considered NR. None of these patients were HCV RNA-negative between weeks 25 and 48.

Sera were collected from the patients before and during treatment and frozen for determination of viral loads by a quantitative HCV RNA PCR assay (COBAS Amplicor HCV Monitor Test v2.0 using a 10-fold dilution method, Roche Diagnostics, Tokyo, Japan), which has a low threshold of quantitation of 5,000 IU/ml and an outer limit of quantitation of 5,100,000 IU/ml. A qualitative test for serum HCV RNA was performed using Amplicor-HCV kit version 2.0 (Roche Diagnostics, Tokyo, Japan) and the results were labeled positive or negative. The lower limit of detection was 50 IU/ml. All testing was performed at a single reference laboratory. The HCV genotype was determined by a type-specific primer from the core region of the HCV genome. Genotyping was carried out as described previously [14].

Criteria for exclusion were [1] clinical or biochemical evidence of hepatic decompensation and advanced cirrhosis identified by ascites, encephalopathy, or hepatocellular carcinoma [2], white blood cell count of less than 3,000/mm^3 and platelet count of less than 50,000/mm^3 [3], concurrent liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive), [4] excessive active alcohol consumption over 60 g/day or drug abuse, [5] severe psychiatric disease, or [6] antiviral or corticosteroid therapy within the 12 months prior to enrollment. Both peginterferon alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 8.5 g/dl, 1,000/mm^3 and 25,000/mm^3, respectively. Treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic problems developed, if continuation of treatment was judged not to be possible by the attending physician, or if the patient no longer desired to continue treatment.

Informed consent

The study protocol was approved by the Institutional Ethics Committee of Shin-Kokura Hospital, and all patients gave informed consent to participate in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice.

Statistical analysis

Differences between viral loads between two groups were analyzed using the Student's *t* test and Mann–Whitney rank-sum test. We conducted analysis using the Kruskal Wallis test for three-group (SVR, TR, and NR) and five-group (RVR, W8EVR, W12EVR, LVR and NVR) comparisons. All statistical analyses were conducted on a Macintosh computer using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA). *P* values of <0.05 were considered to be statistically significant.

Results

Baseline characteristics of patients grouped by SVR, TR and NR

SVR was observed in 72 patients (51.1%), TR in 40 patients (28.4%), and NR in 29 patients (20.6%). The characteristics at enrollment of patients showing SVR, TR, or NR are presented in Table 1. The mean age of SVR, TR, and NR patients was 50.8, 56.6, and 56.0 years, respectively. There were no significant inter-group differences in mean age, gender, or pre-treatment test results (alanine aminotransferase, hemoglobin level, platelet count, and viral load).

Table 1 Baseline characteristics of patients by response (SVR, TR, and NR)

	SVR $n = 72$	TR $n = 40$	NR $n = 29$	Total $n = 141$	*P* value[a]
Age (years)	50.8 (11.3)	56.6 (8.6)	56 (10.4)	53.2 (10.8)	0.084
Male (%)	41 (56)	24 (52)	19 (65)	84 (57)	
Laboratory					
ALT (IU/l)	88 (82)	86 (53)	94 (78)	90 (74)	0.808
Hemoglobin level (g/dl)	14.5 (1.4)	14.6 (1.3)	14.6 (1.0)	14.5 (1.3)	0.917
Platelet count ($\times 10^4$/mm^3)	19 (6)	17 (6)	20 (10)	19 (7)	0.707
HCV RNA loads ($\times 10^3$ IU/ml)	2299 (1634)	2228 (1344)	2390 (1501)	2298 (1524)	0.953
Body mass index (kg/m^2)	23.6 (5.7)	24.8 (2.9)	23.9 (3.6)	24 (4.9)	0.536

Values are represented as means with standard deviation in parentheses or as absolute values with percentages in parentheses

SVR sustained virologic response, *TR* transient response, *NR* non response, *ALT* alanine aminotransferase

[a] Kruskal Wallis Test

Early viral kinetics, RI and RI-2nd relative to SVR, TR and NR

Viral kinetics up to the first two weeks after the start of treatment are shown for the SVR, TR, and NR groups (Fig. 1 and Table 2). The viral load at 24 h for the SVR and TR groups (226,000 and 229,000 IU/ml) was reduced significantly compared to the NR group (523,000 IU/ml) ($P \leq 0.05$). The differences of the viral loads for three groups at weeks 1 and 2 were significant ($P \leq 0.0001$). The viral load of the SVR group at weeks 1 and 2 was significantly lower than that of the TR group ($P < 0.01$ and $P < 0.05$). The former was reduced at week 1 with no increases thereafter, and the viral load at week 2 (76,000 IU/ml) was significantly lower than at 24 h (226,000 IU/ml, $P < 0.01$). The viral load of the TR group rose to 397,000 IU/ml at week 1 and was reduced to 169,000 IU/ml at week 2. This reduction was not significant when compared against that at 24 h (229,000 IU/ml). The viral load of the NR group rose again to 1,206,000 IU/ml at week 1 and was reduced to 615,000 IU/ml at week 2, which was still higher than that at 24 h (523,000 IU/ml).

RI and RI-2nd for the SVR, TR and NR groups are shown in Table 2. RI for the SVR group (0.8) was below 1.0. The differences of the RI for the three groups were significant ($P \leq 0.0001$). RI-2nd for the SVR, TR and NR groups was 0.3, 0.8, and 1.3, respectively, with the highest value observed with the NR group. The differences of the RI-2nd for three groups were significant ($P \leq 0.0001$). RI-2nd for the TR group was significantly higher than for the SVR group ($P < 0.05$).

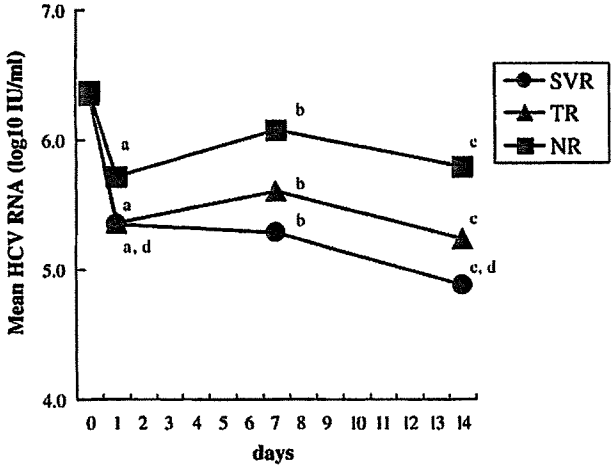

Fig. 1 HCV-RNA kinetics during the first 2 weeks of treatment by SVR (*black circle*), TR (*black triangle*), and NR (*black square*). **a** $P < 0.05$, **b** $P < 0.0001$, **c** $P < 0.0001$ (Kruskal Wallis test), **d** $P < 0.01$ (hour 24 vs. week 2 in SVR). *SVR* sustained virologic response, *TR* transient response, *NR* non-response

SVR rates, early viral kinetics, RI and RI-2nd relative to the timing of HCV RNA negativity

RVR, W8EVR, W12EVR, LVR, and NVR were observed in 26 (18.4%), 31 (22.0%), 31 (22.0%), 24 (17.0%), and 29 (20.6%), respectively. The SVR rate with the RVR, W8EVR, W12EVR, and LVR groups was 96.2% (25/26), 83.9% (26/31), 54.8% (17/31), and 16.7% (4/24), respectively. None in the NVR group exhibited the absence of HCV-RNA at the end of treatment. The HCV RNA kinetics for the RVR, W8EVR, W12EVR, LVR, and NVR groups up to week 2 of treatment are shown in Fig. 2 and Table 3. The viral load of the RVR group was rapidly reduced to 143,000 IU/ml by 24 h, with a further drop to 55,000 IU/ml at week 1. At week 2, the viral load was reduced to 8,000 IU/ml, which was significantly less than that at 24 h ($P < 0.001$). The viral loads for the W8EVR and W12EVR groups were reduced to 186,000 IU/ml and 134,000 IU/ml, respectively, by 24 h but rose to 231,000 IU/ml and

Table 2 Kinetics of HCV RNA during the first 2 weeks of treatment by response (SVR, TR, and NR)

	SVR ($n = 72$)		TR ($n = 40$)		NR ($n = 29$)		P value[a]
	Mean	SD	Mean	SD	Mean	SD	
HCV loads (×1000 IU/ml)							
Before treatment	2299	(1634)[b]	2228	(1344)	2390	(1501)	0.9538
Hour 24	226	(328)	229	(249)	523	(518)	0.0102
Week 1	190	(302)	397	(399)	1206	(811)	<0.0001
Week 2	76	(193)[b]	169	(249)	615	(617)	<0.0001
Rebound index	0.8	(0.9)	2.9	(3.1)	3.1	(1.8)	<0.0001
Rebound index 2	0.3	(0.6)	0.8	(0.7)	1.3	(1.1)	<0.0001

Values represent means with ranges in parentheses

[a] Kruskal Wallis Test

[b] $P < 0.01$ (hour 24 vs. week 2 in SVR)

Abbreviations *SVR* sustained virologic response, *TR* transient response, *NR* non response, *SD* standard deviation

277,000 IU/ml, respectively, at week 1. The viral load of the W8EVR group at week 2 was reduced to 49,000 IU/ml, which was significantly less than that at 24 h ($P < 0.001$). The viral load of the W12EVR group, on the other hand, was 119,000 IU/ml, which was not significantly less than that at 24 h. The viral load of the LVR group at 24 h (563,000 IU/ml) was higher than that of RVR, W8EVR, or W12EVR. The viral load rose further to 674,000 IU/ml at week 1, and although a reduction to 361,000 IU/ml was observed at week 2, it was still significantly greater than with RVR, W8EVR, and W12EVR ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively). The viral load (523,000 IU/ml) of the NVR group at 24 h was similar to that of the LVR group. It rose after one week and was not low in the second week (615,000 IU/ml) compared to that at 24 h. The differences of the viral loads for five groups were significant at hour 24, week 1, or week 2 ($P \leq 0.0001$).

RI and RI-2nd of the RVR, W8EVR, W12EVR, LVR, and NVR groups are shown in Table 3. RI of RVR (0.4) was the lowest and was lower than that of W8EVR (2.3), W12EVR (2.4), LVR (1.5), and NVR (3.1). RI-2nd of NVR (1.3) was the highest, being higher than RVR (0.1), W8EVR (0.5), W12EVR (0.7), and LVR (0.7) ($P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). The mean RI-2nd for other than NVR was below 0.7. The differences of the RI or RI-2nd for five groups were significant ($P \leq 0.0001$).

SVR, TR, and NR rates relative to RI and RI-2nd

The 3 groups (RI-A, RI-B, and RI-C) and 4 groups (RVR, W8EVR, W12EVR, and LVR) were combined and then divided into 12 groups, with SVR, TR, and NR grouped by RI and RI-2 and by RVR, W8EVR, W12EVR, and LVR (Fig. 3). The SVR, TR, and NR rates were 90.2% (46/51), 9.8% (5/51), and 0% (0/51), respectively, with RI-A ($RI \leq 1.0$), 55.6% (25/45), 40.0% (18/45), and 4.4% (2/45), respectively, with RI-B ($RI > 1.0$, RI-2nd < 0.7), and 2.2% (1/45), 37.8% (17/45), and 60.0% (27/45), respectively, with RI-C ($RI > 1.0$, RI-2nd ≥ 0.7). The SVR rate for RI-A and RVR was 90.2% (46/51) and 96.2% (25/26), respectively. The SVR rate for RI-B and W8EVR was 93.3% (14/15). The SVR rate for the patients in these 3 areas was 89.7% (61/68), suggesting that they represent the population for which 48-week treatment is appropriate. Among the 112 patients who became HCV RNA-negative at week 24, 60.7% (68/112) were in the above 48-week regimen area. In particular, the SVR rate (2.2%) from RI-C was very low and the TR and NR rates were 37.8% and 60.0%, respectively. Among W8EVR, W12EVR, and LVR in the range outside that for a 48-week regimen, the SVR rate (25.0%, 11/44) was low but the TR rate (75.0%, 33/44) was high. Thus extension of the treatment period was considered necessary, and a 72-week regimen was recommended.

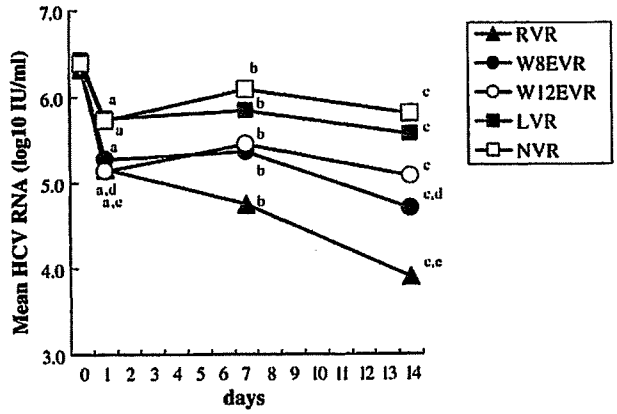

Fig. 2 HCV RNA kinetics by RVR (*black triangle*), W8EVR (*black circle*), W12EVR (*white circle*), LVR (*black square*), and NVR (*white square*) during the first 2 weeks of treatment **a** $P < 0.001$, **b** $P < 0.0001$, **c** $P < 0.0001$ (Kruskal Wallis test), **d** $P < 0.001$ (hour 24 vs. week 2 in W8EVR), **e** $P < 0.001$ (hour 24 vs. week 2 in RVR). *RVR* rapid viral response, *W8EVR*, week 8 early viral response, *W12EVR* week 12 early viral response, *LVR* late viral response, *NVR* non-viral response

Discussion

This is the first study in which SVR, TR, and NR, in response to 48 weeks of peginterferon plus ribavirin treatment, were successfully distinguished in the early stage of treatment. This was possible by using new indices (rebound index: RI, and second rebound index: RI-2nd) calculated from early viral kinetics and the timing of when HCV RNA becomes undetectable. This allows for the TR group to be treated for 72 weeks, potentially raising the SVR rate.

In the treatment of genotype 1 CHC with peginterferon plus ribavirin, relapse occurs in about 30% of patients after the end of treatment [1, 2]. In LVR (late viral responders), in particular, the percentage of relapse is high (59%) after 48 weeks of treatment [7]. It is vital to reduce the relapse rate (TR rate) and raise the SVR rate. This requires (1) dose increase and (2) prolongation of the period of treatment. In Japanese patients, the dose of peginterferon must often be reduced because of the onset of such adverse events as neutropenia, thrombocytopenia, and malaise, and thus dose increase is not a feasible option. The dose of ribavirin must also be reduced in some patients due to ribavirin-induced anemia, and likewise, any increase in dose is not feasible [15]. Thus extending the duration of treatment to 72 weeks is considered necessary, and it becomes essential to distinguish the population for which 48-week treatment is adequate from the population for which 72-week treatment is necessary. In a previous study, HCV RNA-negativity was determined at weeks 12 and 24 [7]. SVR was noted in

Table 3 Kinetics of HCV RNA during the first 2 weeks of treatment by response (RVR, W8EVR, W12EVR, LVR, or NVR)

	RVR ($n = 26$)		W8 EVR ($n = 31$)		W12 EVR ($n = 31$)		LVR ($n = 24$)		NVR ($n = 29$)		P value[a]
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
HCV loads (×1000 IU/ml)											
Before	2102	(1636)[b]	2271	(1536)[c]	2259	(1744)	2625	(1278)	2390	(1501)	0.9172
24 h	143	(182)	186	(191)	134	(95)	563	(558)	523	(518)	0.0007
1 week	55	(66)	231	(213)	277	(325)	674	(503)	1206	(811)	<0.0001
2 week	8	(5)[b]	49	(86)[c]	119	(264)	361	(299)	615	(617)	<0.0001
Rebound index	0.4	(0.3)	2.3	(3.6)	2.4	(2.8)	1.5	(0.6)	3.1	(1.8)	<0.0001
Rebound index 2	0.1	(0.1)	0.5	(0.6)	0.7	(1.4)	0.7	(0.4)	1.3	(1.1)	<0.0001

RVR rapid viral response, *W8EVR* week 8 early viral response, *W12EVR* week 12 early viral response, *LVR* late viral response, *NVR* non viral response, *SD* standard deviation

[a] Kruskal Wallis Test

[b] $P < 0.001$ (hour 24 vs. week 2 in RVR)

[c] $P < 0.001$ (hour 24 vs. week 2 in W8 EVR)

18% even when treatment was continued to week 48 in patients who were HCV RNA-positive at week 12 but HCV RNA-negative at week 24 (LVR) [7]. If treatment is continued for 72 weeks, these patients will receive drugs unnecessarily for an extra 24 weeks. On the other hand, in this study, the SVR rate with W8EVR and W12EVR was 84% and 55%, respectively. The SVR rate for the overall EVR (W8EVR + W12EVR) was 69%, with a relapse rate of 31%. By treating these relapsed patients for 72 weeks, a higher SVR can be expected.

For more effective treatment with peginterferon plus ribavirin, indices besides the currently used index (the time to HCV RNA negativity) should be introduced and evaluated. In this study, we succeeded in differentiating the populations to be treated for 48 and 72 weeks more accurately by measuring the viral loads up to week 2 after the start of treatment and calculating two new indices (rebound index and second rebound index). Unrelated to ribavirin, lowering the dose of peginterferon at the early stage of treatment reduces the SVR rate [16]. In other words, the therapeutic effect of peginterferon, independent of that of ribavirin at the early stage of treatment, is expected to be responsible for SVR and EVR, which is believed to occur before ribavirin takes effect. Early viral kinetics were determined up to week 2, which are believed to express the therapeutic effect of peginterferon. The serum concentration of peginterferon alpha 2b peaks after 24 h, followed by a gradual decline [17, 18]. The viral load is therefore reduced by 24 h but increases in week 1 [19, 20]. A large dose of peginterferon at each administration results in a marked reduction in the viral load at 24 h but the viral load increases in week 1 regardless of the dose. In the responder group, the viral load continues to decline each week thereafter [20]. This trend is also seen with peginterferon monotherapy [17]. On the other hand, in the SVR group, in particular the RVR group, it was noted that a number of patients did not experience an increase in the viral load at week 1. As shown in Fig. 1, in SVR, the viral load does not increase at week 1, while a return of viral loads is seen in TR and NR. The viral loads of SVR and TR were lower than that of NR at week 2. The viral load in week 1 divided by the viral load at 24 h was therefore defined as the rebound index (RI). The RI of SVR is 0.8 (less than 1.0), which is less than that for TR or NR. Among the RI of RVR, W8EVR, W12EVR, LVR, and NVR, only that of RVR was below 1.0. Among the 26 RVR patients, 24 (92%) exhibited RI-A (RI: $\leq$ 1.0) without a rise in week 1 (Fig. 3). The SVR rate with RI-A was 90%. It was believed that this group (RI-A, RI: $\leq$ 1.0) was composed of high responders to peginterferon. Because no decline in the viral load is noted in non-responders after week 2 [20], the viral load at week 2 divided by the viral load at 24 h was defined as the second rebound index (RI-2nd). Compared with SVR and TR, RI-2nd with NR was high. The patients with high RI-2nd were suspected to be poor responders or non-responders to peginterferon. RI-2nd of those other than NVR was below 0.7; and therefore 0.7 was adopted as the reference value for RI-2nd.

Based on the results of our study (Fig. 3), the SVR rate was very high (about 90%) in 3 areas of SVR: RI-A (RI: $\leq$ 1.0), RVR, and RI-B (RI-2: < 0.7) and W8EVR. These are believed to represent the areas for which 48-week treatment is recommended. About 60% of the 112 patients who became HCV RNA-negative within 24 weeks were in this area, and the SVR rate of the remaining 40% was low (25%) while the TR rate was high (75%). It was therefore thought that 72-week treatment is needed for these patients.

In peginterferon and ribavirin treatment, the status of EVR is important. When HCV RNA-negativity is not achieved by week 12, the SVR rate becomes very low [10].

	RVR N=26	W8EVR N=31	W12EVR N=36	LVR N=26	NVR N=29	Total N=148
RI>1.0, RI-2nd≥0.7 RI-C, N=52		△△△	△△△△ ○△△△△△	△△△△△△ ○△△△△△	□□□ □□□□□□ □□□□□□ □□□□□□ □□□□□□	SVR:3.9% TR:44.2% NR:51.9%
RI>1.0, RI-2nd<0.7 RI-B, N=45	○△	○○△ ○○○○○○ ○○○○○○	△△ ○△△△△△ ○○○○○○	△△△△△△ ○○○△△△	□□	SVR:55.6% TR:30.0% NR:4.4%
RI≤1.0 RI-A, N=51	○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○	△ ○○○○○○ ○○○○○○	○○○△△△ ○○○○○○	○△		SVR:90.2% TR:9.8% NR:0.0%
Total N=148	SVR:96.2% TR:3.8% NR:0.0%	SVR:83.9% TR:16.1% NR:0.0%	SVR:47.2% TR:52.8% NR:0.0%	SVR:19.2% TR:80.8% NR:0.0%	SVR:0.0% TR:0.0% NR:100%	SVR:49.3% TR:31.1% NR:19.6%

Fig. 3 SVR, TR, and NR by RI and RI-2nd as well as RVR, W8EVR, W12EVR, LVR, and NVR. *SVR* white circle, *TR* white triangle, *NR* white square, *SVR* sustained virologic response, *TR* transient response, *NR* non-response, *RI* rebound index, *RI-2nd* rebound index second, *RVR* rapid viral response, *W8EVR* week 8 early viral response, *W12EVR* week 12 early viral response, *LVR* late viral response, *NVR* non-viral response

In our study, SVR was low (below 20%) among the LVR who became negative for HCV RNA between weeks 12 and 24. Mangia et al. reported that to raise the SVR rate, treatment for 48 weeks is needed if HCV RNA becomes negative at week 8, while treatment for 72 weeks is needed if HCV RNA negativity is observed at week 12 [21]. In our study, the SVR rate with RVR was 96% and was also very high (84%) with W8EVR achieving HCV RNA negativity between 5 and 8 weeks. On the other hand, the SVR rate was low (55%) in patients who became HCV RNA-negative between weeks 9 and 12. These findings suggested that in treating Japanese patients with CHC with peginterferon plus ribavirin for 48 weeks, EVR should be qualified at week 8 rather than at week 12. Therefore, for evaluation, EVR patients who became HCV RNA-negative by week 8 were classified as W8EVR and those who became HCV RNA-negative by week 12 were classified as W12EVR. Early viral kinetics of both W8EVR and W12EVR indicated a rebound at week 1 but the viral load of W8EVR at week 2 was significantly lower than that at 24 h. On the other hand, the reduction in the viral load of W12EVR at week 2 was not significant when compared against that at 24 h. A significant reduction in the viral load was observed with RVR and W8EVR at week 2 compared to that at 24 h, and the SVR rates were correspondingly very high. It was believed that the reduction in the viral load at week 2 is important.

Real-time PCR assay is now commonly used and is more sensitive for detecting serum HCV than the COBAS Amplicor HCV Monitor assay. Its use may have allowed viral detection for a longer period of time, possibly resulting in the number of RVR and EVR patients being reduced while the SVR rate in RVR and EVR patients was increased. Examination of the SVR rate by the timing of HCV RNA negativity using real time PCR assay will be necessary in the future.

Reduction in the duration of treatment is being investigated for the good responders to peginterferon plus ribavirin treatment. In RVR patients who achieve HCV RNA-negativity at week 4, the SVR rate is reported to be 89% when treatment is continued for 24 weeks [11]. In this study, all patients in the RI-A (RI $\leq$ 1.0) and RVR area became SVR; thus they were believed to be extremely good responders. A more detailed investigation with a larger number of subjects is necessary to elucidate the question of a reduction in the duration of therapy.

The explanation of early viral kinetics by SVR, TR, and NR is highly complex and is impractical in clinical use. In this study, RI and RI-2nd calculated from early viral kinetics were used. It is believed that the simplified RI and RI-2nd are effective indices to determine the therapeutic efficacy of peginterferon therapy alone. By combining these two new indices and the indices for therapeutic efficacy of peginterferon plus ribavirin (RVR, W8EVR, W12EVR and LVR), SVR was distinguished from TR during treatment. With the aid of these indices, it is believed that a more effective peginterferon plus ribavirin treatment will be possible. We used these new indices in this study, and the measurement of HCV RNA levels was conducted using the COBAS Amplicor HCV Monitor assay. Since the range of detection of HCV is narrow with this assay, there were many patients with pretreatment HCV levels above the limit of detection. The timing of HCV RNA negativity and examination based on the HCV levels at week 1 and week 2 needs to be conducted using real time PCR assay in future studies. A larger scale study should be conducted to examine the duration of treatment for patients who are on reduced doses of peginterferon and ribavirin.

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The loss of HBeAg without precore mutation results in lower HBV DNA levels and ALT levels in chronic hepatitis B virus infection

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Abstract
Background The aim of this study was to investigate the correlation between precore (PC)/basal core promoter (BCP) mutations and the viral loads or activity of hepatitis in patients with chronic hepatitis B virus (HBV) infection.
Methods HBV genotypes, PC mutations, BCP mutations, HBV DNA levels, and serological markers of HBV were analyzed in all the patients with chronic HBV infection seen in Fujita Health University Hospital from June 2004 to November 2008 ($n = 215$).
Results HBV genotype was C in 169 patients, B in 16, A in 3, F in 1, and unclassifiable in 5. Among the patients with genotype C, the prevalence of PC wild type was significantly lower in hepatitis B envelope antigen (HBeAg)(−) patients than in HBeAg(+) patients (9.5% versus 49.0%, $P < 0.0001$). Among HBeAg(−) patients, the patients with PC wild type had significantly lower serum viral loads and alanine aminotransferase (ALT) levels compared with those with PC mutant ($P < 0.001$). Among HBeAg(−) patients, the patients with genotype B had lower serum viral loads compared with those with genotype C (3.6 ± 0.9 versus 4.6 ± 1.6, $P < 0.05$), and the prevalence of BCP wild type was significantly higher in those with genotype B than in those with genotype C (58.3% versus 10.8%, $P < 0.05$).
Conclusions Among HBeAg(−) patients with genotype C, the patients with PC wild type had significantly lower viral loads and ALT levels than those with PC mutant. This suggests that the patients with PC wild type may have better prognosis than those with PC mutant among HBeAg(−) patients with genotype C.

Keywords Precore mutation · Basal core promoter mutation · Hepatitis B virus · HBV genotype

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Introduction

Hepatitis B virus (HBV) is one of the most frequent and important causes of chronic viral hepatitis worldwide. It is estimated that approximately 2 billion people have had contact with the virus and more than 400 million people are chronic carriers [1, 2]. Chronic HBV infection is associated with a wide range of clinical manifestation, from an asymptomatic carrier to chronic liver disease, which may lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

Seroconversion from hepatitis B envelope antigen (HBeAg) to its antibody (anti-HBe), either spontaneous or after antiviral therapy, is usually accompanied by a decrease in viral replication and remission of liver disease [3, 4]. However, viral replication and hepatic inflammation persist in about 10% of patients after seroconversion [5, 6]. The exact mechanism of seroconversion has not been fully elucidated, but several mutations in the HBV genome have been reported to be associated with the phenomenon. The mutation of A to G at nucleotide 1896 in the precore (PC) region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of HBeAg [7]. The dual mutation (A1762T and G1764A) in basal core promoter (BCP) region has been shown to reduce the synthesis of HBeAg by suppressing the transcription of

precore mRNA [8, 9]. PC/BCP mutations have been reported to be associated with fulminant hepatitis [10, 11]. Recent study reported that the presence of BCP mutation was an independent risk factor for liver cirrhosis, as well as old age and genotype C infection [12]. However, the relationship of the mutations in PC and BCP region with serum HBV DNA levels, and severity of liver disease is still not fully elucidated.

HBV is classified into eight genotypes designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence [13, 14], and the distribution of HBV genotypes is geographically restricted [15, 16]. Resent studies suggested that HBV genotypes may be related to the rate of recovery from acute HBV infection and the progression of liver disease during chronic HBV infection [17–20]. Genotype C and B are predominant genotypes in Japan, and it has been reported that HBV genotype C is associated with more severe liver diseases than is genotype B [17, 19]. Several studies have shown that genotype C has a higher frequency of the dual mutation in the BCP region than genotype B [5, 18, 19]. However, the prevalence of the mutations in PC and BCP region of these two genotypes with different HBeAg status and its correlation with viral load are not clearly reported.

The aim of this study was to determine whether the mutations in PC and BCP region gave useful information on determining the management of patients with chronic HBV infection. The correlation between these mutations and the viral loads or activities of hepatitis was investigated.

Patients and methods

Patients

This is a retrospective study using stored sera obtained from all the patients with chronic HBV infection seen in Fujita Health University Hospital from June 2004 to November 2008. Chronic HBV infection was defined as persistent seropositivity for HBV surface antigen (HBsAg) for at least 6 months before enrollment. Patients diagnosed with acute hepatitis were excluded. Patients on antiviral therapy were also excluded because of the effects of treatment on serum HBV DNA levels and the possibility that antiviral therapy might have an impact on PC and BCP mutations. All patients were negative for antibodies to hepatitis C virus (HCV), and had no serological markers suggestive of autoimmune disease. Demographic, clinical, and laboratory data were collected during clinic visits. Laboratory results on hepatitis B markers (HBsAg, HBeAg, anti-HBe), liver chemistry including alanine aminotransferase (ALT) values, and platelet count were also recorded. The blood samples were collected during clinic visits and the sera were stored at −80°C as aliquots until use. This study adhered to the guidelines of the 1975 Declaration of Helsinki, and all patients gave informed consent.

HBV genotyping

HBV genotype was determined by restriction fragment length polymorphism (RFLP) method on S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers. The genomic segment between nt256 and nt796 in the S region was amplified by PCR. After a subsequent incubation with HinfI and Tsp509I, genotype was determined from the RFLP patterns on agarose electrophoresis [21, 22].

Quantification of serum HBV DNA levels

Serum HBV DNA level was quantified by PCR assay using an Amplicor HBV monitor kit (Roche Diagnostics, Tokyo, Japan) which had a quantitative range of 2.6–7.6 log copies/ml [23].

Detection of PC and BCP mutations

The stop codon mutation in the PC region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest HBV Pre-C ELMA; Roche Diagnostics, Tokyo, Japan). In principle, G1896 in the wild-type HBV and A1896 in the mutants were determined by mini-sequence reactions using labeled nucleotides that are complementary to either the wild type or mutant. The results were expressed as percentage mutation rate according to the definition by Aritomi et al. [11]. The sample was judged positive for the PC mutation when the mutation rate attained 100% in the present study, and judged mixture status of mutant and wild-type sequence in PC region when the mutation rate was in the range 10–90%. The dual mutation in BCP region was detected with an enzyme-linked specific probe assay (Smitest HBV core promoter mutation detection kit; Roche Diagnostics, Tokyo, Japan) [11]. This kit detects T1762/G1764 or A1762/T1764 by PCR with primers specific for either the wild type or mutant. The results were recorded in three categories: wild, mixed, and mutant types. The detection limits of these kits are both 1,000 copies/ml.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using StatView version 5.0 software

package (SAS, Cary, NC). Statistical analyses were performed using χ^2 and Fisher's extract test for categorical variables. Mann–Whitney U test was used for comparison of continuously distributed variables between two independent groups. Results were considered statistically significant at $P < 0.05$.

Results

Baseline characteristics and HBV genotypes

A total of 215 patients were studied. The study population included 154 (71.6%) men and 61 (28.4%) women; mean age was 48.5 ± 13.7 years (range 18–82 years). All the patients were ethnically Japanese. Clinical diagnoses of the patients were as follows: 67 patients were asymptomatic carriers, 97 had chronic hepatitis, 28 had cirrhosis, and 18 had HCC. At presentation, 73 (34.0%) patients were HBeAg positive.

Serum HBV DNA was detected in 100% (73/73) of HBeAg-positive patients and in 85.2% (121/142) of HBeAg-negative patients after nested PCR. The overall HBV detection rate was 90.2% (194/215). Four HBV genotypes were found: 169 genotype C (87.1%), 16 genotype B (8.2%), 3 genotype A (1.6%), and 1 genotype F (0.5%). In five samples (2.6%), genotype could not be determined. We focused the study on 185 patients with genotype C or genotype B.

The baseline characteristics of the 185 patients with HBV genotype C and B are summarized in Table 1. Genotype B patients had significantly lower ALT values ($P < 0.05$), lower HBV DNA levels ($P < 0.05$), and lower prevalence of HBeAg ($P < 0.05$) compared with genotype C patients.

Table 1 Baseline characteristics of patients with HBV genotype C and genotype B

	Genotype C ($n = 169$)	Genotype B ($n = 16$)	P value
Mean age (years)	46.9 ± 13.1	52.2 ± 15.6	NS
Gender (male:female)	118:51	10:04	NS
Clinical diagnosis			
Asymptomatic carrier	44 (26.1%)	10 (62.5%)	
Chronic hepatitis	82 (48.5%)	5 (31.2%)	
Liver cirrhosis	23 (13.6%)	1 (6.3%)	
Hepatocellular carcinoma	20 (11.8%)	0 (0%)	
ALT (IU/L)	76 ± 101	26 ± 21	<0.05
Platelet count (K/mm^3)	162 ± 61	192 ± 41	NS
HBeAg positive (%)	41.4	0	<0.05
HBV DNA (log copies/ml)	5.6 ± 1.9	3.5 ± 0.9	<0.05

NS not significant

Comparison between HBeAg-positive patients and HBeAg-negative patients

Among the patients with genotype C, HBeAg-positive patients had significantly higher HBV DNA levels ($P < 0.001$) and higher ALT levels ($P < 0.05$) than HBeAg-negative patients (Table 2). The mean level of serum HBV DNA levels ($P < 0.001$) and ALT levels ($P < 0.05$) were significantly higher in HBeAg-positive patients with genotype C than in HBeAg-negative patients with genotype B. Among HBeAg-negative patients, HBV DNA levels were significantly higher in genotype C patients than in genotype B patients ($P < 0.05$).

Prevalence of PC mutation in patients with HBV genotype C

Compared with HBeAg-positive patients, HBeAg-negative patients were significantly less likely to have PC wild type in patients with HBV genotype C ($P < 0.0001$) (Table 3). HBeAg-negative patients with PC wild type had significantly lower HBV DNA levels ($P < 0.05$) and ALT levels

Table 2 Comparison between HBeAg(+) patients and HBeAg(−) patients

	HBV DNA (log copies/ml)	ALT (IU/L)	Platelet count (K/mm^3)
Genotype C			
HBeAg(+) (n=70)	7.1 ± 0.9	111 ± 139	163 ± 64
HBeAg(−) (n=99)	4.5 ± 1.6	51 ± 53	162 ± 60
Genotype B			
HBeAg(+) (n=0)	–	–	–
HBeAg(−) (n=16)	3.5 ± 0.9	26 ± 21	192 ± 41

* $P<0.05$ ** $P<0.001$

Table 3 Prevalence of precore mutation in patients with HBV genotype C

	Prevalence (%)	HBV DNA (log copies/ml)	ALT (IU/L)	Platelet count (K/mm^3)
HBeAg(+) PC-Wild (n=36)	51.47	7.3 ± 0.6	99 ± 135	168 ± 55
HBeAg(+) PC-Mix (n=30)	42.9	7.0 ± 1.0	131 ± 150	151 ± 61
HBeAg(+) PC-Mutant (n=4)	5.7	5.6 ± 2.8	71 ± 58	201 ± 137
HBeAg(−) PC-Wild (n=11)	11.1	3.3 ± 0.9	21 ± 10	194 ± 79
HBeAg(−) PC-Mix (n=26)	26.3	4.9 ± 1.7	54 ± 53	143 ± 53
HBeAg(−) PC-Mutant (n=59)	59.6	4.6 ± 1.4	57 ± 56	163 ± 58
HBeAg(−) PC-UD (n=1)	3.0	2.7 ± 0.2	27 ± 23	188 ± 47

* $P<0.05$ ** $P<0.001$ *** $P<0.0001$

PC precore , *UD* undetectable

Table 4 Prevalence of basal core promoter mutation in patients with HBV genotype C

	Prevalence (%)	HBV DNA (log copies/ml)	ALT (IU/L)	Platelet count (K/mm^3)
HBeAg(+) CP-Wild (n = 17)	24.3	7.5 ± 0.4	122 ± 182	186 ± 62
HBeAg(+) CP-Mix (n = 11)	15.7	6.9 ± 1.1	116 ± 121	177 ± 49
HBeAg(+) CP-Mutant (n = 42)	60	7.0 ± 1.1	105 ± 126	149 ± 66
HBeAg(−) BCP-Wild (n = 13)	13.1	3.7 ± 1.4	39 ± 58	182 ± 47
HBeAg(−) BCP-Mix (n = 2)	2.0	3.5 ± 0.9	26 ± 1	155 ± 27
HBeAg(−) BCP-Mutant (n = 82)	82.9	4.7 ± 1.6	55 ± 53	157 ± 62
HBeAg(−) BCP-UD (n = 2)	2.0	2.6 ± 0.0	26 ± 1	216 ± 30

BCP basal core promoter, *UD* undetectable

Table 5 Prevalence of precore and basal core promoter mutations in patients with HBV genotype B

	Prevalence (%)	HBV DNA (log copies/ml)	ALT (IU/L)	Platelet count (K/mm^3)
HBeAg(+) (n = 0)	–	–	–	–
HBeAg(−) PC-Wild (n = 3)	18.8	3.9 ± 0.8	31 ± 20	192 ± 13
HBeAg(−) PC-Mix (n = 3)	18.8	3.3 ± 0.6	33 ± 16	168 ± 47
HBeAg(−) PC-Mutant (n = 10)	62.4	3.7 ± 1.0	23 ± 22	199 ± 42
HBeAg(−) BCP-Wild (n = 11)	68.7	3.7 ± 0.9	27 ± 23	203 ± 41
HBeAg(−) BCP-Mix (n = 0)	–	–	–	–
HBeAg(−) BCP-Mutant (n = 2)	12.5	3.5 ± 0.6	18 ± 2	184 ± 7
HBeAg(−) BCP-UD (n = 3)	18.8	3.0 ± 0.6	31 ± 20	157 ± 7

PC precore, *BCP* basal core promoter, *UD* undetectable

($P < 0.001$) compared with those with PC mutant. There were no significant differences in the mean level of platelet count between HBeAg-positive patients and HBeAg-negative patients, and among the patients with different patterns of PC sequences.

Prevalence of BCP mutation in patients with HBV genotype C

There was no significant difference in prevalence of BCP mutants between HBeAg-positive patients and HBeAg-negative patients with HBV genotype C (Table 4). There were no significant differences in serum HBV DNA levels, ALT levels, and platelet counts among the patients with different patterns of BCP sequences.

Prevalence of PC and BCP mutations in patients with HBV genotype B

All patients with HBV genotype B were HBeAg negative (Table 5). Among HBeAg-negative patients, genotype B patients were significantly more likely to have BCP wild type than were genotype C patients (68.7% versus 13.1%, $P < 0.001$), while there was no significant difference in prevalence of PC mutants between HBe-negative patients with HBV genotype B and those with HBV genotype C (62.4% versus 59.6%). There were no significant differences in the mean levels of serum HBV DNA, ALT, and platelet counts among the patients with different patterns of PC and BCP sequences. In comparison with genotype C patients, the patients with PC mutant tended to have lower HBV DNA levels and ALT levels in genotype B patients ($P = 0.0753$ and $P = 0.0605$).

Discussion

The present study demonstrated that, among HBeAg-negative patients with genotype C, the patients with PC wild type had significantly lower HBV DNA levels and ALT levels than those with PC mutant. Only a few studies have described the correlation of PC mutations with HBV DNA levels and ALT levels in HBeAg-negative patients. Misawa et al. [24] reported that PC and BCP mutations were significantly more frequent in 18 patients with positive HBV DNA than in 6 patients with negative HBV DNA among HBeAg-negative patients. The results of present study are consistent with their findings, although the present study found no correlation of BCP mutations with HBV DNA levels. On the other hand, Yoo et al. [25] found no correlation of PC mutation with HBV DNA levels or disease severity in HBeAg-negative patients with genotype C. The discrepancy between the present study and the study of Yoo et al. may be due to the difference of the detection method of HBV DNA; Yoo et al. used branched DNA assay which is less sensitive than Amplicor HBV monitor used in the present study.

The mechanism for the loss of HBeAg in patients with PC mutant has been thought to be that HBV with PC mutant cannot produce HBeAg. In the present study, 85.9% of HBeAg-negative patients had PC mixed type or PC mutant, and only 11.1% had PC wild type. This frequency of PC wild type in HBeAg-negative patients roughly coincides with the result of Yoo et al. (6.3%) [25]. The mechanism by which patients with PC wild type lose HBeAg has not been elucidated. Host defense system or mutations in region other than PC region may be responsible. The reason why HBeAg-negative patients with PC wild type had lower HBV DNA levels and ALT levels than those with PC mutant should be elucidated. Since HBeAg-positive patients with PC wild type had significantly higher HBV DNA levels than did HBeAg-negative patients with PC mutant, HBV with PC mutant probably propagates less efficiently than HBV with PC wild type. Therefore, host defense system or mutations in region other than PC region may be responsible for more strongly decreased HBV DNA levels in HBeAg-negative patients with PC wild type than in HBeAg-negative patients with PC mutant. Previous report showed that high serum viral load was the most important predictor of abnormal ALT levels among HBeAg-negative patients [26]. Another study reported an association between serum viral loads and histologic inflammation and fibrosis scores and ALT levels in HBeAg-negative patients [27]. In the present study, HBeAg-negative genotype C patients with PC wild type had lower HBV DNA levels and also lower ALT levels, which probably suggest better prognosis. Thus the follow-up of these patients may be reduced in frequency.

Previous studies showed that HBV genotype C patients had significantly higher serum viral loads, and were more likely to have PC and BCP mutants compared with HBV genotype B patients [5, 15, 17]. Other report also showed that HBV genotype C patients were more likely to have BCP mutants compared with genotype B patients among HBeAg-negative patients [18]. However, these studies have not shown the difference of serum viral loads between HBV genotype C patients and genotype B patients among HBeAg-negative patients. The present study demonstrated that, among HBeAg-negative patients, the patients with genotype B had significantly lower HBV DNA levels than those with genotype C, and that the prevalence of BCP wild type was significantly higher in patients with genotype B than those with genotype C. There was no difference in the prevalence of PC mutation between patients with genotype B and those with genotype C. Either among genotype C patients or among genotype B patients, there was no difference in HBV DNA levels between patients with BCP wild type and those with BCP mutant, although the number of genotype B patients with BCP mutant was too small. Thus the lower HBV DNA levels in genotype B may not correlate with the low prevalence of BCP mutation in genotype B. Further sequence studies on other regions of HBV are necessary to elucidate the mechanism of lower HBV DNA levels in HBeAg-negative genotype B patients.

In contrast to genotype C patients, the patients with PC mutant had similarly low HBV DNA levels and ALT levels compared with those with PC wild type in genotype B patients, although the number of genotype B patients with PC wild type was too small. Actually those with PC mutant tended to have lower HBV DNA levels and ALT levels in genotype B patients than genotype C patients. These findings may explain the lower HBV DNA levels in genotype B patients than in genotype C patients among HBeAg-negative patients. The cause of the difference between the two genotypes is not clear. The differences of specific sequence of HBV or host immune response between the two genotypes may be responsible for the difference. Further studies are needed to elucidate the cause of the difference between the two genotypes.

Recent study reported that HBV genotype B was classified into two subgenotypes called Ba (found in Asia) and Bj (found exclusively in Japan) [28, 29]. We did not determine subgenotypes of HBV genotype B. It was also reported that HBV subgenotype Bj accounted for 93% of carriers of HBV genotype B in Japan, and that higher prevalence rate of HBeAg and BCP mutations were observed in carriers of HBV subgenotype Ba than Bj [29]. For these reasons, most of the patients with HBV genotype B in the present study probably had subgenotype Bj.

The prevalence of genotype C patients and genotype B patients in our study was consistent with that in Japanese population [15], but we acknowledge that the number of genotype B patients was far smaller than that of genotype C patients in the present study. Thus, we may need further investigation such as a case–control study to confirm our results in the future. Furthermore, studies on the exact mechanism of active replication by the presence of PC and BCP mutations are also needed.

In conclusion, we showed that, among HBeAg-negative patients with genotype C, the patients with PC wild type had significantly lower viral loads and ALT levels than those with PC mutant, which probably suggests better prognosis for the patients with PC wild type. It is also suggested that host defense system or mutations in region other than PC region may be responsible for more strongly decreased HBV DNA levels in HBeAg-negative patients with PC wild type than in HBeAg-negative patients with PC mutant. We also showed that, among HBeAg-negative patients, the patients with genotype B had significantly lower viral loads than those with genotype C, and that the prevalence of BCP wild type was significantly higher in patients with genotype B than in those with genotype C. Further studies are needed to confirm our observations and

to determine if HBV genotyping and the mutations in PC and BCP region should be included in the clinical evaluation of chronic HBV infection and in the decision of antiviral therapy.

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Original Article

Liver stiffness measured by transient elastography correlates with fibrosis area in liver biopsy in patients with chronic hepatitis C

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Aim: Liver stiffness (LS) measured by transient elastography (TE) has been reported to correlate with liver fibrosis, which is usually semiquantitatively assessed. In the present study, the fibrosis area was measured by image analysis software in liver biopsy specimens and its correlation with LS was assessed.

Methods: LS was measured by TE in all 165 patients with chronic hepatitis C virus (HCV) infection who underwent liver biopsy consecutively in Fujita Health University Hospital from July 2004 to September 2007.

Results: Fibrosis area was significantly correlated with fibrosis stage as assessed by the Metavir score ($\rho = 0.733$, $P < 0.0001$). The optimal cut-off value of fibrosis area was 1.6% for F > or = 2, 3.1% for F > or = 3, and 3.8–6.4% for F4. LS was significantly correlated with fibrosis stage ($\rho = 0.734$, $P < 0.0001$). The optimal cut-off value of LS was 7.1 kPa for F > or = 2, 9.6 kPa for F > or = 3 and 11.6–16.9 kPa for F4. Multiple linear regression analysis selected fibrosis area ($P = 0.0002$), alanine aminotransferase (ALT) ($P = 0.0237$), γ-glutamyltransferase (γ-GTP) ($P = 0.0114$), prothrombin time ($P = 0.0114$) and hyaluronic acid ($P < 0.0001$) as factors correlating with LS.

Conclusion: The correlation between LS and liver fibrosis was confirmed by the objective measurement of fibrosis area. ALT was significantly correlated with LS, suggesting that inflammatory activity also affects LS values. Despite some limitation, LS measurement is a useful method for the diagnosis of liver fibrosis.

INTRODUCTION

NON-INVASIVE ASSESSMENT OF liver fibrosis is a major objective that has encouraged many new approaches. The prognosis and treatment of chronic liver diseases depend on the stage of liver fibrosis.[1] In chronic viral hepatitis, the presence of significant fibrosis (F > or = 2) indicates the necessity of antiviral therapies, and the outcome of therapy should be assessed by the alleviation of fibrosis stage. Diagnosis of advanced fibrosis is also important, since the risk of hepatocellular carcinoma or bleeding from esophageal varices is high in patients with advanced fibrosis.[2,3] Liver biopsy, the gold standard of assessment of liver fibrosis, is an invasive and expensive procedure, and its accuracy is sometimes questionable because of sampling errors, inadequate specimens and the subjective diagnosis of observers.[4,5]

Recently, transient elastography (TE) with the use of a new apparatus, FibroScan, for the non-invasive measurement of liver stiffness (LS) was developed.[6] LS measured by a FibroScan has been reported to correlate with stage of liver fibrosis in various liver diseases.[6–17]

Liver fibrosis is usually semiquantitatively assessed by the numerical systems of Scheuer,[18] the Metavir group,[19]

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or Ishak *et al.*[20] Recently, direct measurement of the amount of fibrosis in the biopsy specimen by computer-assisted morphometric image analysis has been reported, where the morphometric collagen content was measured quantitatively and correlated well with the score of numerical systems of liver biopsy assessment.[21–23]

In the present study, the proportion of fibrosis area was quantitatively measured by image analysis software in the liver biopsy specimen and the correlation with LS was assessed.

METHODS

Patients

ALL 165 PATIENTS with chronic hepatitis C virus (HCV) infection where liver biopsy was consecutively performed at Fujita Health University Hospital from July 2004 to September 2007 were studied (Table 1). Sections were stained with hematoxylin–eosin (HE) stain and azan stain. Liver biopsy specimens were assessed by a hepatologist (K.Y.) blinded of clinical data according to Metavir score.[24] Fibrosis was staged as: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Activity was graded as A0, none; A1, mild; A2, moderate; and A3, severe activity.

LS measurement

LS measurement by TE was performed with a FibroScan (EchoSens, Paris, France) within a week of liver biopsy. Ten validated measurements were made on each patient. The results were expressed in kilopascals (kPa). Only procedures with 10 validated measurements and a success rate of at least 60% (ratio of the number of successful acquisitions over the total number of acquisitions) were considered reliable. The median value was considered representative of the liver elastic modulus.

Proportion of fibrosis area in the liver biopsy specimens

The proportion of fibrosis area in the biopsy specimens was measured by computer-assisted morphometric image analysis (Fig. 1). Liver biopsy specimens were stained with azan stain. The fibrosis area that was stained blue with azan was marked and measured with the image analysis software, Image Pro Plus 4.0 (Nippon Roper Co, Tokyo, Japan). Each specimen was examined at three different fields or more with the use of a 5X objective.

Statistical analysis

Patients were divided according to fibrosis stage. The groups were compared by χ^2-test and the Mann–Whitney *U*-test with Bonferroni's adjustment. Factors correlating with LS and fibrosis stages were estimated by the Spearmen's ρ coefficient. Factors independently correlating with LS were assessed by multiple regression analysis. The diagnostic performance of LS and fibrosis area was determined in terms of sensitivity, specificity, positive and negative predictive values, positive likelihood ratio, diagnostic accuracy and area under receiver operating characteristics (ROC) curves. Optimal cut-off values between fibrosis categories were determined at the maximum total of sensitivity and specificity with sensitivity of more than 80%. When the positive predictive value was less than 50%, other cut-off values were assessed by lowering the sensitivity less than 80%.

RESULTS

Semiquantitative histological assessment by the Metavir system

THE LIVER BIOPSIES of 165 patients were assessed by the Metavir system. Fibrosis stage was F0 in 14 patients, F1 in 52, F2 in 42, F3 in 33 and F4 in 24 (Table 1). All the patients with F4 were assessed as grade A by the Child–Pugh score. Age ($P = 0.0058$), inflammatory grade ($P < 0.0001$), platelet count ($P = 0.0021$), prothrombin time ($P = 0.0017$) and hyaluronic acid ($P = 0.0224$) differed significantly between F0–1 and F2. Inflammatory grade ($P < 0.0001$), aspartate aminotransferase (AST) ($P = 0.0024$), alanine aminotransferase (ALT) ($P = 0.0371$) and γ-glutamyltranspeptidase (γ-GTP) ($P = 0.0290$) differed significantly between F2 and F3. Platelet count ($P = 0.0351$) differed significantly between F3 and F4.

Fibrosis area in the liver biopsy specimen

The proportion of fibrosis area was significantly correlated with fibrosis stage as assessed by the Metavir system ($\rho = 0.733$, $P < 0.0001$) (Fig. 2). The values of fibrosis area significantly differed between F0–1 and F2 ($P < 0.0001$); between F2 and F3 ($P < 0.0001$); and between F3 and F4 ($P < 0.0001$) (Table 1).

ROC analysis was done to assess the diagnostic value of fibrosis area for different fibrosis stages. Area under

Table 1 Characteristics of 165 patients with chronic hepatitis C virus infection

	All patients ($n = 165$)	F0–1 ($n = 66$)	*P* values of Mann–Whitney *U* test between F0–1 and F2	F2 ($n = 42$)	*P* values of Mann–Whitney *U* test between F2 and F3	F3 ($n = 33$)	*P* values of Mann–Whitney *U* test between F3 and F4	F4 ($n = 24$)
Age (year)†	57 (18–71)	49 (21–69)	$P = 0.0058$	59 (18–71)	NS	61 (22–71)	NS	61 (43–71)
Gender (female/male)‡	73/92	27/39	NS	17/25	NS	19/14	NS	10/14
Fibrosis stage (F0/F1/F2/F3/F4)	14/52/42/33/24	–	–	–	–	–	–	–
Inflammatory grade (A0/A1/A2/A3)	13/73/49/30	13/45/8/0	$P < 0.0001$	0/22/17/3	$P < 0.0001$	0/2/20/11	NS	0/4/4/16
AST (IU/L)†	42 (15–216)	32 (15–148)	NS	33 (16–187)	$P = 0.0024$	66 (23–216)	NS	75 (19–155)
ALT (IU/L)†	56 (9–330)	42 (12–226)	NS	53 (9–178)	$P = 0.0371$	82 (21–330)	NS	91 (16–221)
γ-GTP (IU/L)†	44 (11–556)	30 (11–556)	NS	42 (14–164)	$P = 0.0290$	67 (21–216)	NS	50 (18–195)
Platelet count ($\times 10^4/\mu L$)†	16.3 (6.3–37.1)	18.3 (6.3–37.1)	$P = 0.0021$	15.6 (6.7–26.0)	NS	14.9 (7.7–29.2)	$P = 0.0351$	11.6 (6.5–17.2)
Prothrombin time (%)†	93 (62–120)	99 (72–120)	$P = 0.0017$	93 (71–120)	NS	88 (77–112)	$P = 0.0790$	82 (62–99)
Albumin (g/dL)†	4.3 (3.4–5.0)	4.4 (4.0–5.0)	NS	4.4 (3.5–4.8)	NS	4.2 (3.6–4.6)	NS	4.1 (3.4–4.9)
γ-globulin (g/dL)†	1.5 (0.9–2.7)	1.3 (0.9–2.1)	NS	1.5 (0.9–2.1)	NS	1.6 (1.2–2.4)	NS	1.8 (1.2–2.7)
Hyaluronic acid (ng/mL)†	63 (9–765)	34 (9–328)	$P = 0.0224$	72 (9–427)	NS	96 (9–675)	$P = 0.0683$	145 (59–765)
Fibrosis area (%)†	2.2 (0.2–13.4)	1.0 (0.2–5.4)	$P < 0.0001$	2.2 (0.6–5.2)	$P < 0.0001$	4.3 (1.0–7.6)	$P < 0.0001$	8.0 (0.6–13.4)
Liver stiffness (kPa)†	8.0 (2.8–48.8)	5.4 (2.8–12.5)	$P < 0.0001$	8 (3.7–19.1)	$P = 0.0012$	12.0 (4.3–26.3)	$P = 0.0207$	17.7 (7.4–48.8)

†Data are shown as median (range).
‡Difference of frequency of gender was assessed by χ^2-test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltranspeptidase.

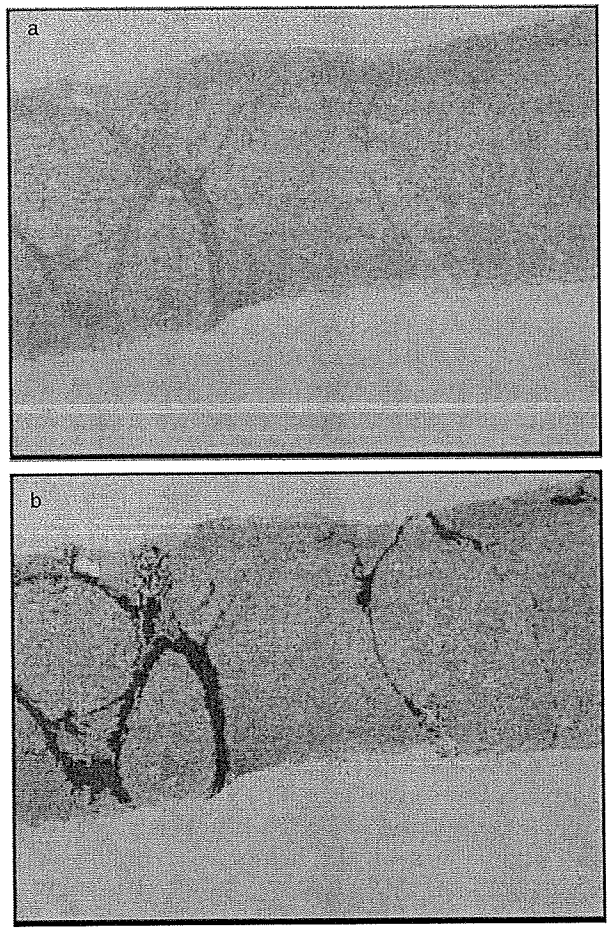

Figure 1 The proportion of fibrosis area in the biopsy specimens was measured by computer-assisted morphometric image analysis. (a) Liver biopsy specimens were stained with azan stain. (b) The fibrosis area that was stained blue with azan was marked and measured with the image analysis software, Image Pro Plus 4.0 imaging software (Nippon Roper Co, Tokyo, Japan). Each specimen was examined at three different fields or more with the use of a 5X objective.

the ROC (AUROC) values (95% confidence interval) were 0.87 (0.81–0.92) for F > or = 2, 0.91 (0.87–0.96) for F > or = 3, and 0.93 (0.85–1.00) for F4. Based on the fibrosis area distribution according to fibrosis stage and ROC curves, the optimal discriminate cut-off values were determined at the maximum total of sensitivity and specificity with sensitivity of more than 80% (Table 2). The cut-off values were 1.6% for F > or = 2, 3.1% for F > or = 3, and 3.8% for F4. The positive predictive value for F4 with the cut-off value of 3.8% was low (45.1%). Thus the higher cut-off values such as 4.9

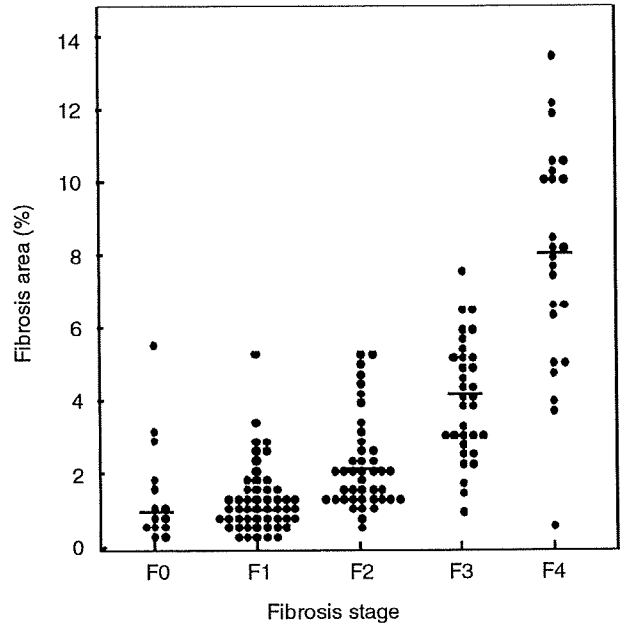

Figure 2 The proportion of fibrosis area was significantly correlated with fibrosis stage, as assessed by the Metavir system ($\rho = 0.733$, $P < 0.0001$).

or 6.4% were further assessed. The positive predictive value with a cut-off value of 6.4% was reasonably high (85.7%), and the sensitivity became slightly lower (75.0%).

Liver stiffness

LS was significantly correlated with fibrosis stage ($\rho = 0.734$, $P < 0.0001$) (Fig. 3). Values of LS signifi-

Table 2 Optimal cut-off value of fibrosis area for each fibrosis stage was determined at the maximum total of sensitivity and specificity with sensitivity of more than 80%. For F4, the higher cut-off values such as 4.9 and 6.4% were further assessed

	F > or = 2	F > or = 3	F4		
Cut-off value (%)	1.6	3.1	3.8	4.9	6.4
Positive predictive value (%)	83.3	78.7	45.1	55.6	85.7
Negative predictive value (%)	72.5	91.3	99.1	96.9	95.8
Sensitivity (%)	80.8	84.2	95.8	83.3	75.0
Specificity (%)	75.8	88.0	80.1	88.7	97.9
Positive likelihood ratio	3.3	7.0	4.8	7.3	35.3
Diagnostic accuracy (%)	78.8	86.7	82.4	87.9	94.5

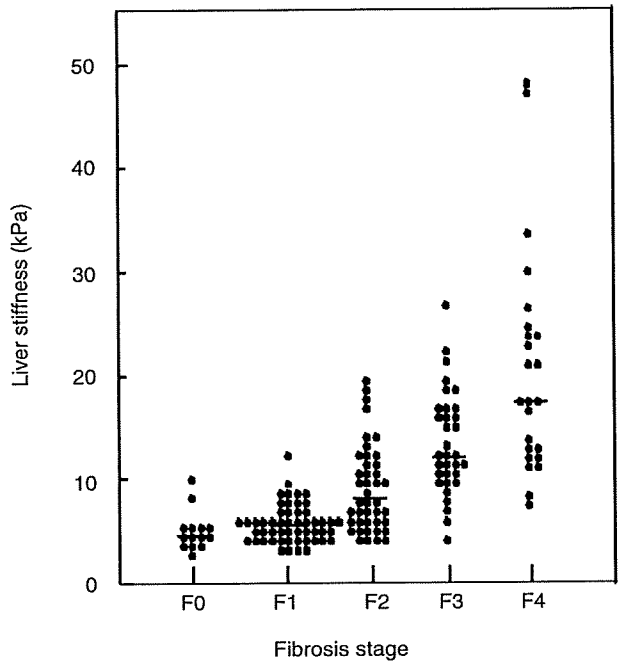

Figure 3 Liver stiffness was significantly correlated with fibrosis stage ($\rho = 0.734$, $P < 0.0001$).

cantly differed between F0–1 and F2 ($P < 0.0001$); between F2 and F3 ($P = 0.0012$); and between F3 and F4 ($P = 0.0207$) (Table 1). LS was significantly correlated with fibrosis area ($\rho = 0.590$, $P < 0.0001$) (Fig. 4).

ROC analysis was done to assess the diagnostic value of LS for different fibrosis stages. AUROC values (95% confidence interval) were 0.88 (0.83–0.93) for F > or = 2 (Fig. 5a), 0.90 (0.86–0.95) for F > or = 3 (Fig. 5b), and 0.90 (0.84–0.96) for F4 (Fig. 5c). Based on the LS distribution according to fibrosis stage and ROC curves, the optimal discriminate cut-off values were determined at the maximum total of sensitivity and specificity with sensitivity of more than 80% (Table 3). The cut-off values were 7.1 kPa for F > or = 2, 9.6 kPa for F > or = 3, and 11.6 kPa for F4. The positive predictive value for F4 with the cut-off value of 11.6 kPa was low (41.5%). Thus the higher cut-off values such as 13.6 kPa or 16.9 kPa were further assessed. The positive predictive value with a cut-off value of 16.9 kPa was slightly higher (55.6%), but the sensitivity became low (62.5%).

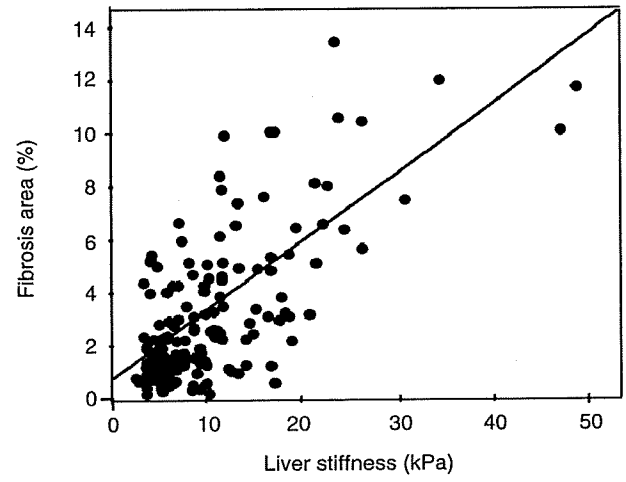

Figure 4 Liver stiffness was significantly correlated with proportion of fibrosis area ($\rho = 0.590$, $P < 0.0001$).

Factors correlating with LS

Other than fibrosis stage and fibrosis area, LS was significantly correlated with age ($P < 0.0001$), inflammatory grade ($P < 0.0001$) (Fig. 6), AST ($P < 0.0001$), ALT ($P < 0.0001$), γ-GTP ($P < 0.0001$), platelet counts ($P < 0.0001$), prothrombin time (%) ($P < 0.0001$), albumin ($P < 0.0001$), γ-globulin ($P < 0.0001$), and hyaluronic acid ($P < 0.0001$) (Table 4). Multiple regression analysis was done to determine the factors independently correlating with LS among the factors without fibrosis stage and AST which highly correlated with fibrosis area or ALT, respectively. Fibrosis area ($P = 0.0002$), ALT ($P = 0.0237$), γ-GTP ($P = 0.0114$), prothrombin time ($P = 0.0114$) and hyaluronic acid ($P < 0.0001$) were selected as factors independently correlating with LS (adjusted R square = 0.547) (Table 4).

To determine the factors correlating with LS in each fibrosis stage, multiple regression analysis was done with the patients with same fibrosis stage. In the patients with F0–1, γ-GTP ($P = 0.0227$) and prothrombin time ($P = 0.0286$) were selected, although the R value of multiple regression analysis was rather low (adjusted R square = 0.112). In those with F2, ALT ($P = 0.0047$) and albumin ($P = 0.0029$) were selected (adjusted R square = 0.362). In those with F3, albumin ($P = 0.0166$) and γ-globulin ($P = 0.0002$) were selected (adjusted R square = 0.512). In those with F4, fibrosis area ($P = 0.0177$) and hyaluronic acid ($P = 0.0349$) were selected (adjusted R square = 0.371).

DISCUSSION

THE PRESENT STUDY demonstrated that LS correlates with the proportion of fibrosis area quantitatively measured in the liver specimens. This finding

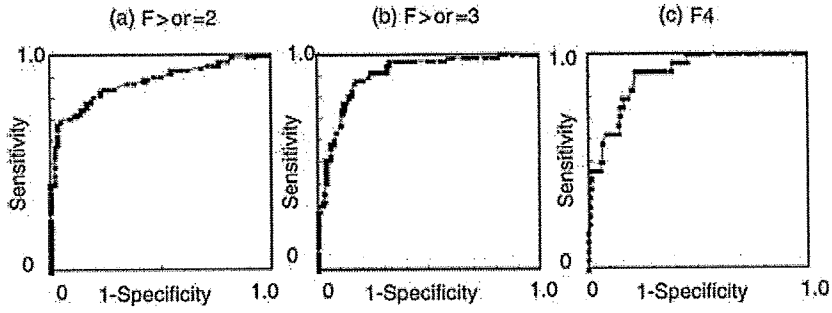

Figure 5 The receiver operating characteristics curve analysis was done to assess the diagnostic value of liver stiffness for different fibrosis stages. The values of area under the receiver operating characteristics curve (95% confidence interval) were 0.88 (0.83–0.93) for F > or = 2 (a); 0.90 (0.86–0.95) for F > or = 3 (b); and 0.90 (0.84–0.96) for F4 (c).

confirmed the correlation between LS and liver fibrosis stage which has been semi-quantitatively measured by numerical systems. In most studies dealing with the correlation between LS and hepatic fibrosis, fibrosis has been measured numerically with the Metavir system,[19] using stages that range from 0 to 4. The numbers actually represent categories of increasing severity of fibrosis based on a combination of location and quantity of scarring, and whether the fibrous tissue forms septa, bridges, or nodules. Thus the numerical systems are not essentially a quantitative method for assessing the amount of fibrosis in the liver, although they are intended to be semi-quantitative. The present study demonstrated that fibrosis area quantitatively measured correlates well with the numerical fibrosis staging and also with LS.

In the present study, the fibrosis areas in the biopsy specimens were measured by computer-assisted morphometric image analysis. Although the methodology is not standardized, a number of publications have described methods for quantifying hepatic fibrosis by image analysis, and all appear to yield similar results.[21–23] Goodman *et al.* reported that the mean morphometric collagen content was 0.0856 in stage 5 (incomplete cirrhosis), and 0.1163 in stage 6 (cirrhosis, probable or definite) of the Ishak score,[23] which correspond with the values of fibrosis area of F4 in the present study. Since the methods were time consuming, and needed the equipment and expertise, they had not been made widely available. However, recently these problems have been gradually fixed. The apparatus for microscopic image acquisition and image analysis software are available in many institutions now, and their functions are being improved. Thus in a study to evalu-

Table 3 Optimal cut-off value of liver stiffness for each fibrosis stage was determined at the maximum total of sensitivity and specificity with sensitivity of more than 80%. For F4, the higher cut-off values such as 13.6 kPa and 16.9 kPa were further assessed

	F > or = 2	F > or = 3	F4		
Cut-off value (kPa)	7.1	9.6	11.6	13.6	16.9
Positive predictive value (%)	86.0	72.5	41.5	44.4	55.6
Negative predictive value (%)	73.6	92.7	98.2	93.8	93.5
Sensitivity (%)	80.8	87.7	91.7	66.7	62.5
Specificity (%)	80.3	82.4	78.0	85.8	91.5
Positive likelihood ratio	4.1	5.0	4.2	4.7	7.3
Diagnostic accuracy (%)	80.6	84.2	80.0	83.0	87.3

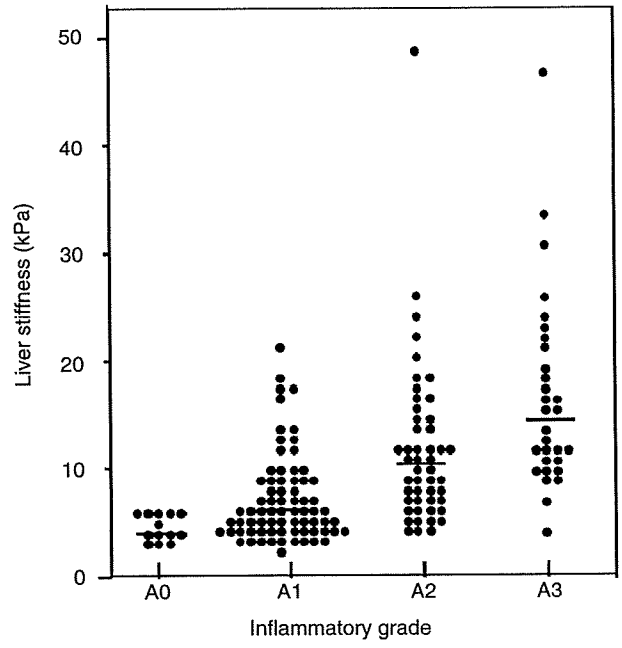

Figure 6 Liver stiffness was significantly correlated with inflammatory grade ($\rho = 0.598$, $P < 0.0001$).

Table 4 Factors correlating with liver stiffness in all patients or patients with each fibrosis stage, as assessed by Spearman's rank correlation test and multiple regression analysis

	All patients				F0–F1			
	Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis	
	ρ	*P*	β	*P*	ρ	*P*	β	*P*
Age	0.483	$P < 0.0001$		NS		NS	–	–
Gender		NS	–	–		NS	–	–
Fibrosis stage	0.734	$P < 0.0001$	–	–	–	–	–	–
Inflammatory grade	0.598	$P < 0.0001$		NS	0.226	$P = 0.0679$		NS
Fibrosis area	0.590	$P < 0.0001$	0.204	$P = 0.0002$		NS	–	–
AST	0.583	$P < 0.0001$	–	–	0.269	$P = 0.0290$		NS
ALT	0.486	$P < 0.0001$	0.064	$P = 0.0237$		NS	–	–
γ-GTP	0.413	$P < 0.0001$	0.061	$P = 0.0114$	0.275	$P = 0.0255$	0.103	$P = 0.0227$
Platelet counts	−0.449	$P < 0.0001$		NS		NS	–	–
Prothrombin time (%)	−0.447	$P < 0.0001$	−0.013	$P = 0.0114$	−0.208	$P = 0.0931$	−0.015	$P = 0.0286$
Albumin	−0.471	$P < 0.0001$		NS		NS	–	–
γ-globulin	0.351	$P < 0.0001$		NS		NS	–	–
Hyaluronic acid	0.599	$P < 0.0001$	0.207	$P < 0.0001$	0.226	$P = 0.0728$		NS
R			0.739				0.373	
Adjusted R square			0.547				0.112	
F			37.4				5.1	
p			$P < 0.0001$				$P = 0.0088$	

Table 4 *Continued*

	F2				F3				F4			
	Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis	
	ρ	*P*	β	*P*	ρ	*P*	β	*P*	ρ	p	β	*P*
Age	0.299	$P = 0.0546$		NS	0.421	$P = 0.0146$		NS		NS	–	–
Gender		NS	–	–		$P = 0.0275$		NS		NS	–	–
Fibrosis stage	–	–	–	–	–	–	–	–	–	–	–	–
Inflammatory grade		NS	–	–	0.324	$P = 0.0655$		NS		NS	–	–
Fibrosis area		NS	–	–	0.357	$P = 0.0413$		NS	0.518	$P = 0.0095$	1.575	$P = 0.0177$
AST	0.492	$P = 0.0009$	–	–		NS	–	–		NS	–	–
ALT	0.520	$P = 0.0004$	0.037	$P = 0.0047$	0.319	$P = 0.0700$		NS		NS	–	–
γ-GTP	0.413	$P = 0.0066$	–	–		NS	–	–	0.360	$P = 0.0838$		NS
Platelet counts		NS	–	–	−0.376	$P = 0.0310$		NS		NS	–	–
Prothrombin time (%)		NS	–	–		NS	–	–		NS	–	–
Albumin	−0.432	$P = 0.0043$	−5.201	$P = 0.0029$	−0.442	$P = 0.0100$	−6.737	$P = 0.0166$	−0.316	$P = 0.0629$		NS
γ-globulin		NS	–	–	0.635	$P = 0.0002$	8.027	$P = 0.0002$		NS	–	–
Hyaluronic acid	0.469	$P = 0.0017$		NS	0.503	$P = 0.0033$		NS	0.394	$P = 0.0121$	0.020	$P = 0.0349$
R			0.602				0.739				0.655	
Adjusted R square			0.362				0.512				0.371	
F			11.1				16.2				7.5	
p			$P = 0.0002$				$P < 0.0001$				$P = 0.0037$	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; R, multiple correlation coefficient; F, ratio of mean square for the model divided by mean square for error, where the mean squares are the respective sums of squares divided by the degrees of freedom; p, probability.