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Original Article

Prolonged treatment with pegylated interferon α 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan

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Aim: This study was conducted to clarify the factors related to sustained virological response (SVR) to pegylated interferon α 2b (PEG-IFN) plus ribavirin (RBV) combination therapy administered for 48 weeks in patients with chronic hepatitis C virus (CHCV) and to evaluate the usefulness of prolonged treatment in patients with late virological response (LVR).

Methods: Of 2257 patients registered at 68 institutions, those with genotype 1 and high viral load were selected to participate in two studies. Study 1 (standard 48-week group, $n = 1480$) investigated SVR-determining factors in patients who received the treatment for ≤52 weeks, whereas study 2 compared SVR rates between patients with LVR who received treatment for either 36–52 weeks (48-week group, $n = 223$) or 60–76 weeks (72-week group, $n = 73$).

Results: In study 1, SVR rate was 44.9%; that in male subjects (50.4%) was significantly ($P < 0.0001$) higher than in female subjects (36.4%). SVR rate significantly ($P < 0.0001$) decreased with 10-year age increments in both sexes. Multivariate logistic regression analysis revealed that age, F score, platelet count, and HCV load were SVR-related factors. In study 2, SVR rate in the 72-week group (67.1%) was significantly ($P = 0.0020$) higher than in the 48-week group (46.2%).

Conclusions: Patients with CHCV genotype 1 infection should be treated with PEG-IFN plus ribavirin combination therapy as early as possible, and 72 weeks' treatment is recommended in patients with LVR regardless of age.

Key words: chronic hepatitis C virus, elderly patients, pegylated interferon, prolonged treatment, ribavirin

INTRODUCTION

THE TOTAL NUMBER of patients infected with the hepatitis C virus (HCV) is estimated at 170 million worldwide, of whom 1.5–1.7 million are Japanese. Treatment of HCV infection began with interferon (IFN) monotherapy before the discovery of HCV in 1989. At that time, responders to treatment were mostly limited to patients with HCV genotypes 2 or 3 infection, which is highly sensitive to IFN. The sustained virological response (SVR: HCV-RNA negative at 24 weeks after end of treatment) to IFN monotherapy in genotype 1 patients known from that time to be difficult to treat was only about 5%. SVR rate has since increased thanks to concomitant administration of the antiviral drug ribavirin (RBV), and with the development of the long-acting

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IFN product pegylated interferon (PEG-IFN) it has increased to 50%.[1–4] Today, PEG-IFN plus ribavirin regimen is internationally recognized as a standard therapy for chronic hepatitis C virus (CHCV) infection.[5,6] Early clinical trials of this regimen focused on specific patient populations. Subsequently, several multinational studies such as WIN-R,[7] HALT-C,[8] EPIC3,[9] and REPEAT Study[10] have been conducted in the general clinical setting. The results of the IDEAL Study[11] directly comparing PEG-IFN α 2a versus PEG-IFN α 2b have also been published. From these studies, variables predictive of SVR have been identified, including ethnicity, sex, age, and weight as demographic parameters, staging and hepatic steatosis as histological parameters, viral load, genotype, NS5A, and core mutation as virologic parameters, alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) as biochemical parameters, and even the timing of viral negativity as a treatment variable.[12–15] More recently, the SVR rate was reported to increase in association with decrease in the relapse rate with 72-week treatment in patients with delayed HCV-RNA negativity.[15,16] However, the majority of patients participating in previous studies in western countries were aged in their 40s on average, and the influence of aging of the patient population has not been studied adequately.

We therefore examined SVR-determining factors with 48-week PEG-IFN α 2b plus RBV combination therapy in the prevailing Japanese clinical setting characterized by increasing numbers of elderly patients. We also compared SVR rate between 48-week and 72-week treatment in patients with late virological response (LVR) defined as achieving HCV-RNA negativity in the period from weeks 13 to 24 after the start of treatment so as to examine the significance of prolonged treatment.

METHODS

Patients

A MULTICENTER STUDY was conducted at 68 institutions in Tokyo and Yamanashi prefectures (PERFECT Study Group; see Appendix I) to survey the actual state of combination therapy with PEG-IFN α 2b (PegIntron; Schering Plough, Kenilworth, NJ) and RBV (Rebetol, Schering Plough) in 2008. A total of 2257 chronic hepatitis C virus (CHCV) patients seen from December 2004 who completed combination treatment by September 2007 were registered regardless of genotype, history of IFN treatment, and ALT levels. The presence of HCV in serum had to be confirmed by Cobas Amplicor HCV Monitor, version 2.0 (Roche Diagnostic, Tokyo) for registration.

Excluded from this study were pregnant or possibly pregnant and lactating women, and patients with severe heart disease, chronic kidney failure or creatinine clearance of ≤50 mL/min, current or history of severe psychiatric disorder, and autoimmune hepatitis.

Demographic characteristics examined included age, sex, height and weight, the presence or absence of diabetes mellitus, hypertension, heavy drinking, and history of IFN therapy and hepatic cancer. Hepatic histological data recorded were stage (F0–F4) and grade (A0–A3). Laboratory tests recorded were ALT, platelet count, albumin, and α-fetoprotein (AFP) before the start of PEG-IFN α 2b plus RBV combination therapy.

As indicated in Figure 1, of the total 2257 patients registered, patients with genotype 1 and high viral load (>100 KIU/mL: Amplicor PCR quantitation) who satisfied the following conditions were included in this study: patients who received treatment for ≤52 weeks (standard 48-week treatment group, $n = 1480$) in study 1, and patients with LVR who received treatment for either 36–52 weeks (48-week treatment group, $n = 223$) or 60–76 weeks (72-week treatment group, $n = 73$) in study 2.

This multicenter study was approved by IRB at each participating institution. The study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from each patient.

Treatment

PEG-IFN α 2b was administered subcutaneously once weekly at a dose of 1.5 μg/kg. Dose reduction and treatment discontinuation followed the instructions given in the package insert, i.e., the dose was reduced by half if WBC decreased to <1500/mm^3, neutrophils to <750/mm^3 or platelet count to <80000/mm^3, and treatment was discontinued if WBC decreased to <1000/mm^3, neutrophils to <500/mm^3 or platelet count to <50000/mm^3. RBV was administered in two divided doses of 600, 800, or 1000 mg/day in patients weighing <60, 60–<80, and ≥80 kg, respectively. Dose reduction and treatment discontinuation followed the package insert, i.e., dose was reduced from 600 mg/day to 400 mg/day, from 800 mg/day to 600 mg/day, or from 1000 mg/day to 600 mg/day if hemoglobin (Hb) concentration decreased to <10 g/dL, and administration was discontinued if Hb decreased to 8.5 g/dL. Duration of treatment was 48 weeks as a rule. In LVR patients who did

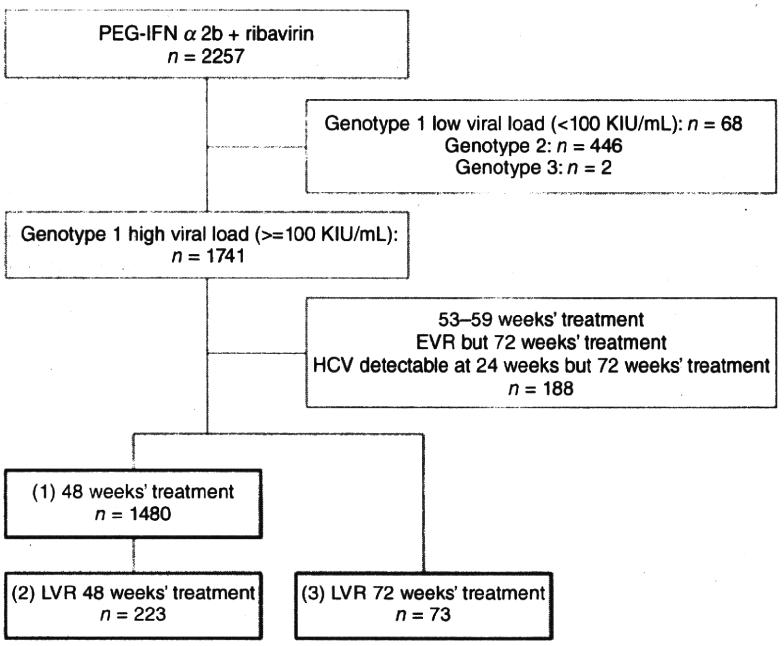

Figure 1 Flow-chart of study subjects. (1) 48 weeks' treatment (48-week standard therapy group): patients with genotype 1 and high viral load who received pegylated interferon α 2b (PEG-IFN α 2b) + ribavirin (RBV) for 52 weeks. Multiple logistic regression analysis was used to evaluate the response to PEG-IFN α 2b + RBV in this group (2) Late virological response (LVR) 48 weeks' treatment: patients with genotype 1 and high viral load who received PEG-IFN α 2b + RBV for 36–52 weeks (3) LVR 72 weeks' treatment: patients with genotype 1 and high viral load who received PEG-IFN α 2b + RBV for 60–76 weeks. SVR rate was compared between LVR 48 weeks' treatment group (2) and LVR 72 weeks' treatment group (3). EVR, early virologic response; HCV, hepatitis C virus.

not achieve HCV-RNA negativity by week 12, treatment could be extended for 48 weeks or longer based on individual patients' desire and investigators' judgment.

Evaluation of response to treatment

Determination of genotype and measurement of HCV-RNA levels were performed at each center. Pre-treatment HCV-RNA levels were determined by Amplicor PCR quantitation. Viral negativity was defined as HCV below detection limit (<50 IU/mL) by Amplicor qualitative analysis (Roche Molecular Systems, NJ).

SVR was defined as HCV below detection limit at 24 weeks after the end of PEG-IFN α 2b plus RBV combination therapy by Amplicor HCV qualitative analysis.

Statistical analysis

All statistical analyses were performed using SAS, version 9.13 (SAS Institute, Cary, NC). Intergroup comparison of SVR rate was performed by Fisher's exact test; that of background variables by Fisher's exact test and Mann–Whitney *U*-test. Trend of SVR rate by age was assessed by Cochran–Armitage test, and intergroup comparison after adjustment of stratification factors was conducted by Mantel–Haenstzel method. Determination of factors associated with SVR was conducted by a stepwise procedure using the results of logistic univariate analysis ($P < 0.2$) into logistic multivariate analysis. All tests were two-sided, with significance level set at $P < 0.05$.

RESULTS

Study 1: SVR-related factors in patients receiving standard 48-week treatment

AS INDICATED IN Table 1 and Figure 1, 1480 subjects (male, $n = 898$ [60.7%]; median age, 57 [range, 13–79] years) were eligible for analysis. SVR rate based on ITT was 44.9%. SVR rate in subjects who completed and who discontinued treatment was 56.5% ($n = 1110$) and 10.3% ($n = 370$), respectively, a statistically significant difference ($P < 0.0001$). SVR rate in male subjects (50.4%; 453/898) was significantly ($P < 0.0001$) higher than in female subjects (36.4%; 212/582). SVR rate significantly ($P < 0.0001$) decreased as age increased by 10 years in both male and female subjects (Fig. 2); the odds ratio for SVR decreasing with 10-year increase in age was 0.688 (95% CI, 0.604–0.784; $P < 0.0001$) in male subjects and 0.546 (0.449–0.663; <0.0001) in female subjects, indicating that the influence of aging was greater in female than in male subjects. There was no bias of older versus younger age among patients who had and had not previously

Table 1 Pretreatment characteristics of chronic hepatitis C virus (CHCV) patients with HCV-1b RNA who received pegylated interferon α 2b + ribavirin standard therapy for 48 weeks

Characteristic	Value (n = 1480)
Sex (male/female)	898/582
Age (years)	57 (13–79)
History of HCC (yes/no/unknown)	8/1405/67
Previous IFN treatment (yes/no/unknown)	459/688/333
Diabetes (yes/no/unknown)	44/480/956
Hypertension (yes/no/unknown)	105/417/958
Ongoing alcohol use (yes/no/unknown)	157/456/867
Grade (A0/A1/A2/A3/unknown)	14/499/478/55/434
Stage (F0/F1/F2/F3/F4/unknown)	36/469/316/176/48/435
ALT (IU/L)	63 (8.4–910)
Platelets (×10⁴/μL)	16.6 (4.3–47.7)
Viral load (KIU/mL)	1900 (100–5100)

Data expressed as median (range). HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; IFN, interferon.

received IFN. Whereas, multivariate logistic regression analysis revealed that older age (<55/≥55 years), degree of progression of hepatic fibrosis (F0–1/2–4), low platelet count (≥16/<16×10⁴/μL), and high viral load (<1900/≥1900 KIU/mL) are resistance factors to SVR (Table 2). In multivariate logistic regression analysis, sex was not selected.

Study 2: usefulness of prolonged treatment in LVR patients

Of the patients who completed standard 48-week treatment, 223 patients (20.0%) showed LVR (Fig. 1), and median duration of treatment was 48 weeks. Compared with patients who exhibited early virologic response (EVR) defined as HCV-RNA negative within 12 weeks after the start of treatment, those with LVR were older (median age, 58 vs 55 years; $P = 0.0043$) and had higher viral load (median, 2700 vs 1620 KIU/mL; $P < 0.0001$) and lower platelet count (median, 16.5 vs 17.3 × 10⁴/μL; $P = 0.0162$). SVR rate based on treatment analysis was 56.5 in all, 79.2% in EVR and 46.2% in LVR, respectively. In multivariate logistic regression analysis of SVR-related factors in LVR patients who completed standard 48-week treatment, age (10-year groups) was selected as a significant factor.

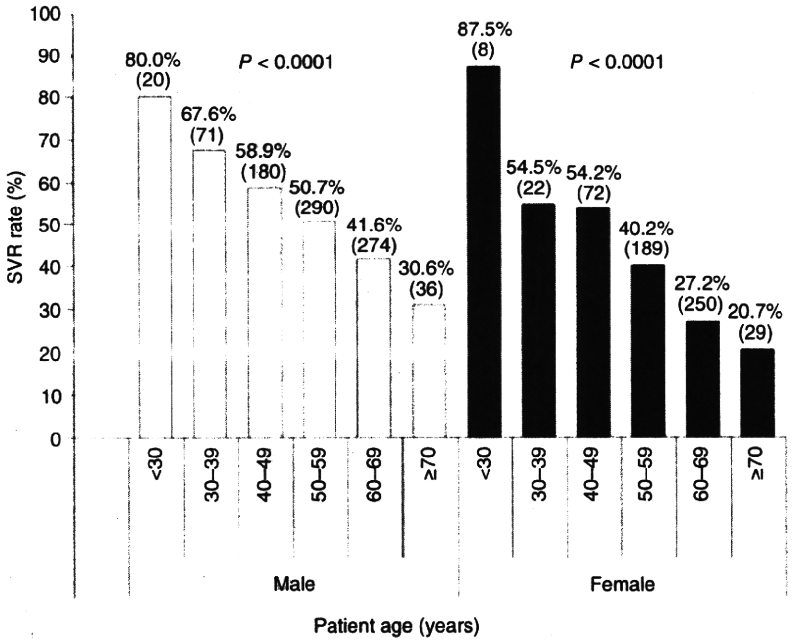

Figure 2 Sustained virological response (SVR) rate to 48 weeks' standard treatment with pegylated interferon α 2b (PEG-IFN α 2b) + ribavirin in male and female patients stratified by age. Cochran–Armitage test was used to study the underlying trend.

Table 2 Independent factors associated with sustained virological response in genotype 1 chronic hepatitis C virus patients who received pegylated interferon α 2b + ribavirin standard therapy for 48 weeks

	Odds ratio	95% confidence interval	*P*-value†
Age <55/≥55 years	0.414	0.293–0.585	<0.0001
Stage 0–1/2–4	0.633	0.442–0.906	0.0124
Platelets <16/≥16 × 10^4/μL	1.876	1.305–2.696	0.0007
Viral load </≥1900 KIU/mL	0.663	0.471–0.935	0.0192

†Multiple logistic regression analysis.

Prolonged treatment was conducted in 73 LVR patients (Fig. 1), with mean duration of 72 weeks. As shown in Table 3, whereas among LVR patients there were significantly ($P = 0.0061$) more female subjects in 72-week group than 48-week group, no intergroup difference of other factors was observed. Overall, SVR rate based on treatment analysis was significantly ($P = 0.0020$) higher in 72-week treatment group than in 48-week treatment group (67.1% [49/73] vs 46.2% [103/223]; Fig. 3A).

When stratified by sex, SVR rate with 48-week and 72-week treatment was 51.4% and 68.6% ($P = 0.0809$) in male subjects and 37.3% and 65.9% ($P = 0.0039$) in female subjects, with SVR in 72-week treatment being significantly higher in female subjects and indicating that, in LVR patients, efficacy comparable to male subjects is achieved in female subjects with 72-week treatment.

In patients aged <55 years SVR rate in the 48- and 72-week treatment groups was 57.6% and 78.9% ($P = 0.1100$) in male subjects and 40.0% and 76.9% ($P = 0.0724$) in female subjects, respectively, with higher SVR rates for the 72-week treatment group (Fig. 3B). In patients aged ≥55 years this parameter was 44.6% and 53.8% ($P = 0.5619$) in male subjects and 37.1% and 60.7% ($P = 0.0425$) in female subjects, respectively, with higher SVR rates for the 72-week treatment group than for the 48-week treatment group as in the case of the younger age group (Fig. 3C).

DISCUSSION

Study 1: SVR-related factors in patients receiving standard 48-week treatment

SVR RATE WITH standard 48-week treatment in this study was 44.9%, roughly equal to the 45% reported in previous clinical trials in Japan.[4,17–19] The present results are also similar to those of clinical trials conducted in patients aged in their mid-40s in western countries and in the general clinical setting.[1–4] Age was

Table 3 Comparison of clinical and virological characteristics between groups receiving pegylated interferon α 2b + ribavirin therapy for 48 and 72 weeks among patients showing late virological response

	48 weeks' group ($n = 223$)	72 weeks' group ($n = 73$)
Sex (male/female)	140/83*	32/41*
Age (years)	58 (21–75)	56 (22–71)
History of HCC (yes/no/unknown)	1/221/11	0/73/0
Previous IFN treatment (yes/no/unknown)	68/113/42	29/32/12
Diabetes (yes/no/unknown)	11/71/141	1/34/38
Hypertension (yes/no/unknown)	18/62/143	6/29/38
Ongoing alcohol use (yes/no/unknown)	17/75/131	6/27/40
Grade (A0/A1/A2/A3/unknown)	2/66/82/6/67	0/21/26/4/22
Stage (F0/F1/F2/F3/F4/unknown)	7/68/45/32/5/66	2/16/20/12/2/21
ALT (IU/L)	61.5 (14–550)	52 (17–254)
Platelets (×10^4/μL)	16.5 (8.5–43.2)	16.6 (4.3–40.2)
Viral load (KIU/mL)	2700 (160–5100)	2100 (130–5000)

Data expressed as median (range). *$P = 0.006$. ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; IFN, interferon.

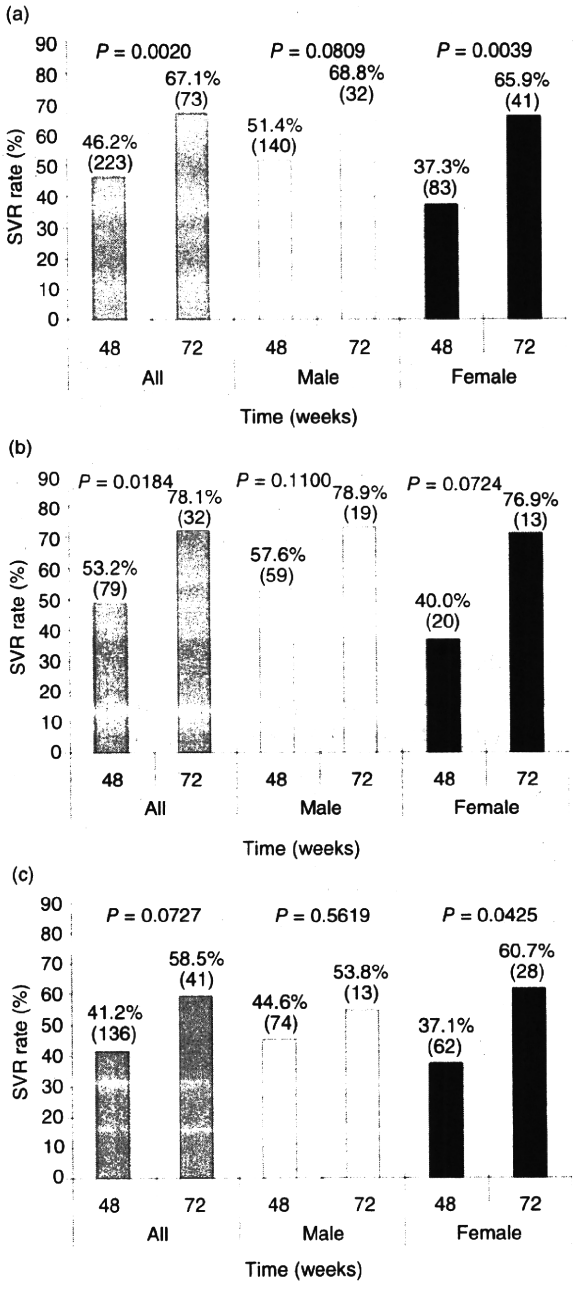

Figure 3 Sustained virological response (SVR) rate based on treatment analysis between groups receiving pegylated interferon α 2b (PEG-IFN α 2b) + ribavirin therapy for 48 and 72 weeks who exhibited late virological response (LVR). (A) Overall; (b) patients aged <55 years; (c) patients aged ≥55 years. Data on age not available for 7 male patients and 1 female patient.

selected among factors for SVR with PEG-IFN plus RBV combination therapy in an aging patient population, the examination of which was the objective of this study, and SVR rate decreased stepwise with 10-year age increase. Of particular note was the greater impact of aging observed in female than male subjects.

Lower efficacy in elderly female patients infected with HCV genotype 1 has already been reported in Japan.[20] A low SVR rate was also observed in elderly female subjects in this study. Although female sex was considered a favorable prognostic factor in some Western studies, there is no established opinion on sex difference. Change associated with aging of the patient population in Japan is considered to account for this phenomenon observed in the present study. This may be due to decrease in compliance among elderly women; on the other hand, however, there was no difference between male and female subjects aged ≥55 years in the rate of completion of treatment. Although the rate of dose reduction of RBV tended to be slightly higher in female subjects (data not shown), the difference was not significant. These findings suggest the influence of factors other than adherence to treatment for the low SVR rate among elderly women. One possible factor for reduced SVR rate among these individuals may be the effect of menopause. In women, insulin resistance begins to worsen after the age of 50 years,[21,22] and this is reported more closely associated with the effect of menopause than age itself.[23]

The presence of insulin resistance has been reported to lower efficacy of PEG-IFN and RBV combination therapy.[24–27] Insulin resistance is also a cause of advanced fibrosis and fatty change of the liver.[28–31] It is possible that such changes combined with other factors associated with metabolic syndrome interact in a complex way to reduce the efficacy of this therapy.[32–35] In fact, the incidence of non-alcoholic fatty liver disease (NAFLD) among elderly Asians was reported higher in women as compared with that in men.[36–38] However, while older age, advanced fibrosis, low platelet count and high HCV load were selected as factors for reduction of SVR rate in our multivariate logistic regression analysis, sex was not selected. It is therefore necessary to examine further the confounding of these selected factors with sex. It also should be taken into consideration that, due to limitations imposed by the retrospective nature of this study, data on factors affecting the efficacy of PEG-IFN plus RBV therapy such as insulin resistance, steatosis, and core mutation are lacking. A large-scale prospective study is

required to examine the lower efficacy observed in elderly women.

Study 2: usefulness of prolonged treatment in LVR patients

EVR (viral load reduced by 2 log or undetected in week 12) has been used for determining continuation or discontinuation of treatment in western countries. Recently, however, EVR was divided into complete EVR (HCV RNA <50 IU/mL at week 12) and partial EVR (>2 log drop in HCV RNA but still detectable [>50 IU/mL]). Fried *et al.*[15] and Berg *et al.*[16] reported that the SVR rate was a high 68–84% in patients showing complete EVR but only 17–29% in those with partial EVR with treatment for 48 weeks. They also reported that treatment for 72 weeks was effective in patients with partial EVR. In the clinical study for health registration in Japan, the SVR rate by timing of HCV-RNA negativity at 4, 12, and 24 weeks was 100%, 71.1%, and 36.4%, respectively, and no patient with HCV-RNA negativity after 25 weeks achieved SVR.[4] With these studies as reference, patients with LVR were defined as those who were positive (>50 IU/mL) at week 12 and became negative (<50 IU/mL) by week 24. To minimize the influence of treatment discontinuation, only patients who completed the standard duration of treatment were selected as subjects in this study. In the comparison of patient background, there was no significant intergroup difference except for a significantly greater number of female subjects in the 72-week treatment group. This finding might be related to the observation that it was already widely believed that efficacy in elderly women in Japan is low and that duration of treatment was at the discretion of individual physicians. Nevertheless, it is noteworthy that the SVR rate was significantly higher in the 72-week treatment group than in the 48-week treatment group and that a high 60% SVR rate was achieved with 72-week treatment in elderly female patients, a population in whom a relatively low SVR was observed with standard 48-week treatment.

This retrospective study had the limitation that duration of treatment was at the sole discretion of each participating physician. A prospective study is necessary to demonstrate whether 72-week treatment in elderly women with LVR is more efficaous than 48-week treatment in male patients. Although the number of younger subjects examined was rather low, it is noteworthy that an SVR rate of >75% was observed with 72-week treatment in both male and female patients. This also should be confirmed by prospective study.

CONCLUSIONS

PATIENTS WITH CHCV genotype 1 infection should be treated with PEG-IFN and ribavirin combination therapy as early as possible. Seventy-two weeks' treatment is recommended in patients with LVR, regardless of age.

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APPENDIX I

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Thrombocytopenia is more severe in patients with advanced chronic hepatitis C than B with the same grade of liver stiffness and splenomegaly

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Abstract
Background and aim The mechanism responsible for thrombocytopenia in chronic liver diseases (CLD) is not yet fully understood. The prevalence of thrombocytopenia has been reported to be higher in patients with hepatitis C virus-related hepatocellular carcinoma (CLD-C) than in those with hepatitis B virus-related hepatocellular carcinoma (CDC-B). We have examined the potential difference in thrombocytopenia between patients with CLD-B and those with CLD-C in terms of liver fibrosis adjustment and splenomegaly.
Methods The study cohort consisted of 102 patients with CLD-B and 143 patients with CLD-C were enrolled. Liver stiffness, which is reported to be well correlated with the degree of liver fibrosis, was measured by transient elastography.
Results The analysis of covariance with liver stiffness as a covariate revealed that the platelet count was lower in CLD-C patients than in CLD-B patients. Following stratification for liver stiffness, thrombocytopenia was found to be more severe in CLD-C patients than CLD-B patients with advanced liver stiffness, whereas the degree of splenomegaly was not significantly different. The plasma thrombopoietin level was not different between CLD-B and CLD-C patients with advanced liver stiffness, and the immature platelet number was lower in CLD-C patients despite thrombocytopenia being more severe in these patients.
Conclusions CLD-C patients with advanced liver stiffness presented with more severe levels of thrombocytopenia than CLD-B patients even with the same grade of splenomegaly. Impaired platelet production rather than enhanced platelet destruction may underlie the mechanism responsible for thrombocytopenia in patients with CLD.

Keywords Liver stiffness · Splenomegaly · Thrombocytopenia · Thrombopoietin · Transient elastography

Abbreviations

ALT	Alanine aminotransferase
ANCOVA	Analysis of covariance
CLD	Chronic liver disease
HBeAg	Hepatitis B envelope antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
IPF	Immature platelet fraction
IQR	Interquartile ranges

Introduction

Advanced chronic liver disease (CLD) has long been known to be accompanied by thrombocytopenia.

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Thrombocytopenia is one of the major problems encountered when treating esophageal varix or hepatocellular carcinoma (HCC), both of which are frequently observed in patients with advanced CLD. Thrombocytopenia also often hampers the treatment of hepatitis C virus (HCV)-related CLD (CLD-C) by interferon [1]. Thus, it is important to clarify the mechanism and establish the therapeutic strategy of thrombocytopenia in CLD. Because cirrhotic liver with advanced tissue fibrogenesis gives rise to portal hypertension, inducing splenic enlargement and congestion [2–5], splenomegaly has been considered to cause thrombocytopenia by increased sequestration and enhanced destruction of platelets [6, 7]. However, the exact pathogenesis of thrombocytopenia in CLD is not yet fully understood [8–11].

Of interest is the fact that the prevalence of thrombocytopenia is higher among patients with HCV-related HCC than in those with hepatitis B virus (HBV)-related HCC [12]. Such evidence suggests that HBV-related HCC may tend to arise in the liver with less advanced chronic injury than in that of patients with HCV-related HCC; it also raises the possibility that thrombocytopenia may be more severe in CLD-C than HBV-related CLD (CLD-B). To examine the latter possibility, it is necessary to compare the platelet count between the two groups with an adjustment for the degree of liver fibrosis or splenomegaly. However, previous studies in this area may have been hindered by the invasiveness of measuring portal blood pressure or liver fibrosis. More recent studies, however, have focused on the non-invasive prediction of liver fibrosis [13–16], taking into account the various limitations of liver biopsy [17–20]. In particular, recent evidence on transient elastography reveals that the liver stiffness values acquired by the elastometer correlate well to the fibrosis stages as determined by liver biopsies [21–24]. Similar optimal cut-off values of liver stiffness have also been reported for the diagnosis of cirrhosis in patients with chronic hepatitis B and C [25, 26].

In the study reported here, we sought to investigate whether the degree of thrombocytopenia in CLD differs according to virological etiology, namely, HBV or HCV. Since most of the patients enrolled in our study were outpatients, we measured liver stiffness in each patient instead of performing liver biopsy to assess the degree of liver fibrosis, which may raise an ethical concern. In addition to the non-invasiveness of the measurement, liver stiffness values may have the merit of being a continuous variate, which has a wider application to statistical analyses than a discrete variate, such as fibrosis stages. Using our measurements of liver stiffness, spleen size, and platelet count obtained for our patients with CLD-B and CLD-C, we compared the degree of thrombocytopenia and splenomegaly with the adjustment for the degree of liver stiffness.

Patients and methods

Patients

A total of 245 consecutive patients with CLD-B or CLD-C who underwent liver stiffness measurements at the Department of Gastroenterology, University of Tokyo Hospital, Tokyo, Japan, between October 2006 and August 2009 were enrolled in this study. CLD-B was diagnosed based on positivity for hepatitis B surface antigen (HBsAg) for at least 6 months. CLD-C was defined by a positive result for serum anti-HCV antibodies and detectable HCV RNA. Exclusion criteria were co-infection of HBV and HCV, co-infection with human immunodeficiency virus, other causes of liver disease, and the existence of ascites, which hinders the liver stiffness measurement. The study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo.

Virological assays

HBsAg was measured using automated chemiluminescence enzyme immunoassay systems (Architect i2000; Abbott Laboratories Diagnostics, Abbott Park, IL) for HBsAg. Anti-HCV antibodies were measured by a third-generation enzyme immunoassay (Architect Anti-HCV; Abbott Laboratories Diagnostics). HCV RNA was assessed by qualitative (sensitivity, 50 IU/mL) or quantitative assays (Cobas Amplicore Hepatitis C Virus Test; Roche Diagnostics Systems, Branchburg, NJ).

Measurement of liver stiffness

Liver stiffness was measured by transient elastography (FibroScan 502; EchoSens, Paris, France) as described previously [22, 23, 27]. Briefly, the measurements were performed in the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position. The median value, calculated with at least ten successful acquisitions in each FibroScan examination, of which the success rate was more than 60%, was adopted and expressed in kilopascals (kPa). On the same day, the spleen size was measured, with the exception of patients with a previous splenectomy, using a common convex probe (3.75 MHz) with an ultrasound machine (SSA-770A Aplio XV; Toshiba Medical Systems, Tochigi, Japan). The spleen size was expressed as the splenic index, calculated as half the maximum craniocaudal

length multiplied by the maximum width of the spleen obtained on longitudinal sections [28].

Platelet count and biochemical assays

Platelet count was determined using a Coulter GEN-S System (Beckman Coulter, Miami, FL) in the clinical laboratory of the hospital. Serum alanine aminotransferase (ALT) activity, total bilirubin concentration, and albumin concentration were measured using an automated analyzer (Hitachi 7170; Hitachi Instruments Service Co, Tokyo, Japan). Prothrombin time was measured using an automated coagulometer (Coagrex 800; Sysmex Co, Kobe, Japan). For the purposes of this study, we adopted the laboratory data acquired on the same day as the liver stiffness measurement. Plasma thrombopoietin concentration was determined in plasma obtained on the day of the liver stiffness measurement, if any, by means of an enzyme-linked immunosorbent assay using a commercial kit (Quantikine Human TPO Immunoassay; R&D Systems, Minneapolis, MN); the reference range was 14–72 pg/mL in plasma [29].

Immature platelet fraction assay

A new automated method to reliably quantify reticulated platelets, expressed as the immature platelet fraction (IPF), was employed for this purpose; IPF was measured using the Sysmex XE-5000 automated hematology analyzer (Sysmex, Kobe, Japan) as previously described [30].

Statistical analysis

Data are expressed as medians with first to third quartile values (interquartile ranges; IQR). Between-group comparisons were made using Fisher's exact test, Mann–Whitney's U test, or an analysis of covariance (ANCOVA) where appropriate. The correlation between two groups, in which the data points were distribution-free, was analyzed using Spearman's rank correlation coefficient (rs). A two-sided P value <0.05 was considered to be statistically significant. Statistical procedures were performed using SAS ver. 9.1 (SAS Institute, Cary, NC).

Results

Patients

The characteristics of the enrolled 245 patients are summarized in Table 1. There were 102 patients with CLD-B and 143 patients with CLD-C. Relative to the CLD-C patients, the CLD-B patients were younger and there was a higher proportion of males. The comparison between the two groups without any adjustment revealed a significant difference in liver stiffness and platelet count, while there was no significant difference in spleen size. CLD-B patients also had a lower serum ALT activity and higher albumin concentration than CLD-C patients. When all of the patients were considered in the analysis, the strongest and most inverse correlation was found between liver stiffness and platelet count ($rs = -0.607$, $P < 0.001$), followed by serum albumin ($rs = -0.566$, $P < 0.001$), and prothrombin time ($rs = -0.505$, $P < 0.001$). There was also a strong correlation between liver stiffness and spleen size ($rs = 0.504$, $P < 0.001$), followed by serum ALT, and then by total bilirubin ($rs = 0.358$, 0.335, respectively, $P < 0.001$). Subsequently, a significant but weak correlation was observed between liver stiffness and age ($rs = 0.201$, $P = 0.002$), which may be explained by the evidence that liver fibrosis advances for a long period of time, especially in CLD-C. The distribution of liver stiffness values in patients with CLD-B and CLD-C is shown in Table 2.

Comparisons of platelet count between CLD-B and CLD-C patients

The possible difference in platelet count between CLD-B patients and CLD-C patients was first examined using ANCOVA to analyze the liver stiffness value, following logarithmic transformation because of its near log-normal distribution, as a covariate. The inter-group difference in platelet count was revealed to be significant ($P < 0.001$), while the interaction between the group and liver stiffness was not ($P = 0.845$). These results indicate that the platelet count is lower in CLD-C patients than in CLD-B patients when adjusted for the degree of liver stiffness.

We then compared platelet count between the two groups after stratification according to liver stiffness. We set four strata of liver stiffness values: first stratum, <5.0 kPa; second stratum, 5.0–10.0 kPa; third stratum, 10.0–15.0 kPa; fourth stratum, ≥15.0 kPa. These cut-off values were adopted from the distribution of liver stiffness values in the patients (Table 2) and also from previously published data showing 4.0–8.8, 9.5, and 11.9–17.6 kPa to be the lower limits of stages F2, F3, and F4, respectively, in the conventional classification of liver fibrosis [24, 31]. Platelet count was significantly higher in CLD-B patients than in CLD-C patients at both the highest and lowest stratum of liver stiffness (Fig. 1a).

Comparisons of spleen size between CLD-B and CLD-C patients

Spleen size was well correlated with liver stiffness in both CLD-B patients ($rs = 0.518$, $P < 0.001$) and CLD-C patients ($rs = 0.538$, $P < 0.001$), suggesting that spleen

Table 1 Characteristics of the 245 patients with chronic liver disease

	Total (n = 245)	HBV-related (n = 102)	HCV-related (n = 143)	HBV vs. HCV P value
Demographics				
Age (years)	61 (53–70)	55 (44–62)	66 (57–73)	<0.001[a]
Male:female	153:92	72:30	81:62	0.032[b]
Viral status				
HBsAg positive		102 [100]	0	
HCV-Ab positive		0	143 [100]	
HCV-RNA positive		Not tested	143 [100]	
Liver stiffness value (kPa)	8.6 (5.4–15.7)	7.8 (4.8–11.8)	10.0 (6.3–17.5)	0.004[a]
Platelet count ($\times 10^4$/mm^3)	15.0 (9.6–19.6)	16.9 (12.4–22.3)	13.5 (8.1–17.8)	<0.001[a]
Splenic index	15.8 (11.7–22.2)	16.0 (11.6–22.9)	15.6 (11.9–21.6)	0.581[a]
Biochemical markers				
ALT (IU/L)	32 (21–53)	27 (20–38)	38 (24–60)	<0.001[a]
Total bilirubin (mg/dL)	0.8 (0.7–1.1)	0.8 (0.6–1.1)	0.9 (0.7–1.3)	0.093[a]
Albumin (g/dL)	4.1 (3.8–4.3)	4.3 (4.0–4.5)	4.0 (3.6–4.2)	<0.001[a]
Prothrombin time (%)	83.1 (74.0–92.7)	84.4 (78.7–92.7)	81.3 (71.2–92.7)	0.070[a]

Data are given as the median with the first to third quartile values (interquartile range, IQR) in round parenthesis or as proportions (n) with the percentage in square parenthesis

Reference ranges: platelet count, 15.5–36.5 $\times$ 10^4/mm^3; splenic index, <20; serum ALT activity, 4–36 IU/L; total bilirubin concentration, 0.3–1.3 mg/dL; albumin concentration, 3.7–4.9 g/dL; prothrombin time, >70.0%

HCV, HBC, Hepatitis C, B; HBsAg, hepatitis B surface antigen; HCV-Ab, hepatitis C antibodies; ALT, alanine aminotransferase

P values <0.05 were considered to be significant

[a] The between-group comparison was made using Mann–Whitney's U test

[b] The between-group comparison was made using Fisher's exact test

Table 2 Distribution of liver stiffness values in patients with CLD-B and CLD-C

Liver stiffness value (kPa)	Patients with CLD-B[a]	Patients with CLD-C[a]	P value
<5.0	27 (26.5)	27 (18.9)	
	4.0 (3.5–4.4)	4.4 (3.7–4.5)	0.253
5.0≤, <10.0	44 (43.1)	44 (30.8)	
	7.5 (5.9–8.4)	7.4 (6.5–8.6)	0.701
10.0≤, <15.0	12 (11.8)	24 (16.8)	
	11.8 (11.8–12.5)	12.0 (11.0–14.0)	0.763
15.0≤	19 (18.6)	48 (33.6)	
	24.1 (20.1–25.6)	21.4 (17.5–27.7)	0.412
Total, n (%)	102 (100)	143 (100)	

CLD-B, -C, HBV- and HBC-related chronic liver disease, respectively

The between-group comparison of liver stiffness value in each stratum was made using Mann–Whitney's U test

[a] Data on liver stiffness are given in each cell as: top line, number (n) of patients, with the percentage in parenthesis; bottom line: median, with the IQR in parenthesis

size is similarly determined throughout the progression of liver stiffness in the two groups. A comparison of spleen size between CLD-B and CLD-C patients after stratification on liver stiffness revealed that spleen size was not significantly different between the two groups in any stratum of liver stiffness (Fig. 1b). Thus, the lower platelet count in the CLD-C patients of our study at the highest stratum of liver stiffness may not be explained by the increased platelet sequestration and destruction caused by splenomegaly.

Comparisons of biochemical markers between CLD-B and CLD-C patients

We then analyzed liver biochemical markers that were also adjusted for the degree of liver stiffness. As shown in

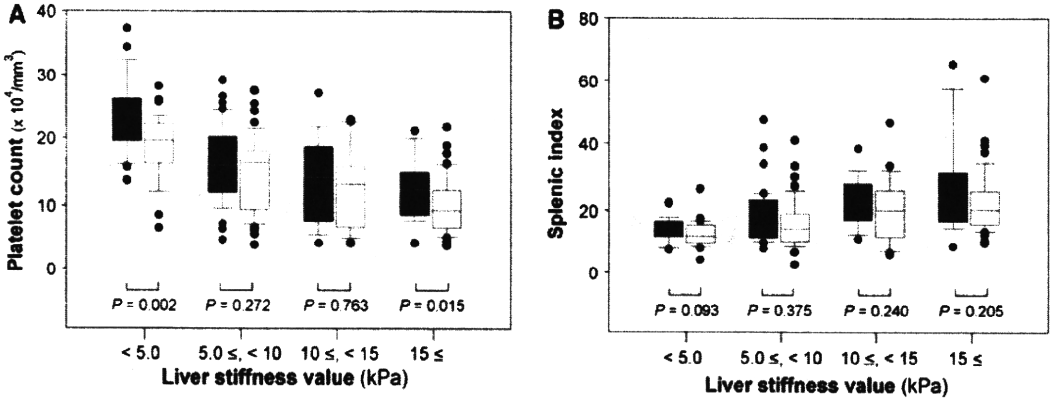

Fig. 1 Comparisons of platelet count or spleen size following stratification for liver stiffness between patients with chronic liver disease related to HBV (*CLD-B*) and HCV (*CLD-C*). Box plots of platelet count (**a**) and splenic index (**b**) in patients with CLD-B (*black boxes*) and CLD-C (*white boxes*). Thrombocytopenia and splenomegaly were defined as a platelet count $<15.5 \times 10^4/mm^3$ and a splenic index of >20, respectively. The *top* and *bottom* of the *boxes* represent the third and first quartile, respectively, with the *length* of the *box* therefore representing the inter-quartile range (IQR). The *horizontal lines inside* the *box* and the *error bars above* and *below* the *box* represent the median and 90th and 10th percentile values, respectively. The data points outside these ranges are also shown as *black dots*. Between-group comparisons were made using Mann–Whitney's *U* test. *P* values <0.05 were considered to be significant

Fig. 2a b, neither serum ALT activity nor total bilirubin concentration was significantly different between the CLD-B and CLD-C patients at any stratum of liver stiffness, including the stratum in which platelet count was different. Compared to CLD-C patients, CLD-B patients had higher serum albumin concentrations in the second to the fourth stratum (Fig. 2c) and higher prothrombin times (%) at the highest stratum of liver stiffness (Fig. 2d). These results suggest that the ability to produce proteins may be more highly impaired in CLD-C patients than in CLD-B patients.

Comparison of thrombopoietin levels between CLD-B and CLD-C patients

The data showing that the platelet count was significantly lower in CLD-C patients than in CLD-B patients at the highest stratum of liver stiffness led us to examine thrombopoietin level. It was possible to measure plasma thrombopoietin level in seven CLD-B patients and nine CLD-C patients at the highest stratum of liver stiffness. The liver stiffness values obtained, 22.6 (IQR 17.4–23.8) kPa and 21.5 (20.9–21.8) kPa in the CLD-B and CLD-C patients, respectively, were not significantly different between these two groups of patients ($P = 0.402$). The platelet count was significantly lower in CLD-C patients ($9.3 \times 10^4/mm^3$; IQR 7.2–10.2) than in CLD-B patients ($13.9 \times 10^4/mm^3$; 12.3–14.1) ($P = 0.007$). However, the plasma thrombopoietin levels in the studied patients were in lower part of the reference range—12.8 (IQR 4.0–15.6) pg/mL in CLD-B patients and 13.8 (6.5–35.5) pg/mL in CLD-C patients—and was not significantly different between the two groups ($P = 0.672$). The plasma thrombopoietin level is regulated by binding to platelets and megakaryocytes; consequently, a low platelet count is known to cause an increase in plasma thrombopoietin level due to a reduced binding to platelets [32–36]. As such, these results raise the possibility that thrombopoietin production may be more severely impaired in CLD-C patients than in CLD-B patients.

Comparison of IPF between CLD-B and CLD-C patients

To determine thrombopoietic activity in CLD-B and CLD-C patients, we also measured the IPF in 11 CLD-B and 13 CLD-C patients at the highest stratum of liver stiffness. IPF measurements have been recently employed to reliably quantify reticulated platelets, and IPF is considered to be a parameter of thrombopoietic activity in evaluating thrombocytopenia [30, 37]. In these 24 patients, the liver stiffness value was 17.6 (IQR 16.6–24.9) kPa in CLD-B patients and 20.9 (18.0–23.9) kPa in CLD-C patients; this difference is not significant ($P = 0.631$). The platelet count was significantly lower in CLD-C patients ($8.7 \times 10^4/mm^3$; IQR 6.1–10.3) than in CLD-B patients ($14.0 \times 10^4/mm^3$; 10.3–18.6) ($P = 0.002$); however, the IPF in CLD-C patients (3.8%; IQR 2.6–4.2) was not significantly higher than that in CLD-B patients (3.6%; 2.2–4.0) ($P = 0.930$). Of note, the number of immature platelets in CLD-C patients ($0.239 \times 10^4/mm^3$; IQR 0.187–0.400) was significantly lower than that in CLD-B patients ($0.396 \times 10^4/mm^3$; 0.373–0.435) ($P = 0.019$). These results suggest that thrombopoietic activity may be more severely impaired in CLD-C patients than in CLD-B patients.

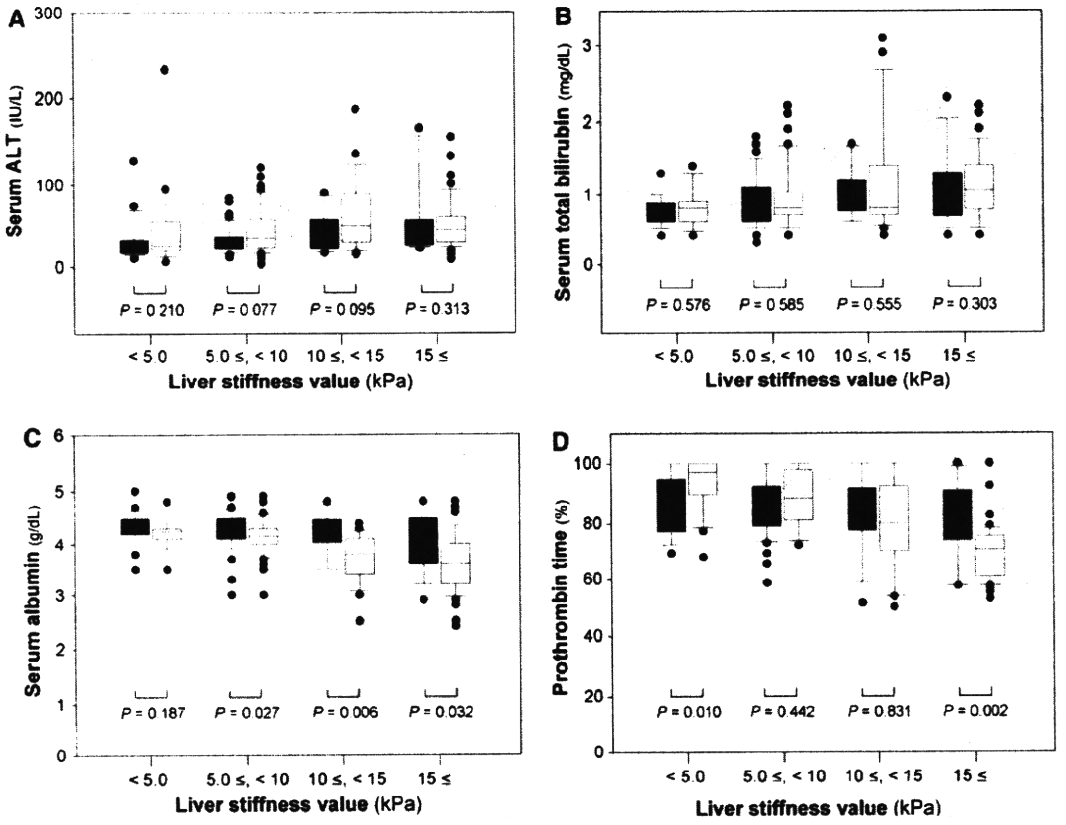

Fig. 2 Comparisons of biochemical markers following stratification for liver stiffness between patients with CLD-B and CLD-C. Box plots of serum alanine aminotransferase (ALT) activity (**a**), total bilirubin concentration (**b**), albumin concentration (**c**), and prothrombin time (**d**) in patients with CLD-B (*black boxes*) and CLD-C (*white boxes*). The *top* and *bottom* of the *boxes* represent the third and first quartile, respectively, with the *length* of the *box* therefore representing the IQR. The *horizontal lines inside* the *box* and the *error bars above* and *below* the *box* represent the median and 90th and 10th percentile values, respectively. The data points outside these ranges are also shown as *black dots*. Between-group comparisons were made using Mann–Whitney's *U* test. *P* values <0.05 were considered to be significant

Discussion

It has been reported that there is no difference in the platelet count between patients with cirrhosis related to HBV and HCV [38]. However, the data of that study were analyzed without stratification for the liver fibrosis stage, liver stiffness, or degree of splenomegaly. In contrast, we have shown here that thrombocytopenia was more severe in our CLD-C patients than in our CLD-B patients with stratification for high liver stiffness. The enrolled patients of the two groups differed in terms of the liver stiffness value, suggesting that the stage of CLD itself may be different. Therefore, in order to compare the severity of thrombocytopenia with adjusting the platelet count for the degree of liver stiffness, we performed ANCOVA and analyzed patients stratified for liver stiffness. Our ANCOVA, with liver stiffness as a covariate, revealed the lower platelet count in our CLD-C patients than in our CLD-B patients, indicating that liver stiffness and virological etiology independently affected the platelet count. In fact, CLD-C patients had a lower platelet count than CLD-B patients at the highest stratum of liver stiffness. These results suggest that virological etiology, namely, HBV or HCV, may distinctly contribute to thrombocytopenia in patients with advanced CLD.

The peripheral platelet count is regulated by the balance of its production and destruction. Because splenomegaly in CLD has been considered to cause thrombocytopenia by increased sequestration and enhanced destruction of platelets [6, 7], we asked the question of whether more splenomegaly may lead to a more severe form of thrombocytopenia in CLD-C patients than in CLD-B patients at the highest stratum of liver stiffness. However, the same degree of splenomegaly was determined at this stratum in both patients, suggesting that a specific mechanism(s) other than enhanced destruction caused by splenomegaly may be