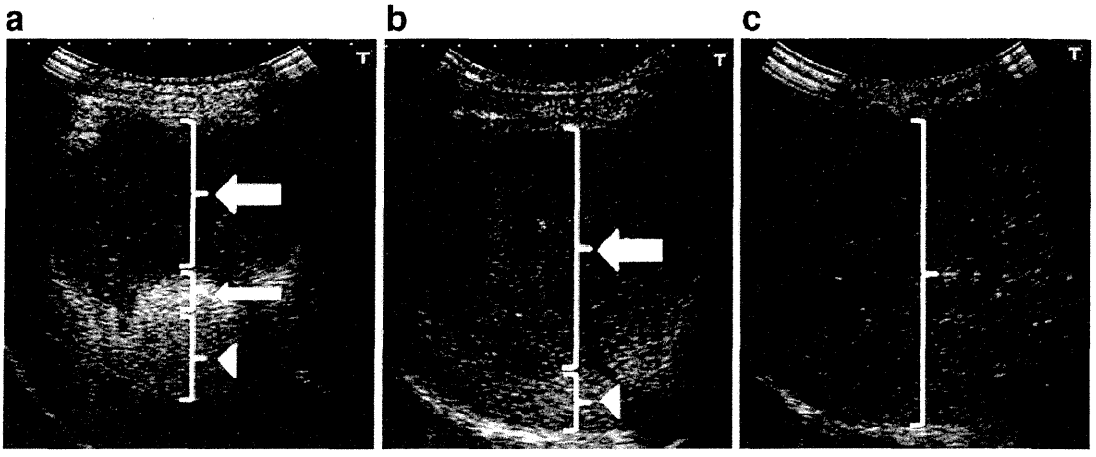

Fig. 3 Three patterns of parenchymal enhancement after IHPE in the 15-min phase. **a** A 60-year-old woman, control subject: Three-layer appearance with a hypo-enhancement band near the liver surface (*thick arrow*), a hyper-enhancement band in the middle (*thin arrow*) and a hypo-enhancement band in the bottom (*arrowhead*). **b** A 58-year-old woman, with hepatitis C-related chronic hepatitis, F3: Two-layer appearance with a hypo-enhancement band as the first layer (*arrow*) and a hyper-enhancement band as the second layer (*arrowhead*). **c** A 56-year-old woman with hepatitis C-related cirrhosis: Monolayer appearance with a single hypo-enhancement layer

Results

Clinical data of the subjects

The study had 202 participants after the exclusion of 8 CLD patients because of inadequate biopsy specimens; 146 subjects for the main study and 56 subjects for the subsequent study (Table 1). Liver samples were obtained from all subjects; percutaneous needle biopsy (16-/18-gauge needle; BARD, Tempe, AZ, USA) in 144 patients without ascites, transjugular liver biopsy (18-gauge needle; Cook, Bloomington, IN, USA) in 12 patients with ascites, and total hepatectomy in two patients. Biopsy samples showed 20±3.3 (mean ± SD, 11–28) mm in length and 21± 5.7 (mean ± SD, 13–66) mm^2 in area. The fibrosis ratio was 3.3±1.6% (0.5–6.4) for F1, 5.4±2.7% (1.8–15) for F2, 11.5± 3.1% (6.2–18) for F3 and 22.2±4.6% (12–35) for cirrhosis. Significant correlation was observed between the fibrosis ratio and the grade of fibrosis by Spearman's correlation coefficient (ρ=0.954, P<0.0001)

Main study results

Parenchymal enhancement after IHPE

Three-layer appearances were found on the post-IHPE sonograms by the initial review: a three-layer appearance showing a hypo-enhancement band near the liver surface with a hyper-enhancement band in the middle and hypo-enhancement band at the bottom, a two-layer appearance showing hypo-enhancement band as the first layer and hyper-enhancement band as the second layer, and a monolayer appearance, showing only hypo-enhancement parenchyma (Fig. 3). The width of band-like structure in the

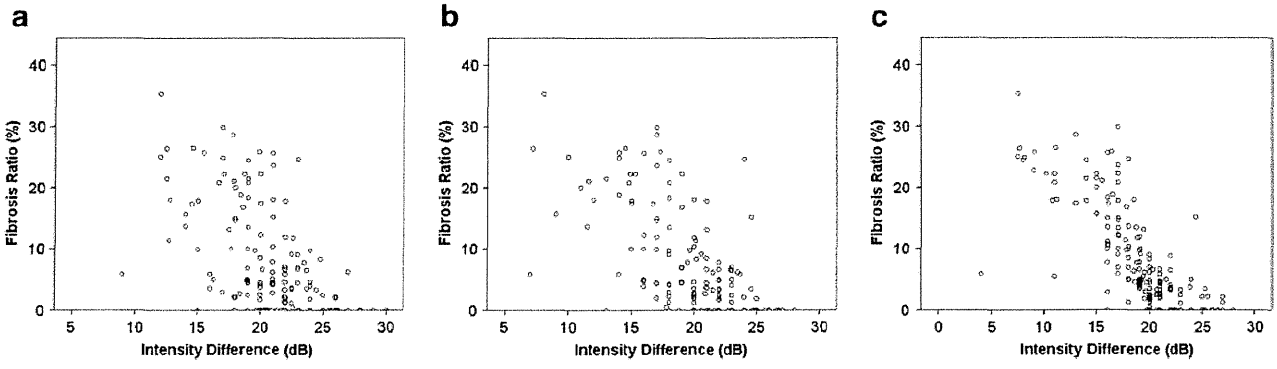

Fig. 4 Correlation between fibrosis ratio and the intensity difference in the 5-, 10- and 15-min phases. **a** 5-min phase: Pearson's correlation coefficient was r=0.56 (P< 0.0001). **b** 10-min phase: Pearson's correlation coefficient was r=0.67 (P<0.0001). **c** 15-min phase: Pearson's correlation coefficient was r=0.76 (P<0.0001). The intensity difference in the 15-min phase showed the closest correlation with the fibrosis ratio among the three phases

three-layer appearance was 49.1±10.4 (20–80) mm in the first layer, 12.9±2.8 (8–18) mm in the second layer and 29.2±12.1 (10–62) mm in the third layer. The deepest level of band-like structure was 63±8.1 (42–90) mm in the first layer and 76±9.9 (52–125) mm in the second layer. Similarly, the width of band-like structure of two-layer appearance showed 67.5±4.2 (60–70) mm in the first layer, and the deepest level of that was 79±5.5 (70–85) mm in the first layer. There was no significant difference in the width or the deepest level of band-like structure between control, chronic hepatitis and cirrhosis groups.

The three-layer appearance was significantly less frequent, and the monolayer appearance was significantly more frequent, in cirrhosis than controls/chronic hepatitis at all phases ($P<0.0001$). However, the layer-appearance did not differ significantly between the controls, F1, F2 and F3. Inter-operator agreement for the layer appearance after IHPE was excellent (kappa=0.824) and inter-reviewer agreement of review results for layer appearance was also excellent (kappa=0.912).

Intensity analysis

Significant correlations were found between the intensity difference and fibrosis grade (Spearman's correlation coefficients: 5-minute phase, $\rho=0.47$, $P<0.0001$; 10-minute phase, $\rho=0.64$, $P<0.0001$, 15-minute phase, $\rho=0.79$, $P<0.0001$)/fibrosis ratio (Fig. 4). The intensity difference in the 15-min phase showed a closer correlation with the fibrosis ratio than the 5-min ($P=0.0009$) and 10-min phase ($P=0.041$). Inter-observer variability for the IHPE technique was 8.7% in the 5-min phase, 7.9% in the 10-min phase and 8.7% in the 15-min phase. The Az value of the intensity difference for cirrhosis at the 15-min phase was significantly higher than those of FIB4 (Table 2). Efficiency of diagnosis in the 15-min phase was 81% for marked fibrosis, 89% for advanced fibrosis and 91% for cirrhosis (Table 3).

There was no significant relationship between the intensity difference (5-/10-/15-min) and the activity grade ($P=0.078$, $P=0.053$, $P=0.15$, respectively). In patients with cirrhosis, there were no significant correlations between the intensity difference in the 5-/10-/15-min phases and the Child-Pugh class ($P=0.18$, $P=0.079$, $P=0.099$), T-BIL ($P=0.082$, $P=0.11$, $P=0.054$), and albumin ($P=0.53$, $P=0.52$, $P=0.95$).

Table 3 Diagnostic accuracy of the intensity difference in the 15-min phase

	Diagnostic accuracy (%)		
	For marked fibrosis (≥F2)	For advanced fibrosis (≥F3)	For cirrhosis
Sensitivity	88	85	97
Specificity	72	91	90
Positive predictive value	78	85	70
Negative predictive value	84	91	99
Efficiency	81	89	91

Subsequent study results

The subsequent study was carried out at the 15-min phase, whose findings were closest to the fibrosis grade. The layer appearance in three different imaging planes was identical in 52 of the 56 subjects (93%).

Variations of intensity difference in the three different imaging planes were 6.3±2.9% (4–13.4) in controls, 6.1±2.2% (2.8–9.4) in F1, 5.1±3.1% (1.5–10.4) in F2, 7.0±2.4% (3.3–10.6) in F3, and 12±4.6% (6.6–18) in cirrhosis. The intensity difference in each imaging plane showed significant correlation with the grade of fibrosis (Spearman's correlation coefficients: first imaging plane, $\rho=0.76$, $P<0.0001$; second imaging plane, $\rho=0.73$, $P<0.0001$, third imaging plane, $\rho=0.77$, $P<0.0001$) and with the fibrosis-ratio (Pearson's correlation coefficient: first imaging plane, $r=0.81$, $P<0.0001$; second imaging plane, $r=0.73$, $P<0.0001$, third imaging plane, $r=0.80$, $P<0.0001$). Fisher's z transformation revealed that there was no significant difference between the intensity difference at the 15-min phase of the main study and that in each of the three imaging planes of the subsequent study; first imaging plane ($P=0.73$), second imaging plane ($P=0.37$) and third imaging plane ($P=0.87$).

Table 2 Comparison of Az values for the diagnosis of marked fibrosis, advanced fibrosis and cirrhosis between the intensity difference in the 15-min phase and FIB4

	For marked fibrosis (≥F2)	For advanced fibrosis (≥F3)	For cirrhosis
The intensity difference in the 15-min phase	0.88 (0.81–0.92)	0.95 (0.91–0.98)	0.97 (0.93–0.99)
FIB4	0.85 (0.78–0.91)	0.89 (0.82–0.94)	0.90 (0.81–0.96)
P-values	0.15	0.057	0.017

Az, area under the receiver operating characteristic curves; marked fibrosis, ≥F2; advanced fibrosis, ≥F3; FIB4, age × AST/(platelet count/$ALT^{0.5}$); AST (IU/L), aspartate transaminase; ALT (IU/L), alanine transaminase

Discussion

As shown in the results, the coefficient of correlation between the fibrosis grade and parenchymal enhancement showed time-dependent change and the closest relationship was detected on the 15-min phase image. This might be explained by the duration time of stability of microbubble distribution in the hepatic sinusoid after the contrast agent injection, i.e. the ratio between the static microbubbles captured in the liver and dynamic microbubbles that are not captured but circulating in the intrahepatic vessel might vary greatly among individual subjects in the 5-min and 10-min phases. Signals originating mainly from captured microbubbles may be suitable for grading hepatic fibrosis.

Kupffer cell may be implicated in the development of hepatic fibrosis [11], and phagocytosis of microbubbles by Kupffer cells is one of the underlying mechanisms for parenchymal enhancement by Sonazoid™. However, in the present study, no significant correlation was found between parenchymal intensity and hepatic function reserve, although previous studies have reported that Kupffer cell function is closely related to hepatocyte function [12]. It suggests that Sonazoid™-induced enhancement might reflect the grade of hepatic fibrosis independently of Kupffer cell function, although continuous study would be necessary to clarify in vivo behaviour of the microbubbles.

Nevertheless, as Sonovue does not have an accumulation property in the liver, previous studies with this agent focused on the haemodynamic evaluation; movement of microbubble in the liver, such as transit time between portal vein and hepatic vein or hepatic vein arrival time [3–5]. However, their results do not seem to separate different fibrosis stages; only discriminate advanced fibrosis (F3–F4) from mild fibrosis (F0–F2), probably because of the variability of the haemodynamic-based study, difficulty of the observation of dynamic microbubble or different property of microbubble.

Unexpectedly, the authors found a unique parenchymal enhancement pattern, a layered appearance. We speculate that an acoustic reaction of the microbubbles against the high power emission accounts for the layer structure. At first, the proximal hypo-intensity band may have a smaller number of microbubbles because of breakdown after high-power emission. If there were numbers of microbubbles in the liver parenchyma, as in the controls, some of them may act as a shelter to protect the distal microbubbles from the ultrasound beam. Consequently, reflection of the ultrasound beam by microbubbles may generate the intermediate hyper-intensity band. Furthermore, reflection of the ultrasound beam at the intermediate band may decrease exposure of microbubbles in the distal parenchyma to the ultrasound beam, which would generate the distal band, resulting in the three-layer appearance. Meanwhile in cirrhosis, because the total amount of intrahepatic microbubbles may be much lower than in the controls, most of the intrahepatic microbubbles could be destroyed by the high power emission. Less reflection of the ultrasound beam results in neither the intermediate nor the distal band being generated and a monolayer appearance. Our results have shown that the layer appearance is significantly effective for differentiating between controls and patients with chronic hepatitis and cirrhosis, although the layer appearance was difficult to differentiate among F1, 2, 3 and 4. The authors strongly recommend that this easy and simple technique should be applied to estimate the degree of liver fibrosis generally while performing the contrast enhanced study.

Diagnostic abilities for moderate or severe grades of fibrosis in the other imaging techniques are almost similar to the results of our technique; the Az value for F3 and/or cirrhosis is 0.84/0.95 by transient elastography [13] and 0.9244 for F2 [14], and 0.962 for F1 and 0.994 for F2 [15] by magnetic resonance elastography. However, the latter has limitations requiring large-scale expensive equipment and inconvenient procedures. Meanwhile, the authors emphasise that contrast-enhanced ultrasound suffers from a drawback of "a requirement of the agent injection". It adds a certain complexity to the simple procedure, and a usage of the agent might make the results variable. Furthermore, it has a risk of causing side-effects though the severe events might be rare. Transient elastography and blood sample marker may have the advantage of simplicity in the acquisition and analysis of the data. In any event, diagnostic accuracy for the mild grade of fibrosis should be improved in the future, and contrast-enhanced ultrasound is expected to play a major role because of its simple and low-cost procedure.

The major limitation of our study is the observation of the limited area of the right lobe. One of the reasons for the selection of this area is that we thought contrast enhancement should be analysed in the area corresponding to parenchyma from which the liver sample is taken, as most of the biopsy procedures were performed in the right lobe. However, assessment of intensity in a much wider area of the liver should be made in the future to overcome the heterogeneous distribution of fibrosis.

In conclusion, the features of microbubbles exposed to high-power ultrasound emission seem to play a role in predicting the grade of hepatic fibrosis. Although further study would be needed to validate the results, our non-invasive and repeatedly available technique may have the potential to improve patient care in the long-term management of CLD.

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Longitudinal changes of the laboratory data of chronic hepatitis C patients with sustained virological response on long-term follow-up

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SUMMARY. There is no study that follows up longitudinal changes in laboratory data of patients with C-viral chronic liver disease (C-CLD) who achieved sustained virological esponse (SVR) with interferon treatment in a long-term study. We investigated the laboratory data in a long-term retrospective cohort study of 581 patients with C-CLD who underwent liver biopsy between January 1986 and December 2005. 467 were treated with interferon and 207 of these patients achieved SVR with follow-up periods of 8.36 ± 5.13 years. Alanine aminotransferase (ALT) levels, albumin levels, platelet counts, and the aspartate aminotransferase (AST)-to-platelet ratio index (APRI) values were serially examined during the follow-up period. None of the 207 patients with SVR exhibited hepatitis C virus (HCV) RNA positivity more than 6 months after the end of IFN treatment. Platelet counts and albumin levels increased only in those with eradication of HCV. APRI values decreased more in patients with SVR than in those with nonsustained virological responses (non-SVR). Patients who achieved SVR and had fibrosis stage 0–1 and 2–4 at enrolment had platelet counts that longitudinally increased by 2.81 ± 3.95 and 5.49 ± 4.53 × $10^3/\mu L$ during the 10-year follow-up period, respectively. Albumin levels continuously increased during the first 2 years by 0.15 ± 0.31 and 0.33 ± 0.37 in fibrosis stage 0–1 and 2–4, respectively and then plateaued. ALT levels decreased rapidly one year after the start of treatment by 110.3 ± 140.0 and 100.5 ± 123.4 in fibrosis 0–1 and 2–4, respectively. HCV RNA negativity persisted in all patients with SVR, and laboratory data including APRI longitudinally improved during the long-term follow-up period.

Keywords: aspartate aminotransferase-to-platelet ratio index, chronic hepatitis C, cohort studies, interferon, platelet counts, sustained virological response.

INTRODUCTION

There are approximately 130–170 million hepatitis C virus (HCV) carriers worldwide, estimated to comprise 3–4% of the population [1,2]. In Japan, the number of HCV carriers was estimated at approximately 1.7 million [2]. HCV infection is persistent in nearly 70% of patients after acute infection, and these patients progress to chronic hepatitis. Thus, chronic inflammation leads to liver cirrhosis and hepatocellular carcinoma (HCC) [3]. Once the infection becomes chronic, the spontaneous viral clearance rate is very low indeed [4], and there was no therapy to eradicate the virus before 1986. Beginning in 1986, interferon-α (IFN-α) was used for the first time to treat patients with chronic HCV infection, and this antiviral therapy enabled eradication of the virus [5]. At present, administration of pegylated IFN with ribavirin is the most common treatment for chronic hepatitis C. Patients with chronic hepatitis C have a high risk of HCC, but sustained virological response (SVR) after IFN treatment induced not only long-lasting normal liver function but also improvement of liver fibrosis and significant reduction in the rate of HCC development [5–7].

There is no study that follows up longitudinally continuous changes in laboratory data of patients with chronic hepatitis C who achieved SVR with IFN treatment in a long-term study. We investigated the laboratory data of such patients in a long-term retrospective cohort study.

Abbreviations: ANOVA, analysis of variance; APRI, AST-to-platelet ratio index; C-CLD, C-viral chronic liver disease; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN-α, interferon-α; SVR, sustained virological response.

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PATIENTS AND METHODS

Patients

Of 853 patients with C-viral chronic liver disease (C-CLD) who underwent liver biopsy at the Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University Hospital between January 1986 and December 2005, 581 patients were enrolled in this study after excluding 272 patients for the reasons shown in Fig. 1. The follow-up period started from the date of liver biopsy in patients who did not receive IFN therapy and from the date when IFN therapy was initiated in those who did receive IFN therapy. The mean duration between the date of liver biopsy and the start of IFN treatment was 5.48 ± 16.18 months.The average age of the 581 patients was 50.52 ± 12.46 years (50.32 ± 12.26 years for males and 50.80 ± 12.77 years for females, $P = 0.485$). Among 581 patients, 467 were treated with IFN, and 207 of these patients achieved SVR (Fig. 1). The average follow-up periods were 8.36 ± 5.13 years among all patients, 8.54 ± 4.91 years among untreated patients, 8.31 ± 5.19 years among treated patients, 8.40 ± 5.31 years among nonsustained response (non-SVR) patients, and 8.20 ± 5.04 years in those with SVR.

Laboratory and imaging examination

Liver biopsy specimens were examined by two independent liver pathology specialists (F.I. and O.Y.) who were blinded to the clinical etiology according to the criteria of Desmet *et al.* [8], with fibrosis staging defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis) and the activity of inflammation being defined as A0 (no inflammation), A1 (mild inflammation), A2 (moderate inflammation), or A3 (severe inflammation).

All patients were positive by second- or third-generation HCV antibody tests. Early patients were diagnosed later with sera stored at −20 °C. Because the methods of quantifying HCV RNA levels varied during the follow-up period, we categorized patients into high titre and low titre groups. Patients who had HCV RNA of ≥100 KIU, ≥100 kc, ≥1.0 Mequiv., or ≥1×10^4 50/μL or who had HCV core antigen levels of ≥30 pg/mL were classified into the high titer group, and the remaining patients were classified into the low titer group [9–12]. The serotype or genotype of HCV RNA was also examined. Laboratory data were examined every 1–3 months. To detect HCC, abdominal ultrasonography was performed every 3–6 months, and if there was a possibility of HCC development, further evaluation was performed such as computed tomography, magnetic resonance imaging, hepatic angiography, or ultrasonography-guided tumour biopsy.

SVR was defined as HCV RNA negativity, determined using the Amplicor quality test or TaqMan method, for more than 6 months after the end of IFN treatment, and any other response was considered a non-SVR. In the early patients treated with IFN, SVR was confirmed later using the Amplicor quality test on stored serum.

We utilized the laboratory data from at least 1 year before death in patients who died and before HCC detection in those who developed HCC during follow-up; we used the laboratory data until just before the start of IFN retreatment in those who were treated with IFN at the last follow-up point.

Statistical analysis

The Wilcoxon signed-rank tests and the analysis of variance (ANOVA) were used to analyze the data. Ratios were examined using Pearson's chi-square test. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Dr. SPSS 2 statistical software package (SPSS Japan Inc., Tokyo, Japan).

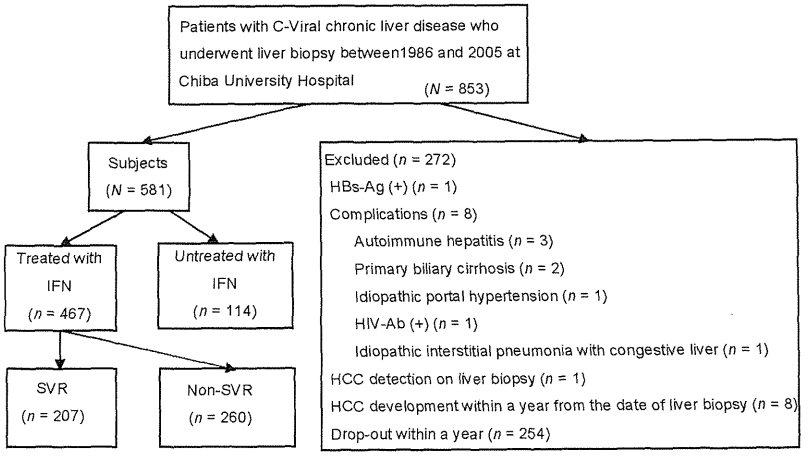

Fig. 1 Flow chart of patients analyzed. IFN, interferon; SVR, sustained virological response; ALT, alanine aminotransferase; HIV, human immunodeficiency virus; HCC, hepatocellular carcinoma.

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. Among 467 patients who received IFN therapy, 100 were treated with IFN-α (38 with SVR), 8 were treated with a sequential combination of IFN-α and IFN-β (3 with SVR), 105 were treated with rIFNα-2a (24 with SVR), 80 were treated with rIFNα-2b (40 with SVR), 9 were treated with IFN-β followed by rIFNα-2b (5 with SVR), 28 were treated with rIFNα-2b and ribavirin (15 with SVR), 17 were treated with IFN-α con1 (12 with SVR), 1 was treated with IFN-α con1 and ribavirin (1 with SVR), 44 were treated with IFN-β (23 with SVR), 26 were treated with pegIFNα-2a (17 with SVR) and 49 were treated with pegIFNα-2b and ribavirin (29 with SVR).

Abnormal ALT level in SVR patients during follow-up

Fourteen (6.76%) of 207 patients with SVR had abnormal ALT levels (≥40 IU/L) during the follow-up period, and they were all male. The highest ALT value in each patient was 116, 62, 51, 51, 48, 46, 44, 43, 42, 42, 42, 41, 41, and 40 IU/L. Abdominal ultrasonography showed that 9 of the 14 patients had fatty liver, of which 1 patient had an alcohol consumption of >20 g/day and 2 patients had body mass index values of >25 kg/m^2. Among the remaining 5 of the 14 patients, 1 had high alcohol consumption and 4 had abnormal ALT levels of unknown cause.

No reappearance of HCV RNA in patients with SVR

None of the 207 patients with SVR exhibited HCV RNA positivity more than 6 months after the end of treatment. The duration between the date of confirmation of SVR and the date of last examination of HCV RNA was 7.5 ± 5.3 years (0–21.3 years). HCV RNA was examined using a TaqMan method in 119 of these patients, and the duration between the date of confirmation of SVR and the date of last examination of HCV RNA was 8.2 ± 5.5 years (0.5–21.3 years).

ALT levels, platelet counts, albumin levels, and AST-to-platelet ratio index values at initiation and end of follow-up

ALT levels significantly decreased in every group at the conclusion of follow-up compared to those before the initiation of follow-up (Fig. 2a). The decreased value in each group was −46.23 ± 125.17 IU/L in untreated patients, −58.02 ± 83.31 in patients with non-SVR, and −112.75 ± 125.17 in those with SVR.

Platelet counts decreased significantly in untreated patients ($P < 0.001$) and patients with non-SVR

Table 1 Patient characteristics at enrolment

	Untreated	IFN treated	Non-SVR	SVR
Patients, *n*	114	467	260	207
Age (years)*	54.1 ± 11.5	49.7 ± 12.6	51.7 ± 11.9	47.5 ± 13.1
Sex (male/female), *n*	53/61 (46.5%/53.5%)	290/177 (62.1%/37.9%)	154/106 (59.2%/40.8%)	136/71 (65.7%/34.3%)
Fibrosis stage: F0/F1/F2/F3/F4	2/55/24/12/21 (1.8%/48.2%/21.1% /10.5%/18.4%)	15/225/105/73/49 (3.2%/48.2%/22.5% /15.6%/10.5%)	5/119/51/51/34 (1.9%/45.8%/19.6% /19.6%/13.1%)	10/106/54/22/15 (4.8%/51.2%/26.1% /10.6%/7.2%)
ALT level (IU/L)*	101.9 ± 120.0	126.7 ± 105.6	121.9 ± 85.9	132.9 ± 126.0
Platelet count (×10^9/L)*	16.0 ± 6.8	17.0 ± 6.1	16.6 ± 6.2	17.6 ± 6.0
Albumin level (g/dL)*	4.1 ± 0.4	4.2 ± 0.4	4.2 ± 0.4	4.3 ± 0.3
APRI*	1.76 ± 1.72	1.78 ± 1.55	1.92 ± 1.63	1.62 ± 1.42
HCV load: high/low, *n*	23/81 (22.1%/77.9%)	142/305 (31.8%/68.2%)	44/203 (17.8%/82.8%)	98/102 (49.0%/51.0%)
HCV serotype: 1/2, *n*	78/23 (77.2%/22.8%)	324/127 (71.8%/28.2%)	211/42 (83.4%/16.6%)	113/85 (57.1%/42.9%)
Serotype1 and high viral load/others, *n*	62/35 (63.9%/36.1%)	227/208 (52.2%/47.8%)	172/70 (71.1%/28.9%)	55/138 (28.5%/71.5%)

IFN, interferon; SVR, sustained virological response; ALT, alanine aminotransferase; APRI, aspartate aminotransferase to platelet ratio index; HCV, hepatitis C virus.
*Data are shown as mean ± SD.

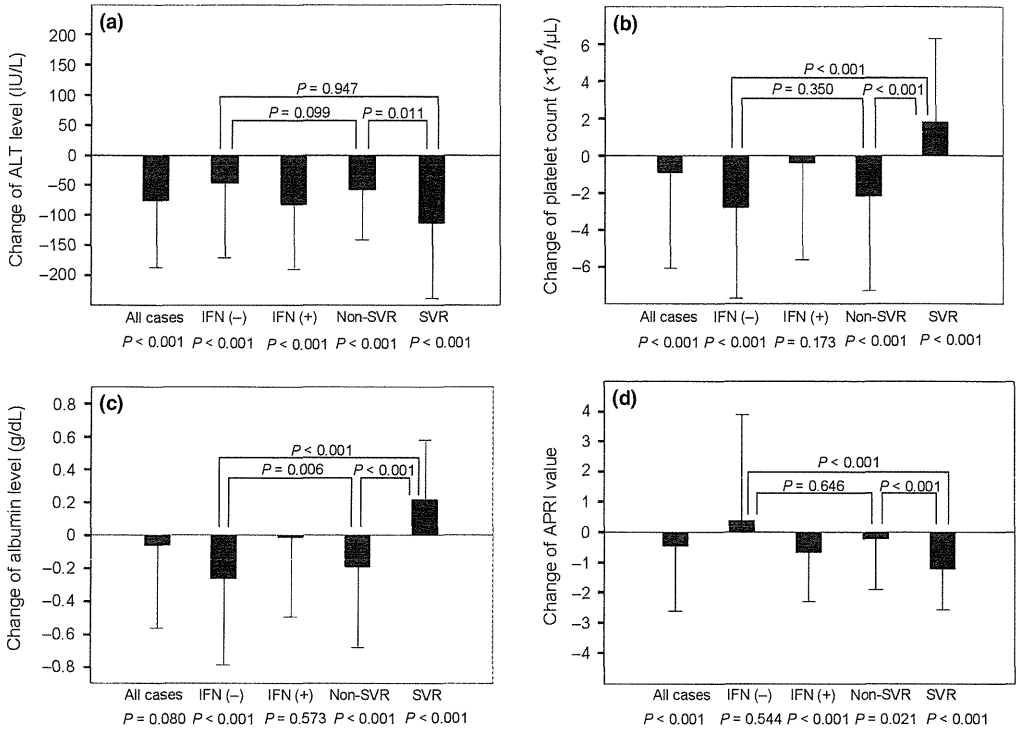

Fig. 2 Changes in (a) ALT levels, (b) platelet counts, (c) albumin levels and (d) APRI values according to IFN treatment. *P* values represent comparisons between the values at the start of observation and those at the end of observation by using the Wilcoxon signed-rank test. Changes in values were compared using the ANOVA followed by *post hoc* tests for multiple comparisons (Tukey's test). ALT, alanine aminotransferase; IFN, interferon; SVR, sustained virological response.

($P < 0.001$) but increased in those with SVR ($P < 0.001$) (Fig. 2b). The degree of change in each group was −2.83 ± 4.84 in untreated patients, −2.21 ± 5.07 in those with non-SVR, and +1.82 ± 4.41 in those with SVR, indicating that platelet count increased only in those with eradication of HCV. There was a significant difference in values between untreated and SVR patients ($P < 0.001$), and between patients with non-SVR and those with SVR ($P < 0.001$) but not between untreated and non-SVR patients ($P = 0.350$), suggesting that platelet counts decreased in non-SVR patients at the same pace as observed in untreated patients and only increased in patients with SVR.

Albumin levels decreased significantly in untreated patients ($P < 0.001$) and in those with non-SVR ($P < 0.001$) but increased significantly in those with SVR ($P < 0.001$) (Fig. 2c). The degree of change in albumin levels in each group was −0.26 ± 0.53 in untreated patients, −0.19 ± 0.49 in those with non-SVR, and +0.21 ± 0.37 in those with SVR, indicating that albumin levels increased only in those with eradication of HCV. There was a significant difference in values between untreated and non-SVR patients ($P = 0.006$), suggesting that albumin levels decreased in untreated patients at a greater rate than in non-SVR patients.

AST-to-platelet ratio index (APRI) has been reported to be useful for evaluating liver fibrosis [13], and high APRI values could indicate progression of liver fibrosis. In our study, APRI values tended to increase in untreated patients, and decrease significantly in IFN-treated patients, especially in those with SVR and even in those with non-SVR (Fig. 2d). The decrease in the APRI value in patients with SVR was greater than that in those with non-SVR (−1.208 ± 1.335 *vs* −0.297 ± 1.399, $P < 0.001$).

Serial changes in ALT levels, platelet counts, albumin levels, and APRI values in patients with SVR

Next, the serial changes in ALT levels, platelet counts, albumin levels, and APRI values were analyzed in the 207 patients with SVR. Histological examination before IFN treatment showed F0–F1 fibrosis in 116 (F0: 10, F1: 106) and F2–F4 fibrosis in 91 (F2: 54, F3: 22, F4: 15) of these patients. Laboratory data were obtained for 193 patients after 6 months (F0–F1: 110, F2–F4: 83), for 194 patients after 1 year (F0–1: 111, F2–4: 83), for 190 patients after 2 years (F0–F1: 105, F2–F4: 85), for 165 patients after 4 years (F0–F1: 95, F2–F4: 70), for 115 patients after 7 years (F0–F1: 68, F2–F4: 47), and for 77 patients after 10 years (F0–F1: 52, F2–F4: 25).

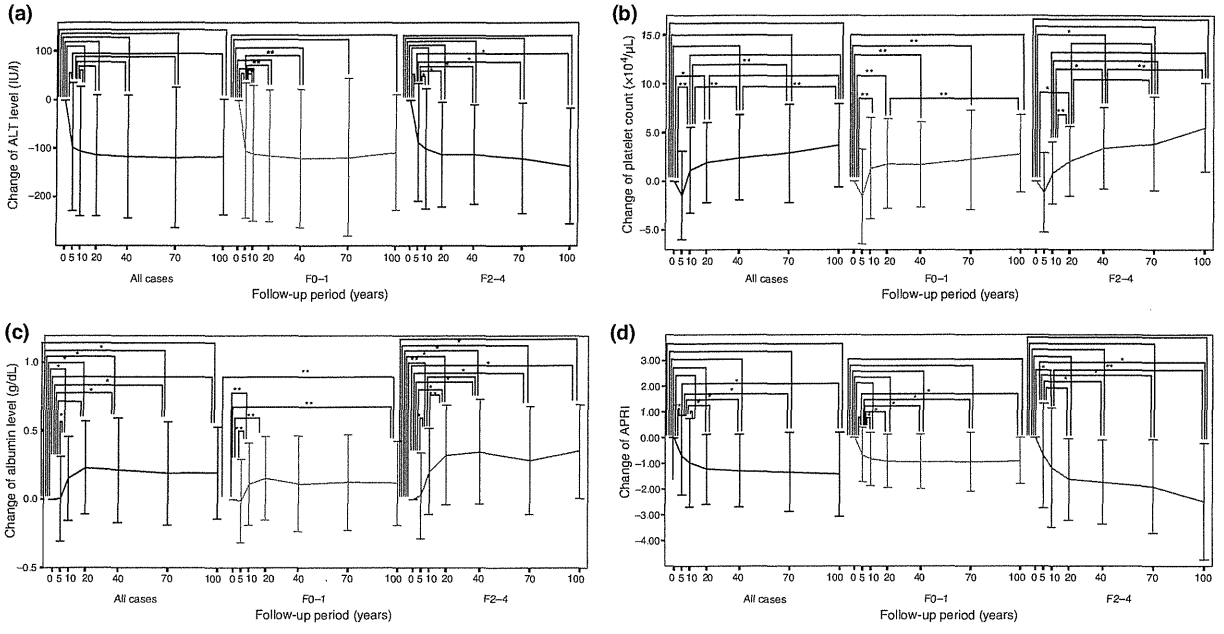

Fig. 3 Serial changes in (a) ALT levels, (b) platelet counts, (c) albumin levels and (d) APRI values in patients with SVR. Changes in values were compared using the one-way repeated-measures ANOVA. No asterisk, $P < 0.001$; $^{*}P < 0.01$; $^{**}P < 0.05$. ALT, alanine aminotransferase; APRI, aspartate aminotransferase to platelet ratio index.

ALT levels decreased rapidly 6 months after the initiation of treatment and gradually over the next 6 months by 110.3 ± 140.0 and 100.5 ± 123.4 in F0–F1 and F2–F4, respectively. After that, ALT levels nearly plateaued in F0–F1 patients but continued to decrease gradually in F2–F4 patients (Fig. 3a).

Platelet counts continued to increase significantly for 10 years after IFN treatment, especially in F2–F4 patients. The mean platelet count was 14.57 ± 4.57 before IFN treatment, and the increase in this value was +0.84 ± 3.22 at 1 year, +2.03 ± 3.58 at 2 years, +3.38 ± 4.20 at 4 years, and +5.49 ± 4.53 at 10 years after the start of IFN treatment (Fig. 3b). The mean platelet count was 18.85 ± 5.26 at 10 years after the start of IFN treatment in F2–F4 patients. In F0–F1 patients, platelet counts markedly increased 1 year after IFN therapy and gradually increased thereafter with smaller changes compared to F2–F4 patients (+1.34 ± 5.18 at 1 year, +2.81 ± 3.95 at 10 years after the start of IFN treatment, respectively). The mean platelet count was 19.89 ± 5.98 before IFN treatment and 22.12 ± 5.79 at 10 years after the start of IFN treatment in F0–F1 patients.

Albumin levels increased only during the first 2 years in F0–F1 and F2–F4 patients (+0.15 ± 0.31 and +0.33 ± 0.37, respectively). The mean albumin level was 4.35 ± 0.27 and 4.18 ± 0.37 before IFN treatment, and 4.48 ± 0.24 and 4.49 ± 0.33 at 2 years after the start of IFN treatment in F0–F1 and F2–F4 patients, respectively. Albumin levels did not increase beyond 2 years of follow-up (Fig. 3c).

APRI values significantly decreased for the first 2 years in F0–F1 patients (−0.93 ± 1.03 in the 2 years after the start of IFN treatment compared to the baseline value of 1.19 ± 1.01 before IFN treatment), whereas in F2–F4 patients, APRI values tended to decrease continuously throughout the follow-up period (−1.64 ± 1.59 in the 2-year period after the start of IFN treatment, −2.50 ± 2.26 in the 10-year period after the start of IFN treatment compared to the baseline value of 2.15 ± 1.67 before IFN treatment) (Fig. 3d).

DISCUSSION

We showed favourable outcomes regarding laboratory data of chronic hepatitis C patients who achieved SVR. During a long-term follow-up period of a mean 7.5 years (0–21.3 years), reappearance of HCV RNA in serum was detected in none of the 207 patients with SVR. Previous reports on long-term follow up studies of patients who achieved SVR have shown that the rate of relapse of HCV RNA is relatively low but not rare [14–24]. In contrast, reports that HCV RNA was not detected in serum for long periods are few. Tsuda *et al.* reported that HCV RNA was not detected in serum in 38 patients over a 4.4–12.0-year (median 6.8 years) observation period [25]. Adamek *et al.* reported the nonreappearance of HCV RNA in the sera of 78

patients for 0.5–5.3 years (median, 1.8 years) [26]; George *et al.*, in 150 patients for 1.0–7.8 years (median, 5.1 years) [27]; Formann *et al.*, in 187 patients for 1.0–14.3 years (mean, 2.4 years) [28]; and Maylin *et al.*, in 344 patients for 0.5–18 years (mean, 3.3 years) [29]. In all of these reports excluding that of Maylin *et al.*, the patient numbers were smaller and the observation periods were shorter than those in our study. The study by Maylin *et al.* had larger numbers than our study, but the observation period in our study was more than twofold larger. In addition, in our study, HCV RNA was examined in 119 of 207 patients by using the TaqMan PCR method, one of the most specific and sensitive tests, 0.5–21.3 years (8.2 ± 5.5 years) after the confirmation of SVR, and HCV RNA was not detected in any of these patients. No report mentioned the use of TaqMan methods except our study.

McHutchinson *et al.* reported that 7 (2%) of 400 patients with SVR had detectable HCV RNA in liver biopsy specimens, and 2 patients had reappearance of serum HCV RNA 12 months after therapy [30]. HCV might replicate in extrahepatic tissues such as bone marrow and lymphocytes, as the antisense strand of HCV RNA was detected in these organs [31–34]. In our study, some patients with SVR exhibited elevated ALT levels without any definite cause, and there is a possibility of an effect of residual HCV in the liver.

ALT levels decreased in all patients during the follow-up period. Platelet counts and albumin levels decreased in untreated patients and non-SVR patients, and they increased only in patients with SVR. These results may indicate that the progression of liver fibrosis is different between untreated or non-SVR patients and SVR patients. Toccaceli *et al.* reported that histological improvement was achieved in 44% of 112 SVR patients after 36–76 months of follow-up [35], and Shiratori *et al.* reported that improvement of liver fibrosis was obtained in 59% of 183 SVR patients after a mean follow-up period of 3.7 years [36]. However, Shindo *et al.* reported that the fibrotic stage did not significantly improve in non-SVR patients after a follow-up period of 2 years [37]. Our investigation was based on the laboratory data in serum, and our data were in accordance with these reports, indicating that the improvement of hepatic fibrosis could be surmised by transition of common serum data.

Serial changes in ALT levels, platelet counts, and albumin levels in patients with SVR according to the hepatic fibrosis stage at enrolment showed that ALT levels decreased for only 12 months after initiation of IFN treatment and almost plateaued for 10 years. Platelet counts continued to increase significantly for 10 years, especially in F2–F4 patients. Therefore, serial changes in platelet counts may be useful to estimate the long-term improvement of liver fibrosis after achievement of SVR. In contrast to platelet counts, albumin levels increased significantly only for the first 2 years. Previous studies reported that ALT levels normalized in most patients after SVR [18], but there are few reports analyzing consecutive serum data. George *et al.* followed 150 patients with SVR, and 136 of these patients (91%) were followed over 3 years [27]. They found no differences in ALT levels, albumin levels, and platelet counts at the last follow-up point compared to those at 6 months after the end of IFN treatment. However, our data revealed gradual increases in platelet counts after IFN treatment, especially in patients with advanced fibrosis. There is no study that follows up longitudinal changes in laboratory data of patients with C-CLD who achieved SVR with interferon treatment in a long-term study except our study.

Liver biopsy is the most reliable examination to confirm the fibrotic stage of the liver, but it has some risk of complications, sampling variability, procedural discomfort, and added cost. Recently, noninvasive methods have been developed to evaluate liver fibrosis by ultrasonography, such as Fibroscan [38] or real-time tissue elastography [39], but not all institutions can perform these examinations because they lack the necessary equipment. Hyaluronic acid is superior in evaluating liver fibrosis independently, but this examination is not performed at every institution. Wai *et al.* reported that APRI was useful to distinguish between F0–F1 and F2–F4 fibrosis by AUROC 0.80, and a cut-off value of 1.5 showed that a positive predictive value of 88% was obtained for the diagnosis of F2–F4 fibrosis [13]. There are some reports that improvement in liver fibrosis for a few years after SVR was determined by liver biopsy as previously explained. However, there is no report showing that liver fibrosis improved for 10 long years continuously in SVR patients by any method, and we proved that by using platelet counts and APRI, the consequential indices of fibrosis.

In conclusion, we showed a favourable clinical course in patients who achieved SVR. HCV RNA remained negative in all patients, and laboratory data including APRI showed longitudinal improved during the long-term follow-up period.

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DISCLOSURE

None of the authors have any conflicts of interest or financial disclosures to declare.

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Acute Liver Failure in an Antimitochondrial Antibody-Positive 63-Year-Old Man

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Key Words
Primary biliary cirrhosis · Acute liver failure · Autoimmune hepatitis · Overlap syndrome

Abstract
Antimitochondrial antibody (AMA) is one of the representative features of primary biliary cirrhosis (PBC). PBC is a female-dominant disease usually presenting intrahepatic bile duct destruction, cholestasis and fibrosis with or without chronic nonsuppurative destructive cholangitis. We presented the case of a 63-year-old man with acute liver failure who had AMA, pronounced alanine aminotransferase elevation and high bilirubinemia. We administered corticosteroids and rescued this patient without liver transplantation. It is well known that some patients within the spectrum of autoimmune liver disease present with characteristics of both PBC and autoimmune hepatitis. Although corticosteroids may be associated with a significant worsening of adverse events in patients with PBC, if acute liver failure in AMA-positive cases is progressive, the administration of corticosteroids has to be considered, as well as the preparation of urgent liver transplantation.

Introduction

Autoimmune hepatitis (AIH) patients usually present elevated serum aminotransferase levels (3–10-fold increase), marked hypergammaglobulinemia (typically IgG), positive titers of autoantibodies, histological findings of interface hepatitis and portal plasma cell infiltration [1], although atypical cases also exist [2–4]. Typical primary biliary cirrhosis (PBC) patients are females in the age range

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of 30–65 years, presenting with biochemical signs of cholestasis and the presence of antimitochondrial antibody (AMA), and they are asymptomatic or suffer from fatigue or pruritus [5–9]. Diagnosis can be made in patients who have elevated alkaline phosphatase (ALP) levels of at least 6 months duration, in combination with the presence of AMA (≥1:40) [5]. Serum aminotransferase levels usually are only slightly elevated, whereas IgM concentration is typically increased [5].

Some patients within the spectrum of autoimmune liver disease present with characteristics of both PBC and AIH and are commonly classified as having an 'overlap syndrome' [5, 10]. In AIH patients, AMA is occasionally seen at low titers, but an AMA anti-pyruvate dehydrogenase complex-E2 (AMA-M2) pattern, which is specific to PBC, is rarely detected [5]. Muratori et al. [11] reported that only 2% of type 1 AIH patients were AMA-positive. Overlap cases are usually selected either on the basis of biochemical and serological findings or of histological features, although limitations due to biopsy size and sampling error should always be kept in mind in the assessment of a liver biopsy [5].

The acute and fulminant forms of AIH were recognized by the International Autoimmune Hepatitis Group in 1992, when it codified diagnostic criteria and waived the requirement for 6 months of disease activity to establish the diagnosis [1, 4], although it is still difficult to diagnose and treat the acute and fulminant forms of AIH [2, 4, 12]. We present a case of acute liver failure with overlap features of both PBC and AIH who was successfully treated with corticosteroids without liver transplantation.

Case Report

A 63-year-old Japanese man was referred to Chiba University Hospital at the end of April 2011 for the treatment of acute liver failure. Blood tests showed serum alanine aminotransferase (ALT) 2,415 IU/l, aspartate aminotransferase (AST) 3,253 IU/l, ALP 804 IU/l, total bilirubin 12.7 mg/dl, and prothrombin time international normalized ratio 1.65. Two weeks earlier, liver dysfunction had first been diagnosed at his regional clinic. At that time he had developed fatigue, pruritus and jaundice. Clinical data at admission to our hospital are shown in **table 1**. His medical history consisted of mild cerebral infarction 8 years before and surgery for inflammation of the right middle ear in his twenties. He had been taking medicines such as fenofibrate, allopurinol, diclofenac sodium, valsartan, nilvadipine and aspirin for hyperlipidemia, hyperuric acid, lumbar herniated disk and hypertension for more than 1 year. He occasionally drank alcohol and had no family history of liver diseases or autoimmune disorders. Physical examination on admission revealed jaundice but no consciousness disturbance. Computed tomography showed minimal ascites and mild hepatosplenomegaly with a thickened gall bladder wall (**fig. 1**). Doppler ultrasound color flow imaging showed inversion of venous flow, suggesting portal hypertension, although endoscopic findings did not reveal any esophageal or gastric varices. There were no positivities of hepatitis viral markers (table 1). Possible reactivation of herpes simplex virus and drug-induced liver injury were not completely ruled out at that time. Because of the positivity for antinuclear antibody and AMA, especially AMA-M2, we considered him to have autoimmune liver disease such as AIH or overlap syndrome with characteristics of both PBC and AIH, and so the administration of corticosteroids was initiated [2]. He was first treated with 1,000–125 mg of methylprednisolone daily for 10 days, and then with 60–5 mg prednisolone daily for approximately 110 days. He also started taking 600 mg of ursodeoxycholic acid daily for about 90 days. Total bilirubin continued to rise up to 31.2 mg/dl, but then recovered. He was released from our hospital 120 days after admission. After his discharge, he was treated daily with 5 mg of prednisolone and 600 mg of ursodeoxycholic acid.

During his hospitalization, 70 days after the start of corticosteroid treatment, transjugular liver biopsy was performed (**fig. 2**). The liver biopsy specimen showed the architecture of the liver as being preserved, indicating no cirrhosis, but revealed submassive hepatic necrosis, findings compatible with

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overlap syndrome with characteristics of both PBC and AIH. The AIH score was 16 after completion of corticosteroid treatment, pointing to AIH compatibility [1].

One year later, he was well but complained of pruritus. His liver function tests were improved (ALT 11 IU/ml, AST 22 IU/ml, total bilirubin 0.6 mg/dl, IgM 166 mg/dl, and IgG 1,176 mg/dl), but his antinuclear antibody and AMA were still positive. Doppler ultrasound color flow imaging showed no inversion of venous flow and no ascites.

Discussion

We present a 63-year-old Japanese man with acute liver failure and positivity for AMA-M2 who was successfully treated with corticosteroids. Before admission he had no symptoms, and ALP was not examined. However, ALP levels were still elevated in mid-October 2011, AMA-M2 was still positive (8 IU/ml), and he had intractable pruritus, suggesting that PBC also existed. We diagnosed him as overlap syndrome having AIH and PBC features.

PBC-AIH overlap syndrome is a clinical entity characterized by the occurrence of both conditions at the same time in the same patient [13]. We and others previously reported the consecutive occurrence of AIH and PBC [9, 13]. It is very important for patients with acute liver failure such as the present case to be treated as soon as possible. We used corticosteroid therapy and made preparations for urgent liver transplantation [14, 15].

Corticosteroids may be associated with a significant worsening of osteoporosis in patients with PBC [5–7]. We previously reported that corticosteroids are effective for AIH with acute presentation [2] and that they are also effective for overlap syndrome having AIH and PBC features with acute transaminase elevation [10]. Ichai et al. [12] indicated that there is a point beyond which AIH cannot be salvaged by drug therapy, and this point can be defined only by assessing the immediate response to corticosteroid treatment [4]. This assessment can be made over a 2-week interval, which is sufficiently short to avoid the infectious complications associated with protracted immunosuppressive therapy and liver failure [4, 12]. In our case, prothrombin time recovered relatively soon after the administration of corticosteroids. Corticosteroid therapy remains appropriate for severe, immediately life-threatening and fulminant AIH, although the treatment should be limited to 2 weeks or less [4].

We present a case of acute liver failure with overlap features with both PBC and AIH, who was successfully treated with corticosteroids without liver transplantation. In conclusion, it seems important to finely judge the corticosteroid use for atypical cases of autoimmune liver diseases with acute presentation over shorter periods. Of course, further studies are urgently needed.

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Disclosure Statement

The authors have nothing to disclose.

Table 1. Laboratory data on admission to Chiba University Hospital

White blood cells	10,300/μl	Gamma-glutamyl transpeptidase	116 IU/l
Hemoglobin	13.1 g/dl	Total bilirubin	18.0 mg/dl
Platelets	38.2 × 10^4/μl	Direct bilirubin	13.5 mg/dl
PT-INR	1.33	Immunoglobulin M	286 mg/dl
PT%	49%	Immunoglobulin G	1,928 mg/dl
Ammonia	71 μg/dl	HBsAg	–
Total cholesterol	88 mg/dl	Anti-HBs	–
Blood urea nitrogen	12 mg/dl	Anti-HBc	–
Creatinine	0.56 mg/dl	IgM-HBc	–
C-reactive protein	2.9 mg/dl	HBV DNA	–
Alpha-fetoprotein	9.0 ng/ml	IgM-HA	–
PIVKA-II	37 mAU/ml	Anti-HCV	–
Total protein	6.5 g/dl	HCV RNA	–
Albumin	3.3 g/dl	Antinuclear antibody	×640
Aspartate aminotransferase	3,334 IU/l	AMA	×40
Alanine aminotransferase	2,602 IU/l	AMA-M2	14 IU/ml
Lactate dehydrogenase	568 IU/l	Anti-smooth muscle antibody	–
Alkaline phosphatase	795 IU/l		

AMA = Antimitochondrial antibody; Anti-HBc = hepatitis B core antibody, total; Anti-HBs = hepatitis B surface antibody; Anti-HCV = hepatitis C virus antibody; HBsAg = hepatitis B surface antigen; HBV DNA = hepatitis B virus DNA; IgM-HA = hepatitis A antibody immunoglobulin M; IgM-HBc = hepatitis B core immunoglobulin M antibody; M2 = pyruvate dehydrogenase complex-E2 (PDC-E2); PIVKA-II = protein induced by vitamin K absence II; PT-INR = prothrombin time international normalized ratio.

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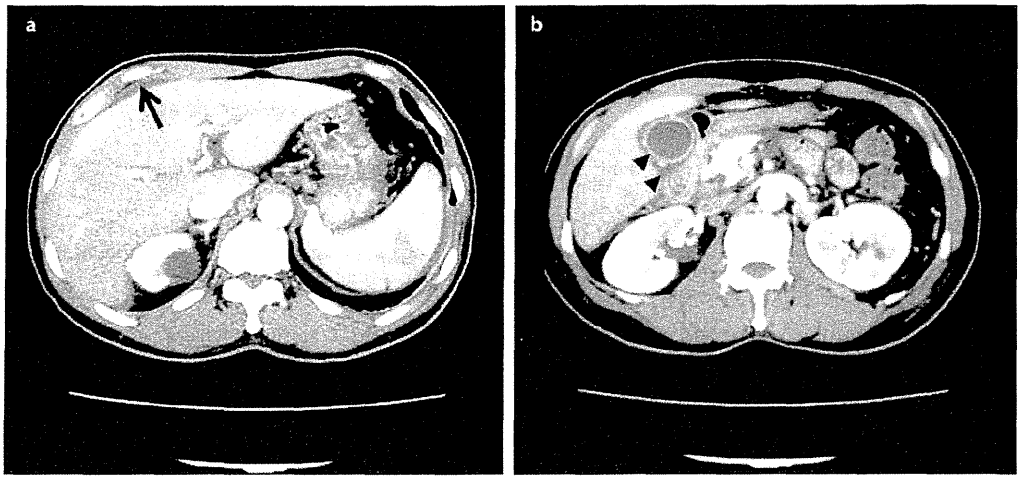

Fig. 1. Computed tomography on admission showed minimal ascites (arrow) and mild hepatosplenomegaly (**a**) with a thickened gall bladder wall (arrowheads) (**b**). These indicated severe liver injury.

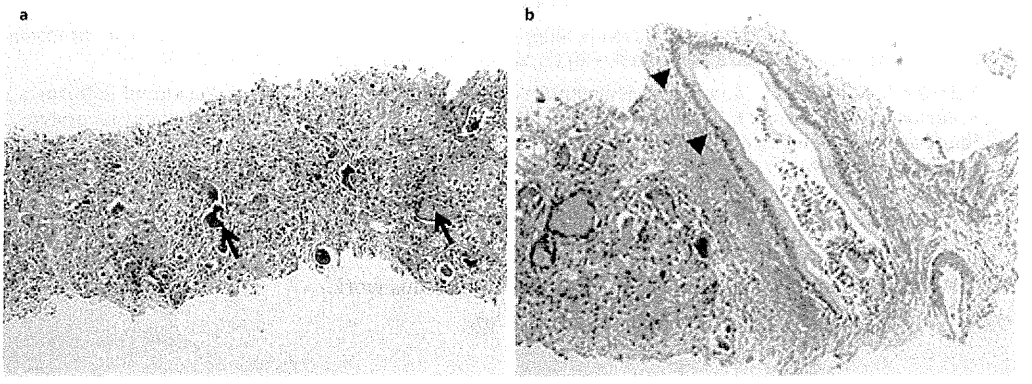

Fig. 2. Liver biopsy showed submassive hepatic necrosis and cholestasis (arrows) (hematoxylin and eosin; original magnification 40×) (**a**) and florid bile duct regions (arrowheads) (hematoxylin and eosin; original magnification 200×) (**b**). The architecture of the liver was preserved, indicating no cirrhosis, but revealed submassive hepatic necrosis, findings compatible with overlap syndrome with characteristics of both PBC and AIH.

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Article

Roles of ITPA and IL28B Genotypes in Chronic Hepatitis C Patients Treated with Peginterferon Plus Ribavirin

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Abstract: It has been reported that inosine triphosphatase (ITPA) gene variants protect against ribavirin-induced anemia in patients treated for chronic hepatitis C. IL28B variants also influence the treatment response of peginterferon plus ribavirin treatment in these patients. In the present study, we examined how ITPA and IL28B genotypes have clinical impacts on treatment-induced hematotoxicities and treatment response in HCV-infected patients treated with peginterferon plus ribavirin. ITPA genotypes (rs1127354 and rs6051702) and IL28B genotype (rs8099917) were determined by TaqMan SNP assay. We compared clinical background, treatment course and treatment response in terms of these genotypes. Only IL28B rs8099917 major type could predict sustained virological response. ITPA rs1127354 major type leads to significantly greater ribavirin-induced anemia than ITPA rs1127354 minor type between days 0 and 84. We noticed that IL28B rs8099917 minor genotype was associated with higher reduction of neutrophils and platelets. ITPA rs1127354 is useful for the prediction of ribavirin-induced anemia in the early phase after the commencement of peginterferon plus ribavirin treatment and IL28B rs8099917 is useful for the prediction of sustained virological response. Use of the combination of these two genotypes could lead to safe and effective treatment of chronic hepatitis C patients.

Keywords: anemia; HCV; IL28B; ITPA; SNP; sustained virological response

1. Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and a leading cause of end-stage liver disease worldwide [1]. The current standard therapy is based on a combination of peginterferon and ribavirin, but this treatment leads to only about 50% sustained virological response (SVR) in patients with HCV genotype 1 and high viral loads, who were mostly null-responders or relapsers [2]. Recently, the direct-acting antiviral (DAA) agents boceprevir and telaprevir were licensed for the treatment of HCV infection [3], and these drugs might be more powerful tools for HCV-infected patients.

Interleukin 28B (IL28B) variants influence the treatment response of peginterferon plus ribavirin treatment in HCV-infected patients [4–8]. Genome-wide association study has revealed a strong relationship between single-nucleotide polymorphisms (SNPs) near IL28B on chromosome 19 and null virological response in the treatment of patients with HCV genotype 1 in Australian [4], Japanese [5] and other populations [6]. Baseline plasma interferon-gamma inducible protein 10 kDa (IP-10 or CXCL10) is significantly associated with IL28B-related SNPs, and augments the level of predictiveness of the first-phase decline in HCV RNA, rapid virological response (RVR) and final treatment outcome [9,10]. Further studies will be needed to reveal the mechanism concerning IL28B and the response to interferon.

It has also been reported that inosine triphosphatase (ITPA) gene variants protect against ribavirin-induced hemolytic anemia in chronic hepatitis C patients [11]. Proposed mechanisms of action for ribavirin against HCV include (1) direct effect against HCV RNA-dependent RNA polymerase [12], (2) induction of misincorporation of nucleotides leading to lethal mutagenesis [13,14], (3) depletion of intracellular pools via inhibition of inosine monophosphate dehydrogenase [15], (4) alteration in the cytokine balance from a Th2 profile (anti-inflammatory) to a Th1 profile (pro-inflammatory) [16], and (5) potentiating the effect of interferon via up-regulation of genes involved in interferon signaling [17,18]. Clinical studies provide strong evidence for the benefit of ribavirin in combination with DAAs for both interferon containing and sparing regimens [18].

In the present study, we examined how ITPA and IL28B genotypes clinically contribute to treatment-induced hematotoxicities and treatment response in HCV-infected patients treated with peginterferon plus ribavirin. We found that IL28B rs8099917 minor genotype was associated with greater reduction of neutrophils and platelets. Use of a combination of these genotypes could lead to a safe and effective treatment for chronic hepatitis C patients. It is conceivable that these variants may modulate treatment responses as well as treatment pathways, and the result of this study might show the way of the future direction of these gene variants in treatment or drug development.