to the amino acid sequences of the HCV core, E1, and NS5A regions reported to be associated with the outcome of antiviral therapy.

Methods: A total of 107 patients with HCV genotype 1b were investigated. All patients received combination therapy with PEG-IFN alpha-2b and ribavirin. Amino acids 70 and 91 (core), 139 (E1), and 2209–2248 (NS5A) of HCV were analyzed by direct nucleotide sequencing.

Results: The reduction in HCV RNA concentration at 24 h after a single administration of conventional IFN-alpha and after the start of combination therapy was significantly less marked, and rates of complete early virologic response, end-of-treatment response, and sustained virologic response (SVR) were significantly lower (all $P < 0.0001$) in patients with glutamine at amino acid 70 ($n = 29$) than in those with arginine at that position ($n = 70$). We found no differences associated with the other amino acid positions. Amino acid 70 was an independent factor for the responses to the therapy in multivariate analysis.

Conclusion: The identity of amino acid 70 of the HCV core region affected the sensitivity to IFN; patients with glutamine at amino acid 70 of HCV showed resistance to IFN. Consequently, it strongly affected the outcome of combination therapy with PEG-IFN and ribavirin in Japanese patients with HCV genotype 1b.

Introduction

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] The rate of sustained virologic response (SVR), which indicates the eradication of the hepatitis C virus (HCV) is around 50% in patients infected with HCV genotype 1, which is more resistant to this therapy than genotypes 2 or 3; the recommended duration of the PEG-IFN/ribavirin treatment period differs between patients with HCV genotype 1 and those with genotype 2 or 3.[1,2]

Many studies have been performed to elucidate the viral factor determining the sensitivity or resistance to the IFN-based antiviral therapy, especially for HCV genotype 1. Several amino acid substitutions have been reported to be associated with the efficacy of IFN-based antiviral therapy in patients infected with HCV genotype 1b.[3–9] In the late 1990s, Enomoto *et al.* reported that mutations in the amino acids at positions 2209–2248 of the NS5A region of HCV were closely associated with the efficacy of IFN monotherapy;[3] however, these results proved controversial.[10–22] Very recently, a few studies have reported an association between other amino acid substitutions and the rate of SVR by the PEG-IFN/ribavirin therapy in patients with HCV genotype 1b.[7,8] However, the influence of these amino acid substitutions on the sensitivity to IFN or the outcome of PEG-IFN/ribavirin combination therapy has not been fully established.

In the present study, the authors investigated the association of four HCV amino acid substitutions (70 and 91 of the core region, 139 of the E1 region, and 2209–2248 of the NS5A region), with IFN sensitivity. Its association with the combination therapy PEG-IFN and ribavirin was also investigated in Japanese patients chronically infected with HCV genotype 1b, and having high pretreatment HCV RNA concentration.

Patients and methods

Patients

A total of 148 patients with chronic hepatitis C and without cirrhosis received antiviral combination therapy with PEG-IFN and ribavirin at the Ogaki Municipal Hospital, Ogaki, Japan, between July 2005 and June 2007. Among them, 109 patients had been infected with HCV genotype 1b and had pretreatment HCV RNA concentration > 100×10^3 IU/mL, as assessed by quantitative polymerase chain reaction (PCR) assay (Amplicor HCV Monitor Test, version 2.0; Roche Molecular Systems, Pleasanton, CA, USA). Of those 109 patients, 107 patients were enrolled in the study (two patients declined to enroll). The clinical characteristics of study patients are listed in Table 1. The patient group was comprised of 52 males (48.6%) and 55 females (51.4%), with a mean age of 58.9 ± 9.0 years. Twenty-two patients (20.6%) had previously received blood transfusion. Although 32 patients (29.9%) had a history of previous antiviral therapy by monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin, no patients had a history of the combination therapy with PEG-IFN and ribavirin. The average pretreatment HCV RNA concentration was $1760 \pm 1139 \times 10^3$ IU/mL. In 102 patients who underwent pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[23] was F0 in 5 patients (4.9%), F1 in 61 patients (59.8%), F2 in 24 patients (23.5%), and F3 in 12 patients (11.8%), respectively. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. No patients were alcohol abusers or intravenous drug users.

Table 1 Clinical characteristics of study patients ($n = 107$)

Age (years)	58.9 ± 9.0
Sex (female/male)	55 (51.4)/52 (48.6)
Body weight (kg)	59.1 ± 10.2
History of interferon therapy (naive/retreatment)	75 (70.1)/32 (29.9)
History of transfusion (–/+)	85 (79.4)/22 (20.6)
Alanine aminotransferase (IU/L)	65.8 ± 64.9
Aspartate aminotransferase (IU/L)	55.9 ± 44.3
Gamma-glutamyl transpeptidase (IU)	53.7 ± 53.6
Alkaline phosphatase (IU/L)	265.2 ± 86.4
Albumin (g/dL)	4.14 ± 0.35
Total bilirubin (mg/dL)	0.69 ± 0.28
White blood cell count (/μL)	5201 ± 1197
Hemoglobin (g/dL)	14.0 ± 1.4
Platelet count ($\times 10^3$/μL)	166 ± 51
Liver histology-activity (A0/A1/A2/A3)†	2 (2.0)/55 (53.9)/36 (35.3)/9 (8.8)
Liver histology-fibrosis (F0/F1/F2/F3)†	5 (4.9)/61 (59.8)/24 (23.5)/12 (11.8)
HCV RNA concentration ($\times 10^3$ IU/mL)	1760 ± 1139
Reduction of peginterferon dose	29 (27.1)
Reduction of ribavirin dose	49 (45.8)
Response (SVR/relapse/NR)	39 (36.5)/38 (35.5)/30 (28.0)

Percentages are shown in parentheses.
†Liver biopsy was not performed in five patients.
HCV, hepatitis C virus; NR, no response; SVR, sustained virologic response.

Single administration test of conventional interferon alpha to evaluate sensitivity to interferon

All patients were underwent a single administration test of conventional IFN alpha more than 2 weeks before the start of the combination therapy to evaluate the sensitivity of HCV to IFN in each patient. They received intramuscular administration of 6 mega-units of standard IFN alpha-2b (Intron A; Schering-Plough, Tokyo, Japan). The concentration of HCV RNA was measured before and 24 h after the single administration test and the reduction of serum HCV RNA was calculated.

Combination therapy with peginterferon and ribavirin

For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough) weekly and ribavirin (Rebetol, Schering-Plough) daily according to the manufacturer's recommendations. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤ 45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing > 45 kg and ≤60 kg were given 80 μg, those weighing > 60 kg and ≤75 kg were given 100 μg, those weighing > 75 kg and ≤90 kg were given 120 μg, and those weighing > 90 kg were given 150 μg. Patients weighing ≤ 60 kg were given 600 mg of ribavirin per day, those weighing > 60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing > 80 kg were given 1000 mg of ribavirin per day. All patients were scheduled to undergo 48 weeks of treatment; longer durations were not considered in this study. Serum HCV RNA concentration was measured every 4 weeks on an outpatient basis. The presence of HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems; detection limit, 50 IU/mL) to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay (detection limit, 615 IU/mL). Patients were classified into categories as follows: rapid virologic response (RVR) was defined as undetectable serum HCV RNA at 4 weeks from the start of the combination therapy. Complete early virologic response (cEVR) was defined as undetectable serum HCV RNA within 12 weeks of the start of the therapy. End-of-treatment response (ETR) was defined as undetectable serum HCV RNA at the end of the treatment period (i.e. 48 weeks after the start of the therapy). Sustained virologic response (SVR) was defined as undetectable serum HCV RNA at 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, following ETR. Null-response (NR) was defined as positive serum HCV RNA throughout the treatment period and thereafter.

Measurements of sequences of amino acids 70 and 91 of core region, amino acid 139 of E1 region, and amino acid sequence 2209–2248 of NS5A region of hepatitis C virus

Amino acids 70 and 91 of the core region of HCV, amino acid 139 of the E1 region of HCV, and amino acid sequence of 2209–2248 of the NS5A region of HCV were analyzed by direct nucleotide sequencing of each region according to previous reports.[3,7,8] Hepatitis C virus was defined as wild type for the amino acid sequence of 2209–2248 of the NS5A region when the number of amino acid substitutions in comparison with HCV-J strain[24] was 0 or 1, and as non-wild type when the number of substitutions was greater than 1.

Statistical analyses

Quantitative values are shown as mean ± SD. Between-group differences were analyzed by the chi-square test. Differences in quantitative values between the two groups were analyzed by the Mann-Whitney *U*-test. Multivariate analysis was performed for baseline factors that affected the response to the combination therapy using a logistic regression model. The factors analyzed were age, sex, body weight, history of previous IFN therapy, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase, serum alkaline phosphatase, serum albumin, serum total bilirubin, white blood cell count, hemoglobin, platelet count, hepatitis activity grade (A0 and A1 *versus* A2 and A3), grade of liver fibrosis (F0 and F1 *versus* F2 and F3), pretreatment HCV RNA concentration, amino acids 70 and 91 of the HCV core protein, amino acid 139 of HCV E1 protein, and amino acid sequence 2209–2248 of the HCV NS5A protein. All statistical tests were two-tailed; $P < 0.05$ was accepted as statistically significant.

The study protocol was approved by the institutional review board and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients prior to the study for use of the laboratory data.

Results

All 107 patients completed the entire treatment duration; no patient dropped-out of the study. Although reduction of PEG-IFN dose and ribavirin dose were experienced by 29 patients (27.1%) and 49 patients (45.8%), respectively, no patient discontinued either PEG-IFN or ribavirin. Eight patients (7.5%) had achieved RVR and 40 patients (37.4%) had achieved cEVR. HCV RNA was undetectable in 77 patients (72.0%) at the end of treatment (ETR). Outcomes following combination therapy were SVR in 39 patients (36.5%), relapse in 38 patients (35.5%), and NR in 30 patients (28.0%). Amino acid position 70 of the HCV protein core region was Arginine (Arg, reportedly associated with a good response to IFN) in 70 patients (65.4%) and Glutamine (Gln, reportedly associated with a poor response) in 29 patients (27.1%). Of the other eight patients, five had Histidine at that position, and in the other three HCV with both Arg and Gln at this position was detected. Amino acid position 91 of the HCV core region was Leucine (Leu, reportedly associated with a good response to IFN) in 76 patients (71.0%) and Methionine (Met, reportedly associated with a poor response) in 29 patients (27.1%). In the remaining two patients, HCV with both Leu and Met at this position was detected. Amino acid position 139 of the HCV E1 region was Threonine (Thr, reportedly associated with a good response to IFN) in 55 patients (51.4%) and Alanine at this position (Ala, reportedly associated with a poor response) in 40 patients (37.4%). From the remaining patients, the amino acid at position 139 was Serine in two patients; in four patients HCV with both Thr and Ala was detected; in HCV from four patients this position was deleted. In two patients we failed to amplify this region. At amino acid range 2209–2248 of the HCV NS5A region, HCV of 61 patients (57.0%) was wild type and that of 46 patients (43.0%) was non-wild type. There was no difference in baseline characteristics, including the rate of patients with reduction of PEG-IFN dose or ribavirin dose, between patients with Arg and Glu at amino acid position 70, between patients with Leu and Met at amino acid position 91, between patients with Thr and Ala at amino acid position 139, and between patients with non-wild type and wild type at amino acids range 2209–2248, respectively (data not shown).

Pretreatment HCV RNA concentration, and reduction in HCV RNA concentration at 24 h after the single administration of conventional interferon and after the start of combination therapy according to amino acid sequences

Pretreatment HCV RNA concentration, the reduction of HCV RNA concentration at 24 h after a single administration test of conventional IFN, and the reduction of HCV RNA concentration at 24 h after the start of combination therapy with PEG-IFN and ribavirin was compared between patients according to the amino acid sequence of the HCV (Table 2). Pretreatment HCV RNA concentration in patients with Arg at position 70 was significantly higher than in those with Gln at that position ($P = 0.0260$). Pretreatment HCV RNA concentration in patients with the wild type sequence at position 2209–2248 was significantly higher than that in patients with the non-wild type sequence ($P = 0.0002$). We found no difference in pretreatment HCV RNA concentration according to the identities of amino acids 91 or 139. The reduction of HCV RNA concentration in patients with Arg at position 70 was significantly more marked than in patients with Gln at that position ($P < 0.0001$). We found no difference in reduction of HCV RNA concentration after a single administration of conventional IFN according to the identities of amino acids 91 or 139, or the 2209–2248 sequence. Similarly, the reduction of HCV RNA concentration at 24 h after the start of combination therapy in patients with Arg at position 70 was significantly greater than that in patients with Gln at position 70 ($P = 0.0025$). We found no difference in reduction of HCV RNA concentration after a single administration of conventional IFN according to the identities of amino acids 91 or 139, or the sequence 2209–2248.

Response to combination therapy with peginterferon and ribavirin

The responses to combination therapy according to amino acid identity are summarized in Table 3. The rates of cEVR, ETR, and SVR were significantly higher in patients with Arg than in those with Gln at position 70 (all $P < 0.0001$), whereas we found no

Table 2 Association between amino acid substitutions and pretreatment HCV RNA concentration, reduction in HCV RNA concentration at 24 h after the single administration of conventional interferon (IFN) alpha-2b, and reduction in HCV RNA concentration at 24 h after the start of combination therapy with peginterferon (PEG-IFN) and ribavirin

	Core: amino acid70		Core: amino acid91	
	Arg ($n = 70$)	Gln ($n = 29$)	Leu ($n = 76$)	Met ($n = 29$)
Pretreatment HCV RNA concentration ($\times 10^3$ IU/mL)	1943 ± 1294	1410 ± 895*	1755 ± 1151	1731 ± 1107
Reduction in HCV RNA concentration at 24 h after single administration (log_{10})	1.50 ± 0.45	0.95 ± 0.69**	1.41 ± 0.60	1.21 ± 0.49
Reduction in HCV RNA concentration at 24 h after combination therapy (log_{10})	1.34 ± 0.52	0.97 ± 0.57***	1.25 ± 0.54	1.30 ± 0.60

	E1: amino acid139		NS5A: amino acids 2209–2248	
	Thr ($n = 55$)	Ala ($n = 40$)	Non-wild ($n = 46$)	Wild ($n = 61$)
Pretreatment HCV RNA concentration ($\times 10^3$ IU/mL)	1809 ± 985	1759 ± 1374	1290 ± 851	2115 ± 1205****
Reduction in HCV RNA concentration at 24 h after single administration (log_{10})	1.34 ± 0.64	1.40 ± 0.53	1.40 ± 0.60	1.32 ± 0.57
Reduction in HCV RNA concentration at 24 h after combination therapy (log_{10})	1.21 ± 0.53	1.24 ± 0.61	1.29 ± 0.57	1.25 ± 0.55

Mean ± SD.

*$P = 0.0260$; **$P < 0.0001$; ***$P = 0.0025$; ****$P = 0.0002$.

Ala, Alanine; Arg, Arginine; Gln, Glutamine; Leu, Leucine; Met, Methionine; Thr, Threonine.

Table 3 Association between amino acid substitutions and responses to combination therapy with peginterferon and ribavirin

	Core: amino acid70		Core: amino acid91		E1: amino acid139		NS5A: amino acids 2209–2248	
	Arg ($n = 70$)	Gln ($n = 29$)	Leu ($n = 76$)	Met ($n = 29$)	Thr ($n = 47$)	Ala ($n = 34$)	non-wild ($n = 46$)	wild ($n = 61$)
RVR	6 (55.7)	0	6 (43.4)	2 (44.8)	1 (46.8)	4 (38.2)	7 (15.2)	1 (1.6)**
cEVR	39 (55.7)	1 (3.5)*	33 (43.4)	13 (44.8)	22 (46.8)	13 (38.2)	22 (47.8)	24 (39.3)
ETR	58 (82.9)	12 (41.4)*	58 (76.3)	18 (62.1)	36 (76.6)	23 (67.6)	35 (76.1)	42 (68.9)
SVR	33 (47.2)	1 (3.5)*	31 (40.8)	8 (27.6)	19 (40.4)	11 (32.3)	21 (45.7)	18 (29.5)
Relapse	25 (35.6)	11 (37.9)	27 (35.5)	10 (34.5)	17 (36.2)	12 (35.4)	14 (30.4)	24 (39.3)
NR	12 (17.2)	17 (58.6)*	18 (23.7)	11 (37.9)	11 (23.4)	11 (32.3)	11 (23.9)	19 (31.1)

*$P < 0.0001$; **$P = 0.0229$.

Ala, Alanine; Arg, Arginine; cEVR, complete early virologic response; ETR, end-of-treatment response; Gln, Glutamine; Leu, Leucine; Met, Methionine; NR, no response; SVR, sustained virologic response; Thr, Threonine.

difference in the rates of cEVR, ETR, and SVR according to the amino acids at position 91 or 139, or the sequence 2209–2248. In contrast, the NR rate was significantly lower in patients with Arg at position 70 than in those with Gln at that position ($P < 0.0001$). We found no difference in the rates of cEVR, ETR, or SVR according to the identities of amino acids 91 or 139, or the sequence 2209–2248. The results were the same when we focused on 72 treatment-naïve patients (data not shown; see Table S1).

Univariate and multivariate analyses for baseline factors affecting response to combination therapy with peginterferon and ribavirin

Univariate and multivariate analyses were conducted for baseline factors that could affect cEVR, ETR, or SVR. In univariate analysis, serum gamma-glutamyl transpeptidase level ($P = 0.0025$), serum albumin level ($P = 0.0008$), and the amino acid at position 70 of the HCV core region (Arg *versus* Gln, $P < 0.0001$) were significantly associated with cEVR, and platelet count ($P = 0.0707$) was associated with cEVR but not significantly. In multivariate analysis, the identity of amino acid 70 of the HCV core region ($P = 0.0013$), serum albumin level ($P = 0.0265$), and serum gamma-glutamyl transpeptidase level ($P = 0.0308$) independently affected the rate of cEVR (Table 4). Serum gamma-glutamyl transpeptidase level ($P = 0.0004$), serum albumin level ($P = 0.0015$), white blood cell count ($P = 0.0490$), and the identity of amino acid 70 ($P < 0.0001$) were significantly associated with ETR, and serum alkaline phosphatase level ($P = 0.0814$) was associated with ETR but not significantly by univariate analysis. By multivariate analysis, the identity of amino acid 70 ($P = 0.0010$) and serum gamma-glutamyl transpeptidase level ($P = 0.0055$) independently affected ETR (Table 5). In the analysis of factors affecting SVR, serum albumin level ($P = 0.0069$), platelet count ($P = 0.0238$), amino acid 70 of the HCV core region ($P < 0.0001$) were significantly associated with SVR, and serum gamma-glutamyl transpeptidase level ($P = 0.0832$) and the liver fibrosis grade (F0 and F1 *versus* F2 and F3, $P = 0.0998$) were associated

Table 4 Multivariate analysis for factors affecting complete early virologic response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Gamma-glutamyl transpeptidase	by 1 IU	−0.0162	0.0075	4.66	0.0053 (0.0000–0.3807)	0.0308
Albumin	by 1.0 g/dL	2.3638	1.0650	4.93	181.33 (2.4858–25744)	0.0265
Platelet count	by 1.0 × 10^3/μL	0.0179	0.0554	0.10	1.5106 (0.1243–19.838)	0.7460
Core-70	1: Arginine				1	
	2: Glutamine	−1.7453	0.5429	10.33	0.0305 (0.0016–0.1719)	0.0013

Table 5 Multivariate analysis for factors affecting end-of-treatment response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Alkaline phosphatase	by 1 IU/L	−0.0028	0.0037	0.59	0.3034 (0.0137–6.6841)	0.4428
Gamma-glutamyl transpeptidase	by 1 IU	−0.0162	0.0058	7.72	0.0053 (0.0001–0.1738)	0.0055
Albumin	by 1.0 g/dL	1.2821	0.7700	2.77	16.787 (0.7440–626.72)	0.0959
White blood cell count	by 1/μL	0.0004	0.0002	3.09	12.603 (0.8685–269.45)	0.0787
Core-70	1: Arginine				1	
	2: Glutamine	−0.9882	0.3006	10.80	0.1386 (0.0400–0.4347)	0.0010

Table 6 Multivariate analysis for factors affecting sustained virologic response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Gamma-glutamyl transpeptidase	by 1 IU	−0.0015	0.0057	0.07	0.6201 (0.0121–20.764)	0.7962
Albumin	by 1.0 g/dL	1.4230	0.8788	2.62	22.888 (0.6793–1381.2)	0.1054
Platelet count	by 1.0 × 10^3/μL	0.0511	0.0556	0.84	3.2365 (0.2734–44.588)	0.3585
Fibrosis	1: F0/F1				1	
	2: F2/F3	0.0661	0.2976	0.05	1.1413 (0.3557–3.7626)	0.8242
Core-70	1: Arginine				1	
	2: Glutamine	−1.5097	0.5364	7.92	0.0488 (0.0026–0.2661)	0.0049

with SVR but not significantly by univariate analysis. By multivariate analysis, only amino acid 70 of the HCV core region independently affected SVR ($P = 0.0049$, Table 6).

Discussion

In the present study, we validated the association of several reported amino acid substitutions with IFN sensitivity and with the response to combination therapy with PEG-IFN and ribavirin. We found that total rate of SVR in the present study was considerably lower (36.5%) in comparison to previous reports on PEG-IFN and ribavirin combination therapy for patients with HCV genotype 1b. The reason for this was unknown. It could be partly because patients with a history of previous IFN therapy (retreatment cases) were included to the study population, partly because we focused on patients with higher pretreatment HCV RNA concentration, and partly because all patients completed the treatment at 48 weeks without elongation. In addition, more patients with HCV strain resistant to IFN might be included in the study patients, although we enrolled all patients who fulfilled the inclusion criteria.

We found a significant difference in pretreatment HCV RNA concentration according to the amino acid sequence 2209–2248 of the HCV NS5A protein. The original report[3] of an association between the amino acid sequence 2209–2248 of HCV NS5A and the response to IFN monotherapy showed a higher pretreatment HCV RNA concentration in patients with the wild type sequence of the HCV NS5A protein. Our results are in agreement with this result. We found a significant difference in the reduction in HCV RNA concentration at 24 h after a single administration of conventional IFN-alpha, according to the amino acid at position 70 of the HCV core protein. The reduction of HCV RNA concentration after a single administration of conventional IFN was significantly less marked in patients with Gln at position 70. The reduction after a single administration of conventional IFN was considered to reflect the resistance to IFN, and this result suggests a difference in sensitivity to IFN between HCV with Arg at position 70 and that with Gln at position 70. In previous studies, Jessner *et al.*[25,26] and Boulestin *et al.*[27] reported the importance of the decrease in serum HCV RNA concentration at 24 h after a single administration of conventional IFN in distinguishing HCV strains that are resistant to IFN-based antiviral therapy against HCV. We suggest that HCV with Glutamine at position 70 might be resistant to IFN-based therapy.

In the association between amino acid identities and the response to the combination therapy, the identity of the amino acid at position 70 was solely and strongly associated with cEVR, ETR, and SVR. In addition to the effect of the substitution of amino acid 70 of the HCV core region, Akuta *et al.*[8,28,29] reported the effect of the substitution of amino acid 91 of the HCV core region and Donlin *et al.*[7] suggested the effect of the substitution of amino acid 139 of the HCV E1 region on the response to the combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1b. More recently, Mori *et al.*[30] reported the effect of amino acid sequence 2209–2248 of the HCV NS5A region, in addition to the effect of amino acid 70 on the response to combination therapy in Japanese patients with HCV genotype 1b. In contrast to these reports, we did not find an association between these substitutions and the response to the combination therapy. The reason for this discrepancy is unknown. It might be partly because we focused on patients with high pretreatment HCV RNA concentration.

The predictive value for SVR of Arg at position 70 was not high (47.2%). In contrast, the predictive value for the lack of SVR of Gln at position 70 was extremely high (96.5%). The influence of amino acid identity at position 70 was therefore closely related to the resistance to IFN of HCV with Gln at position 70. Recently, Okanoue *et al.* reported the close association of amino acid identity at position 70 not with SVR but with the lack of SVR.[31] Our results supported their findings and, in addition, our results showed the direct resistance to IFN of HCV with Gln at position 70.

There are several limitations of the present study. The patients studied were all Japanese. The effect of amino acid 70 on combination therapy with PEG-IFN and ribavirin should therefore be verified in other ethnicities. Also, all patients studied had high pretreatment HCV RNA concentration ($>100 \times 10^3$ IU/mL). If HCV genotype 1b with amino acid 70 of Gln is resistant to IFN, the lower rates of cEVR, ETR, or SVR will be maintained in patients with HCV genotype 1b but with lower pretreatment HCV RNA concentration. This should also be clarified in the future. In addition, there are no reported mechanisms which could explain the effect of this amino acid substitution on the effectiveness of the combination therapy against HCV. This area should be studied in the future.

In conclusion, the results of the present study show that the substitution of amino acid 70 of the HCV core region was strongly associated with resistance to IFN and was independently associated with the response to the combination therapy with PEG-IFN and ribavirin in Japanese patients with HCV genotype 1b, presumably due to this resistance to IFN of HCV with Gln at position 70. The determination of the identity of amino acid 70 of the HCV core region can be useful for prediction of the resistance to combination therapy in this patient subpopulation, and can be useful for the decision of the indication of this therapy. Further studies in larger patient populations including multiple ethnicities will be needed to validate the significance of these amino acid substitutions and others which have been reported.

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Supporting information

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

Table S1. Association between amino acid substitutions and responses to combination therapy with peginterferon and ribavirin in treatment-naïve patients.

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HEPATOLOGY

Prevalence and clinical characterization of patients with acute hepatitis B induced by lamivudine-resistant strains

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acute hepatitis, genotype, hepatitis B virus, lamivudine.

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Abstract

Background and Aims: Acute hepatitis caused by lamivudine (LMV)-resistant strains has not been reported, and the clinical impact of LMV-resistant strains on acute hepatitis is not known. The aim of this study was to investigate the molecular and clinical characteristics of patients with acute hepatitis B caused by LMV-resistant strains.

Methods: Forty-five patients with acute hepatitis B were studied. Hepatitis B virus (HBV) subgenotypes and LMV-resistance mutations were determined by direct sequencing of the preS and polymerase regions, respectively.

Results: HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected in patients with acute hepatitis. LMV-resistance mutations were detected in two patients. LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2 who had acute self-limited hepatitis. The other patient with LMV-resistance mutations (L180M, M204V) was infected with subgenotype A2 and had severe hepatitis.

Conclusion: LMV-resistant strains are rare, but they are starting to be found in patients with acute hepatitis B. Surveillance for detecting drug-resistant HBV strains would be important for clinical practice.

Introduction

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV).[1] HBV infection causes a variety of clinical courses, such as self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma.[2] Therefore, HBV infection is one of the most important global health problems. Most countries have performed universal vaccination to prevent HBV infection, but only high-risk groups, such as health-care workers and household contacts of HBV carriers, have received HBV vaccination in Japan.[3] Therefore, acute hepatitis is still a major problem in Japan. The frequencies of HBV strains that are rare in Japan have increased among Japanese patients with acute hepatitis B.[4–6] The distributions of the HBV strains in acute hepatitis are variable due to the changing social environment. Along the same lines, a study investigated acute hepatitis B induced by lamivudine (LMV)-resistant HBV strains, but acute hepatitis caused by an LMV-resistant strain has not been found, and the clinical impact of LMV-resistant strains on acute hepatitis is still unknown.[7] Surveillance of HBV strains associated with acute hepatitis B has been continued, and LMV-resistant strains have begun to be detected in patients with acute hepatitis B. Thus, the present study reports the clinical characteristics of patients in Japan with acute hepatitis B caused by LMV-resistant HBV strains.

Materials

Forty-five Japanese patients with acute hepatitis B who were treated at Nagoya University Hospital, Ogaki Municipal Hospital, Tosei Hospital, Yokkaichi Hospital, and Fujita Health University Hospital were enrolled in this study between January 2006 and September 2008. The patients were 37 men and eight women, with a mean age of 38.6 ± 12.9 years (range, 18–84 years). There were no patients who had received HBV vaccine. Acute hepatitis B was diagnosed as follows. Each patient had high titers of hepatitis B surface antigen (HBsAg) and immunoglobulin (Ig)M class antibody against HBV core antigen, elevated serum levels of alanine aminotransferase and absence of antibodies against other causative viruses, such as hepatitis A virus, hepatitis C virus, Epstein–Barr virus and cytomegalovirus. It was necessary to discriminate