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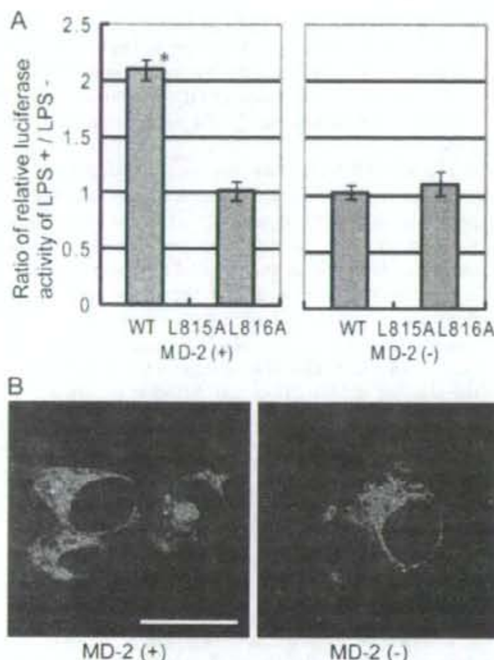

FIGURE 4. **Leucines at positions 815–816 of TLR4 are responsible for impairment of LPS responsiveness and plasma membrane expression.** *A*, the LPS stimulation assay was done for TLR4 (L815AL816A)-EGFP as in Fig. 2. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* value for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$). *B*, TLR4 (L815AL816A)-EGFP expression in HEK293T cells was observed by laser confocal microscopy. *Bar*, 20 μm.

FIGURE 5. **Alignment of the cytoplasmic domain of EGFP fusion TLR4 amino acid-replacement mutants used in this study.** TLR4 (L813A) signifies a mutant with leucine replaced with alanine at position 813. Others are named in the same manner. The amino acids are *colored* as in Fig. 1. All amino acids are designated using the single-letter code.

was reasonable to explore whether leucines 815 and 816 need to be adjacent to each other. We created five genotypes of single amino acid mutants of TLR4: TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP. We excluded the amino acid at position 814 from the analysis, because the amino acid in position 814 of wild-type TLR4 is alanine. The amino acid sequence alignment of wild-type TLR4 and the single amino acid replacement mutants is shown in Fig. 5. DNA sequences were confirmed by sequencing.

As was done with truncation mutants, we measured NF-κB activity of wild-type TLR4-EGFP, TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP in response to LPS stimulation. All mutants except TLR4 (L815A)-EGFP showed responsiveness to LPS stimulation with coexpression of MD-2 (Fig. 6*A*). Without MD-2, no genotype of TLR4-EGFP responded to LPS stimulation (Fig. 6*B*). LPS stimulation was performed in an identical manner as with truncation mutants.

We analyzed the subcellular distribution of single amino acid mutants of TLR4-EGFP with and without MD-2 coexpression by fluorescence microscopy. TLR4 (K813A)-EGFP and TLR4 (D817A)-EGFP showed a similar fluorescence pattern to the wild-type, which localized at the plasma membrane when coexpressed with MD-2. No genotypes of TLR4-EGFP localized on the plasma membrane without MD-2 (Fig. 7). The cells transfected with TLR4 (L815A)-EGFP coexpressed with MD-2 did not show plasma membrane fluorescent pattern. Also, TLR4 (L815A)-EGFP showed comparatively weaker fluoresce than other mutants, possibly due to lower expression of the protein. Fluorescence of TLR4 (L816A)-EGFP with MD-2 was ambiguous as for the plasma membrane expression. Some kind of membranous structure was observed in the cytoplasmic area, but the intensity of the plasma membrane green fluorescence was obscure. Together with the results from the LPS stimulation experiment, the leucines at positions 815 and 816 are considered to play important roles in signal transduction and/or subcellular distribution of TLR4.

Because EGFP consists of 239 amino acids, which is about one-third the size of the complete TLR4 protein, the experimental results obtained using TLR4-EGFP could have been influenced by the presence of the EGFP fused at the C terminus of TLR4. To rule out this possibility, we tested the functional integrity of both TLR4 (L815A) and TLR4 (L816A) with and without EGFP at the C terminus. Reporter assays were performed under the same conditions except that the shorter tag, FLAG-His_6, which has only 21-amino acid tags at the C terminus, was fused to TLR4 in place of EGFP. There was no difference

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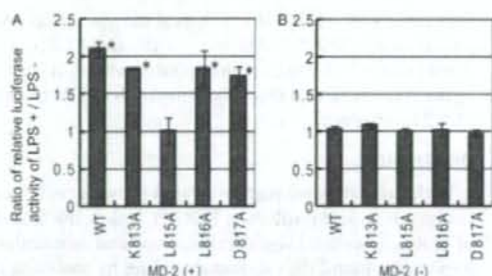

FIGURE 6. **Leucine at position 815 of TLR4 is pivotal for LPS responsiveness as measured by NF-κB luciferase assay.** *A*, HEK293T cells were transfected with single amino acid replacement mutants of the human TLR4-EGFP fusion protein plasmid, human MD-2 plasmid, and luciferase reporter and control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. *B*, instead of MD-2, an empty vector was cotransfected with TLR4-EGFP plasmid and reporter assay vectors. LPS stimulation was done as in *A*. All results were expressed in the ratio of relative luciferase activity with LPS stimulation to that without the stimulation as in Fig. 2. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), p = 0.002; TLR4 (K813A)-EGFP/MD-2 (+), p = 0.000; TLR4 (L816A)-EGFP/MD-2 (+), p = 0.018; and TLR4 (D817A)-EGFP/MD-2 (+), p = 0.007).

between EGFP-tagged proteins and FLAG-His_6-tagged proteins in the relative pattern of responsiveness against LPS stimulation (Fig. 8*A*). Because CD14 is also important for LPS recognition by TLR4, we examined the effect of CD14 coexpression on the phenotypic changes of the mutants (17, 18). Coexpression of CD14 did not change the phenotypes of wild-type TLR4, TLR4 (L815A), and TLR4 (L816A) in terms of LSP responsiveness (data not shown).

Cell surface expressions of the wild-type, L815A mutant, and L816A mutant TLR4-FLAG- His_6 fusion proteins were also examined. Live cells transfected with wild-type TLR4, the L815A mutant or the L816A mutant as well as human MD-2 and CD14 were biotinylated on the cell surface, and the biotinylated proteins were affinity-purified and subjected to Western blotting. Fig. 8*B* shows the marked difference in cell surface expression of wild-type and mutants L815A and L816A. Note that biotinylated proteins have additional residues on every amine of the extracellular domain, which leads to a band shift during electrophoresis. Although both mutants were detected far less than the wild-type on the cell surface, comparatively more L816A mutant was expressed on the plasma membrane than L815A mutant, and the amount of L815A mutant seemed to be negligible compared with the wild type. These results may clarify the ambiguity of the microscopic observation of TLR4 (L815A) and TLR4 (L816A). Plasma membrane expression of TLR4 was impaired when the leucine at 815 or 816 was replaced to alanine. But the leucine at 815 is more critical, and the mutant L816A may show the weaker phenotypic change.

To further investigate the characteristics of the TLR4 (L815A) mutant, we performed an immunoprecipitation assay of wild-type and mutant TLR4. Cells were transfected with a human MD-2-FLAG-His_6 expression vector and either the wild-type or the mutant (L815A) TLR4-EGFP expression vector. Anti-TLR4 monoclonal antibody (clone HTA125), anti-GFP polyclonal antibody, or anti-FLAG monoclonal antibody

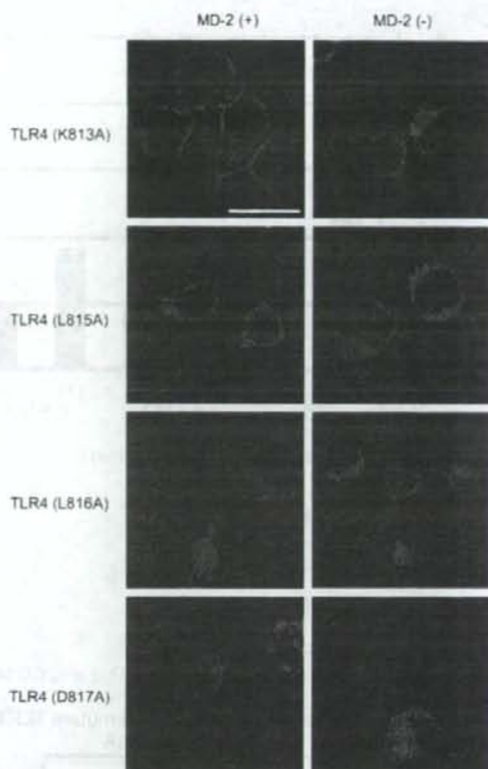

FIGURE 7. **Leucines at the position 815 and 816 of TLR4 are responsible for full plasma membrane expression.** Cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Each genotype of TLR4-EGFP was cotransfected with human MD-2 plasmid or empty vector. *Bar*, 20 μm.

was added to the lysate and precipitated with Protein G-Sepharose beads. Collected proteins were eluted and subjected to Western blotting. The results are shown in Fig. 8C. TLR4 (L815A) was not immunoprecipitated with anti-TLR4 antibody (HTA125). HTA125 antibody was raised against TLR4-expressing cells (9) and recognizes the extracellular portion of TLR4. This result suggests that the amino acid replacement at position 815 may cause a change in the extracellular portion of TLR4 and/or that the replacement may also inhibit cell surface expression of the mutant protein. On the other hand, both wild-type TLR4-EGFP and mutant TLR4-EGFP were immunoprecipitated with anti-GFP polyclonal antibody, which recognized EGFP. However, of the two bands of TLR4, the heavier band seems to be somewhat faint in the mutant, whereas in the wild type the heavier band is at least as dense as the lighter one. TLR4 can be detected as two separate bands in a Western blot (19), especially under transient transfection conditions. The difference in proportion of the heavy and light bands between wild-type and mutant TLR4 may suggest that there is some difference in glycosylation. Furthermore, wild-type TLR4 was coprecipitated with MD-2-FLAG-His_6, but the mutant TLR4 could not be detected (Fig. 8C, *lanes 4* and *8*). Because MD-2 is

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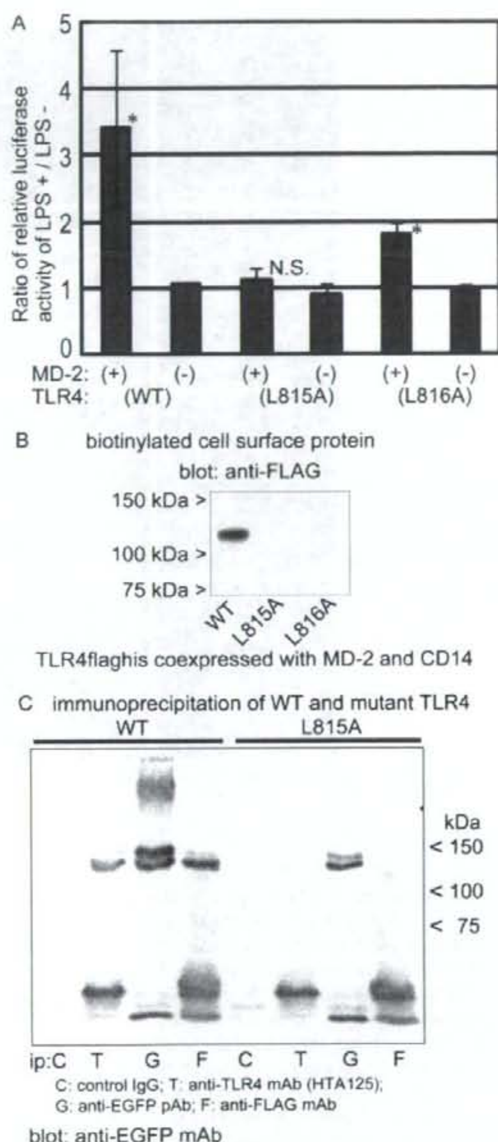

FIGURE 8. *A*, TLR4 mutants L815A and L816A with and without EGFP fusion exhibit the same phenotypes in LPS responsiveness and plasma membrane expression. HEK293T cells were transfected with the wild-type, the L815A or L816A mutant TLR4flaghis plasmid plus the human MD-2 plasmid and luciferase reporter, or control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* values for * are: TLR4 (WT) flaghis/MD-2 (+), $p = 0.046$; TLR4 (L816A) flaghis/MD-2 (+), $p = 0.003$). *N.S.*: not significant. *B*, wild-type and mutant TLR4s L815A and L816A were tagged by biotinylation of the cell surface proteins and affinity-purified. Human MD-2 and CD14 were coexpressed. TLR4 was visualized by immunoblotting using an anti-FLAG monoclonal antibody (*mAb*). Faint bands below 100 kDa are considered to be unbiotinylated intracellular TLR4 proteins that were not washed off during the process. Samples from TLR4 (WT), TLR4 (L815A), and TLR4 (L816A), respectively, were prepared from the same number of cells as for the biotinylation experiment. *C*, immunoprecipitation with antibodies further reveals the characteristics of TLR4 (L815A). Anti-TLR4 monoclonal antibody (HTA125) does not precipitate the mutant TLR4, whereas anti-GFP polyclonal antibody (*pAb*) precipitates both wild-type and mutant TLR4. Mutant TLR4 was not coprecipitated with MD-2-FLAG-His_6. Lysates were prepared from cells transiently expressing wild-type or mutant TLR4-EGFP and MD-2-FLAG-His_6.

associated with TLR4 (9), it is logical to expect that immunoprecipitating MD-2-FLAG-His_6 with anti-FLAG antibody should cause TLR4 to be coprecipitated with it. It is suggested by the result here that the association of the TLR4 mutant with MD-2 is impaired.

DISCUSSION

In this research, we performed mutagenesis analyses of particular amino acid residues in TLR4 to explore the mechanisms of TLR4 intracellular signal transduction and subcellular distribution. We found the candidate residues by analyzing truncation mutants of TLR4 in the cytoplasmic region, in which both signaling and normal subcellular distribution of TLR4 are disturbed. Because we are focusing on a common mechanism for the impaired signaling and distribution, we finally picked a single amino acid mutant that does not respond to LPS stimuli, as measured with NF-κB reporter luciferase assay, and one that does not localize on the plasma membrane. TLR4 (L815A) is a mutant that meets these conditions, and our results suggest that the leucine at position 815 of TLR4 is required for both signal transduction and plasma membrane localization.

The best known single amino acid mutant of TLR4 is TLR4 (P712H) known as the *Lps^d* mutation in the C3H/HeJ mouse, which corresponds to position 714 in this study of human TLR4 (5, 6, 20). Mice carrying this mutation opened up the rediscovery of TLR4 as a key player in innate immunity. Because this proline residue at this position is within the TIR domain and is conserved among TLRs or TLR4s of other species, it is assumed that the residue plays an important role in TLR4 function. The association of TLR4 (P712H) with its adapter proteins is reported to be intact, and the explanation for the functional impairment of TLR4 (P712H) is not clear (21–23).

Some single amino acid variants are found in humans, and these are related to the incidence or prognosis of some infections and other diseases. A growing body of data suggests that the ability of certain individuals to respond properly to TLR4 ligands may be impaired by single-nucleotide polymorphisms within TLR4 genes (24). The D299G and T399I alleles of the TLR4 gene have been associated with increased risk of severe infections (25).

By clarifying the subcellular component where the mutant protein is retained, or by clarifying to which compartment the mutant is not delivered, the abnormal intracellular sorting that is caused by the mutation in TLR4 (L815A) could be elucidated more precisely. Usually a sorting signal motif is comprised of several amino acids. In this regard, if the leucine at position 815 is a part of a motif, there should be other amino acids that are also members of the motif. Although replacement of leucine with alanine at position 816 did not cause an apparent signal transduction impediment, plasma membrane expression of TLR4 (L816A) was impaired to a certain extent. Positive

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response to LPS stimulation by TLR4(L816A) could be attributable to this small amount of expression on the plasma membrane. Mutagenesis analyses of neighboring amino acids of the leucine at 815 were not definitive, but the results could be suggestive that the adjacent leucine at 816 may work together with the leucine at 815. Leucines at position 815 and 816 could be in the same motif, and the leucine at position 816 may be less critical.

Several proteins have been reported to be involved in TLR4 cell surface expression. Heat shock protein gp96 is necessary for TLR4 association with MD-2 in the ER and for subsequent cell surface expression (26). PRAT4A and PRAT4B are associated with TLR4 and regulate TLR4 cell surface expression (27, 28). In embryonic fibroblasts of MD-2 knockout mice, TLR4 localization on the cell surface is severely impaired, and most TLR4 is retained in the ER or Golgi apparatus (15). MD-2 binds to TLR4 at its extracellular domain and is essential for LPS recognition by TLR4 (29). Although proteins such as CD14 and LPS-binding protein are reported to have important roles in LPS recognition by TLR4, in an *in vitro* setting HEK293T cells gain LPS responsiveness by introducing only TLR4 and MD-2 genes when measured by NF-κB reporter assay (9, 30). Without transfection, HEK293 cells do not express TLR4, MD-2, or CD14, which are involved in LPS-induced intracellular signaling (31, 32). In this study, we show that the association of the TLR4 mutant and MD-2 is impaired (Fig. 8C).

Post-translational modification is another important factor for TLR4 function. Asparagine residues in the extracellular portion of TLR4 need to be glycosylated for plasma membrane expression of TLR4 (15, 19, 33). TLR4·MD-2 association is necessary for this glycosylation as well. The difference in the proportion of the heavy band to lighter band between wild-type and L815A mutant TLR4 immunoprecipitated with anti-GFP polyclonal antibody suggests that there may be some difference in glycosylation between wild-type and L815A mutant TLR4 (Fig. 8C). Although leucine at position 815 is located in the cytoplasmic tail of TLR4, we speculated that substitution of leucine at position 815 may cause a conformational change in the extracellular portion of the protein, which may interfere with the association between L815A mutant TLR4 and MD-2, leading to inhibition of glycosylation and cell surface expression of the mutant protein. Further investigation may reveal the mechanism involved in this phenotypic change in TLR4 (L815A), which would lead to better understanding of the mechanism of wild-type TLR4 signaling and trafficking.

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Original Article

Evidence of oxidative stress as a cofactor in the development of insulin resistance in patients with chronic hepatitis C

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Aim: The mechanisms by which metabolic disorders develop in patients with chronic hepatitis C are unknown. Our study aimed to test whether oxidative stress contributes to these mechanisms.

Methods: The index of homeostasis model assessment–insulin resistance (HOMA–IR) and serum and hepatic levels of thioredoxin (Trx), which are markers of oxidative stress, were evaluated in 203 biopsy-proven chronic hepatitis C patients with hepatitis C virus (HCV) genotype 1 or 2 infection. HOMA–IR and Trx levels were compared with baseline values after phlebotomy in 23 patients.

Results: HOMA–IR and serum Trx levels were significantly correlated with disease stage (HOMA–IR, $P < 0.00001$; Trx, $P < 0.0001$) and independently predicted fibrosis scores (HOMA–IR, $P < 0.05$; Trx, $P < 0.005$). Steatosis (%) was significantly correlated with HOMA–IR ($P < 0.00005$) and Trx ($P < 0.001$) stage ($P < 0.00001$). Serum Trx levels were significantly correlated with HOMA–IR ($P < 0.05$), even after adjustment for body mass index ($P < 0.05$). Furthermore, the mRNA levels of hepatic Trx were significantly correlated with HOMA–IR ($P < 0.05$) and independently-predicted HOMA–IR ($P < 0.05$). The alanine aminotransferase ($P < 0.00001$), Trx ($P < 0.05$), and HOMA–IR ($P < 0.05$) serum levels decreased significantly after phlebotomy; these effects were similar even in non-responders to interferon.

Conclusion: Oxidative stress contributed to the development of IR irrespective of obesity in patients with HCV genotype 1 or 2 infection. This study could contribute to our understanding of how metabolic disorders develop and how they should be treated in chronic hepatitis C patients.

Key words: hepatitis C virus, insulin resistance, oxidative stress, steatosis, thioredoxin

INTRODUCTION

CHRONIC HEPATITIS C progresses to cirrhosis and eventually to hepatocellular carcinoma (HCC).[1] Although interferon (IFN)-based antiviral therapy has achieved great advances, it can not eradicate hepatitis C virus (HCV) in approximately 50% of patients infected with the genotype 1 strain,[1] which is highly prevalent in Japan. Therefore, other therapeutic strategies remain important, and efforts to understand the pathogenesis are required.

Metabolic disorders have recently been implicated in the pathogenesis of chronic hepatitis C.[2–6] HCV-infected patients with hepatic steatosis exhibit clinical features associated with metabolic syndromes,[3] and glucose intolerance is considered to represent an extra-hepatic manifestation of HCV infection.[2,4] Furthermore, grades of steatosis are reported to predict rapid fibrosis progression,[5] and diabetes increases the risk of HCC.[6] From these findings, insulin resistance (IR), a central cause of metabolic syndromes,[7] has been described as a risk factor in advanced staged chronic hepatitis C patients,[2,8] as seen in non-alcoholic steatohepatitis.[9] Thus, insulin signaling could be an important target for

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the management of patients with HCV infection; however, how IR develops is not well understood.

In this study, we focused on the role of oxidative stress, another key player in progressive liver injury in patients with chronic hepatitis C infection[10] in the development of IR. Because steatosis results in the overproduction of reactive oxygen species (ROS),[11] and ROS may exacerbate hepatic insulin sensitivity,[12] we hypothesized a close relationship between IR and oxidative stress. Therefore, we retrospectively analyzed the index of IR[13] and the serum and hepatic levels of thioredoxin (Trx), which are markers of oxidative stress,[14] in 203 patients with HCV infection. We also investigated whether relieving hepatic oxidative stress could improve IR among these patients.

METHODS

Patients

CHRONIC HEPATITIS PATIENTS who underwent liver biopsies in our institute between April 2003 and March 2006 were selected according to the following criteria: no excessive alcohol intake (more than 40 g/week), as assessed by interview (at least on 3 occasions); positive serum HCV-RNA, as confirmed by reverse transcription–polymerase chain reaction (RT–PCR); infection with HCV genotype 1 (1a, 1b) or 2 (2a, 2b); no history of antiviral therapy nor treatment with steatosis-inducing drugs within the 12 months before the study; negativity for hepatitis B surface antigen or antibodies to HIV; and an absence of other forms of chronic liver disease. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. The serum HCV-RNA level was determined using the AMPLICOR GT HCV Monitor (Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes 1 and 2 were determined by a serologic genotyping assay.[15] Serogroups 1 and 2 in this assay correspond to genotypes 1 (1a, 1b) and 2 (2a, 2b). Informed written consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

Laboratory determination

After a 12-h overnight fast, venous blood samples were drawn to determine alanine aminotransferase (ALT), γ-glutamylcysteine transpeptidases, fasting plasma glucose (FPG), insulin (IRI), triglyceride, and ferritin levels. These parameters were measured using standard techniques from clinical chemistry laboratories. The index of IR was calculated only in patients without overt diabetes (FPG >126 mg/dL), according to the homeostasis model assessment (HOMA).[13] The formula for IR was as follows: HOMA-IR = FPG (mg/dL) × IRI (IU/mL)/405. HOMA-IR was only calculated in patients without diabetes ($n = 189$).

Measurement of Trx

The levels of serum Trx were used as a marker of hepatic oxidative stress, as reported previously.[14] For the measurements of Trx concentrations, serum and liver biopsy specimens were stored at −80°C until use. The serum Trx levels were then measured using a commercial, sensitive enzyme-linked immunosorbent assay kit (REDOX BIOSCIENCE, Kyoto, Japan), as described previously.[14] All measurements were made in duplicate and average values were used for the statistical analysis.

The hepatic levels of Trx were measured by real-time PCR. Total RNA was isolated from biopsy specimens using the RNeasy kit (Qiagen, Hilden, Germany). The PCR mixture contained first-strand cDNA and specific primers for human Trx: sense, 5′-CTGCTTTTCAG GAAGCCTTG-3′ and antisense, 5′-ACCCACCTTTTGT CCCTTCT-3′. PCR was performed using the Light Cycler 2.0 System (Roche, Mannheim, Germany), and the mRNA levels of Trx were normalized to those of β-actin.

Histological evaluation

Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin–eosin, Masson's trichrome, and Perl's Prussian blue. Degrees of hepatic fibrosis (stage) were scored as follows: F0 = none, F1 = portal expansion, F2 = bridging fibrosis, F3 = bridging fibrosis with lobular distortion, and F4 = cirrhosis. Degrees of inflammation (grade) were scored as follows: A0 = none, A1 = mild, A2 = moderate, and A3 = severe. Steatosis was assessed according to the percentage of hepatocytes containing fat droplets. The degree of iron loading was graded using a Perl's score of 0–4, as described previously.[16]

Phlebotomy

Phlebotomy was initiated to relieve iron-induced oxidative stress in 23 patients. All patients showed elevated serum ferritin levels and/or persistent abnormal ALT levels, and none showed anemia (hemoglobin <11.0 g/dL). They underwent phlebotomy (300–400 mL) either biweekly or monthly until serum ferritin levels were <20 ng/mL. Thereafter, the serum Trx levels and HOMA-IR were compared with baseline values in each

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individual. However, treatments were terminated irrespective of serum ferritin levels when blood hemoglobin concentrations decreased to less than 10 g/dL.

Statistical analysis

The relationships between variables were analyzed using the Spearman's correlation coefficient by rank, and a partial correlation coefficient was calculated to remove the influence of confounding variables. Values after phlebotomy were compared with baseline values using a Wilcoxon rank sum test. All analyses were performed using SPSS software for Windows, version 14.0 (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 was considered significant.

RESULTS

General characteristics of and histological findings in patients

OF THE 309 HCV-infected patients who underwent liver biopsies, 203 patients met the criteria. Because many excessive drinkers among the male patients were excluded from the analysis, the number of females exceeded that of males in the study population. A summary of the clinical data for the liver biopsy findings in these patients is shown in Tables 1 and 2. Of the 203 patients that qualified, body mass index (BMI) was greater than 25 (kg/m^2) in 57 patients (28%), and 124 patients (61%) had a varying degree of hepatic steatosis, as shown in Table 2. Iron staining was performed in only 198 patients; a varying degree of iron loading was observed in 71 patients. Fourteen patients (7%) suffered from type 2 diabetes mellitus. The fibrosis scores of these patients were F1 in two patients, F2 in six, F3 in five, and F4 in only one. Seventy patients had received IFN-based antiviral therapy before the study and this treatment had failed to eradicate HCV.

Table 1 Baseline characteristics of patients

	Mean values of clinical data
Age	56.0 ± 11.9
Male/Female	73/146
BMI (kg/m^2)	22.9 ± 3.1
IFN: yes/no	70/133
ALT (IU/L)	75.5 ± 59.3
γ-GTP (U/L)	56.0 ± 51.0
FPG (mg/dl)	96.8 ± 13.1
HOMA-IR	2.3 ± 1.4
Ferritin (ng/ml)	174.2 ± 161.0
TG (mg/dl)	99.5 ± 50.5
Plt (×10^4/ml)	17.4 ± 5.3
HCV-RNA (KIU/ml)	1516 ± 1484.7
Serogroup 1/2	162/41
Trx (ng/ml)	30.4 ± 15.4

Data are expressed as mean ± standard deviation.
ALT, alanine aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; γ-GTP, γ-glutamylcysteine transpeptidases; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance; IFN, interferon; Plt, platelet; TG, triglyceride; Trx, thioredoxin.

Table 2 Histological findings on liver biopsy

	No. patients
F0/F1/F2/F3/F4	3/72/71/51/6
A0/A1/A2/A3	2/79/89/33
Steatosis:	
None	79
<10%	54
<30%	53
<60%	17
Iron load:	
Grade 0/1/2/3	127/33/25/13

Data are expressed as number of patients.

Predictors of the fibrosis score

The stage was significantly correlated with age, BMI, grade, grades of steatosis, iron score, ALT levels, platelet counts, ferritin levels, HOMA-IR, and serum Trx levels (Table 3). In a multiple regression analysis, grade, HOMA-IR, and serum Trx levels were shown to be

Table 3 Variables correlated with fibrosis scores

	Coefficient	Univariate	Multivariate
Age	$r = 0.163$	$P = 0.019$	$P = 0.931$
BMI	$r = 0.199$	$P = 0.004$	$P = 0.920$
Grade	$r = 0.869$	$P < 0.00001$	$P < 0.00001$
Steatosis	$r = 0.412$	$P < 0.00001$	$P = 0.761$
Iron score	$r = 0.155$	$P = 0.030$	$P = 0.437$
ALT	$r = 0.416$	$P < 0.00001$	$P = 0.259$
Plt	$r = -0.376$	$P < 0.00001$	$P = 0.119$
Ferritin	$r = 0.189$	$P = 0.010$	$P = 0.227$
HOMA-IR	$r = 0.406$	$P < 0.00001$	$P = 0.043$
Trx	$r = 0.365$	$P = 0.00006$	$P = 0.003$

Multiple regression analysis was used to analyze variables independently correlated with fibrosis scores.
ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; Plt, platelet; Trx, thioredoxin.

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independently correlated with stage (Table 3). Although the grade of steatosis is reported to predict rapid fibrosis progression,[5] it was not an independent variable in the multivariate analysis. Considering that IR is a major cause of hepatic steatosis,[11] HOMA-IR should be more significant than steatosis in this model.

Relationship between grades of steatosis and HOMA-IR or serum Trx levels

Steatosis has been considered to independently contribute to the progression of fibrosis in patients with chronic hepatitis C.[5] Therefore, we focused on the relationships between steatosis and either IR or oxidative stress. We found that grades of steatosis were significantly correlated not only with HOMA-IR, but also with serum Trx levels (HOMA-IR; $r = 0.344$, $P = 0.00002$; Trx; $r = 0.3$, $P < 0.001$). These findings suggested that oxidative stress could have a significant role in fibrosis progression through steatogenesis. We then focused on the relationship between IR and oxidative stress.

Relationship between HOMA-IR and serum Trx levels

HOMA-IR was significantly correlated with serum Trx levels (Fig. 1a: $r = 0.262$, $P = 0.012$) and BMI (Fig. 1b:

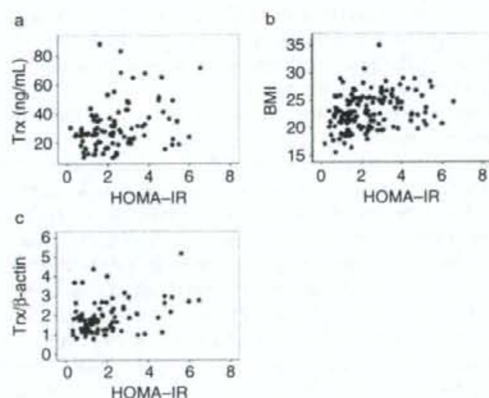

Figure 1 Correlation between homeostasis model assessment-insulin resistance (HOMA-IR) and serum levels of thioredoxin (Trx) (a), body mass index (BMI) (b), and mRNA levels of Trx (c). Both serum Trx levels and BMI were significantly correlated with HOMA-IR (serum Trx levels; $r = 0.262$, $P = 0.012$; BMI; $r = 0.302$, $P = 0.0002$). HOMA-IR was also significantly correlated with hepatic Trx levels ($r = 0.273$, $P = 0.014$).

Table 4 Factors correlated with HOMA-IR in subgroup patients ($n = 101$)

	Coefficient	Univariate	Multivariate
Hepatic Trx	$r = 0.273$	$P = 0.014$	$P = 0.011$
Grade	$r = 0.233$	$P = 0.038$	$P = 0.170$
Steatosis	$r = 0.286$	$P = 0.010$	$P = 0.251$
ALT	$r = 0.287$	$r = 0.010$	$r = 0.517$

Multiple regression analysis was used to analyze variables independently correlated with HOMA-IR.
ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment-insulin resistance; Trx, thioredoxin.

$r = 0.302$, $P = 0.0002$). After adjustment for the effect of each variable using a corrected correlation coefficient, a significant relationship with HOMA-IR still remained for both serum Trx levels ($r = 0.244$, $P = 0.02$) and BMI ($r = 0.284$, $P = 0.006$). These results indicated that IR was attributable to oxidative stress, irrespective of obesity.

Relationship between HOMA-IR and hepatic Trx levels

Since Trx is known to be ubiquitously expressed,[17] we compared the mRNA levels of hepatic Trx with HOMA-IR in 101 patients whose liver biopsy specimens were available. The mRNA levels of Trx were significantly correlated with HOMA-IR (Fig. 1c: $r = 0.273$, $P = 0.014$). Among these patients, HOMA-IR also significantly correlated with grade, steatosis, and ALT levels (Table 4). In a multiple regression analysis, only the level of hepatic Trx was independently correlated with HOMA-IR (Table 4).

Effects of phlebotomy on ALT and serum Trx levels and HOMA-IR

All patients completed treatment without a significant change in body weight (age; 60.8 ± 10.8 kg, male/female; 15/8, BMI; 25.3 ± 2.6 kg/m^2, F0/F1/F2/F3/F4; 3/8/8/4, serogroup 1/2; 20/3). Nine patients had experienced IFN therapy before phlebotomy, whereas 14 patients had not experienced IFN therapy because of either old age or personal reasons. Changes in the serum levels of ALT, Trx, ferritin, HOMA-IR in the 23 patients that received phlebotomy are summarized in Table 5. Overall, the serum levels of ALT, Trx, and HOMA-IR were significantly decreased after phlebotomy compared with baseline values ($P < 0.00001$, $P = 0.023$, $P = 0.022$, respectively). These results indicated the efficacy of phlebotomy on insulin sensitivity as well as on liver function

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Table 5 Changes in ALT, Trx, ferritin, and HOMA-IR after phlebotomy ($n = 23$)

	Before	After	Difference
BMI (kg/m²)	23.6 (19.1–29.4)	24.0 (19.1–29.4)	NS
AST (IU/L)	67.0 (21–527)	51.0 (32–129)	$P < 0.005$
ALT (IU/L)	42.5 (27–121)	29.5 (24–53)	$P < 0.00001$
γ-GTP (IU/L)	89.0 (29–287)	60.5 (23–218)	$P < 0.0005$
Trx (ng/ml)	36.1 (20.2–79.4)	26.7 (18.1–32.7)	$P = 0.023$
Ferritin (ng/ml)	409.5 (125–1028)	20 (20–53)	$P < 0.00001$
HOMA-IR	3.5 (0.9–4.6)	2.4 (0.8–3.7)	$P = 0.022$

Data are expressed as medians (±range). Wilcoxon signed-ranks test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, glutamylcysteine transpeptidases; HOMA-IR, homeostasis model assessment-insulin resistance; Trx, thioredoxin.

tests in chronic hepatitis C patients. Furthermore, we analyzed the effects of phlebotomy on HOMA-IR in patients with a history of past IFN therapy and found that there were significant decreases in HOMA-IR (from 4.2 [3.7–4.6] to 2.9 [2.3–3.7], $P = 0.043$).

DISCUSSION

THE PRESENT STUDY shows that oxidative stress is an independent factor in the development of IR in patients with chronic hepatitis C, and validates the beneficial effect of phlebotomy on insulin sensitivity. To our knowledge, our report is the first to show a direct relationship between IR and oxidative stress in patients with HCV. We excluded alcohol drinkers, patients treated with steatosis-inducing drugs, and patients infected with HCV genotype 3a[18] from our analysis, as these are confounding factors affecting steatosis.

In general, the development of IR and steatosis is due to host-associated factors (e.g. obesity). The molecular mechanism underlying IR involves dysregulation of insulin-stimulated tyrosine phosphorylation of insulin receptor substrates (IRS).[19] This is achieved by phosphorylation of serine/threonine residues in IRS by either increased or decreased levels of adipokines associated with obesity (such as tumor necrosis factor [TNF]-α and adiponectin), thereby inhibiting tyrosine phosphorylation.[19] However, a high prevalence (61%) of steatosis, despite a low prevalence (28%) of obesity (BMI >25 kg/m²) or diabetes (7%), indicated that there are mechanisms regulating insulin sensitivity other than obesity. In our study, HOMA-IR was significantly correlated with serum Trx levels, independent of BMI. Furthermore, the hepatic Trx levels independently predicted HOMA-IR in subgroup patients. Thus, hepatic oxidative stress directly contributes to IR in chronic hepatitis C patients.

Our hypothesis is supported by the following findings. First, chronic hepatitis C is characterized by oxidative stress-induced liver injury.[10,14,20] The overproduction of ROS could result from inflammatory cells,[10] iron overload,[20] and presumably the direct association of HCV core protein with mitochondria in hepatocytes.[21] In addition, steatosis, a prominent feature of chronic hepatitis C,[2–5] could result in oxidative stress.[11] Second, the increased abundance of ROS inhibits tyrosine phosphorylation of IRS in hepatocytes via the activation of stress-sensitive pathways, such as the c-Jun N-terminal kinase (JNK)[12] and nuclear factor (NF)-κB[22] pathways. JNK directly phosphorylates serine/threonine residues in IRS,[12] while NF-κB inhibits its tyrosine phosphorylation via the induction of TNF-α.[22] The failure of hepatic insulin signaling subsequently leads to systemic IR.[12]

The question arising from this correlation between IR and oxidative stress is how metabolic disorders and liver injury can develop simultaneously in patients with HCV infection. One possible mechanism could be an interaction between IR and oxidative stress. IR results in hepatic steatosis,[11] which leads to increased ROS production concomitant with an increase in the number of inflammatory cells[10] and/or iron overload.[23] Conversely, ROS could exacerbate insulin sensitivity to promote steatosis,[11,12] and could promote the recruitment of inflammatory cells and fibrosis through lipid peroxidation products.[24,25] Thus, IR, steatosis, and oxidative stress could be involved in a feedback loop that exacerbates liver injury. This hypothesis is supported by the findings that HOMA-IR was significantly correlated with the serum and hepatic Trx levels, and both the HOMA-IR and serum Trx levels were significantly correlated with grades of steatosis.

Finally, we employed phlebotomy to validate the interaction between IR and oxidative stress, because

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phlebotomy is useful for reducing hepatic oxidative stress.[20] Although phlebotomy is known to improve liver function tests in patients with HCV infection, its efficacy on insulin metabolism has not been well documented. Therefore, our findings provide new insight into the efficacy of phlebotomy. Notably, phlebotomy significantly improved HOMA-IR, even in patients who had been refractory to IFN. However, the long-term outcome of phlebotomy was unclear in this study, and a follow-up study should be performed.

In conclusion, we demonstrated an association between oxidative stress and IR in patients infected with HCV genotype 1 or 2. Our findings will contribute to our understanding of how metabolic disorders can develop in patients with chronic hepatitis C. Antioxidative therapy is a promising treatment to improve the pathogenesis of HCV.

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Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

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Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naive. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum $Hb \leq 8.5$ g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum $Hb \leq 8.5$ g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

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of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm³ and platelets ≥10⁵/mm³ within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Crnn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm³)	5317 ± 1207
Neutrocytes (/mm³)	2778 ± 902
Platelets (×10⁴/mm³)	17.4 ± 4.0
RBC (×10⁴/mm³)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 µg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

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body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = $32.3 \times BW \times (1 - 0.0094 \times age) \times (1 - 0.42 \times sex)/Scr$ (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel–Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

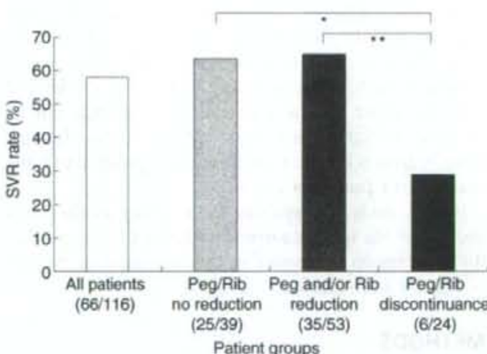

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (▩), patients without dose reduction; (■), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: $^*P = 0.003$; $^{**}P = 0.001$.

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Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
$20 \le CL/F$ ($n = 12$)	67% (8/12)	25% (3/12)	8% (1/12)	0
$15 \le CL/F < 20$ ($n = 23$)	57% (13/23)	30% (7/23)	13% (3/23)	0
$10 \le CL/F < 15$ ($n = 39$)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
$CL/F < 10$ ($n = 42$)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

$P = 0.031$ (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
$20 \le CL/F$ ($n = 12$)	92% (11/12)	12% (1/12)	0
$15 \le CL/F < 20$ ($n = 23$)	83% (19/23)	17% (4/23)	0
$10 \le CL/F < 15$ ($n = 39$)	72% (28/39)	23% (9/39)	5% (2/39)
$CL/F < 10$ ($n = 42$)	50% (21/42)	43% (18/42)	7% (3/42)

$P = 0.009$ (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs ($P = 0.003$), and the group with reduction of PegIFN and/or ribavirin ($P = 0.001$).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the $CL/F \ge 20$, 13% (3/23) for the $15 \le CL/F < 20$, 23% (9/39) for the $10 \le CL/F < 15$, and 26% (11/42) for the $CL/F < 10$ group. Ribavirin did not have to be discontinued due to severe anemia among patients with $15 \le CL/F$, but did for the 18% (2/11) of those with $CL/F < 10$ and 22% (2/9) of those with $10 \le CL/F < 15$. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of $CL/F < 10$, and 5% (2/39) of the group of $10 \le CL/F < 15$. No patients of the group of $CL/F \ge 15$ showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four-weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin ($n = 53$), 1.6 ± 1.2 g/dL among those with dose reduction ($n = 39$), and 1.8 ± 1.0 g/dL among

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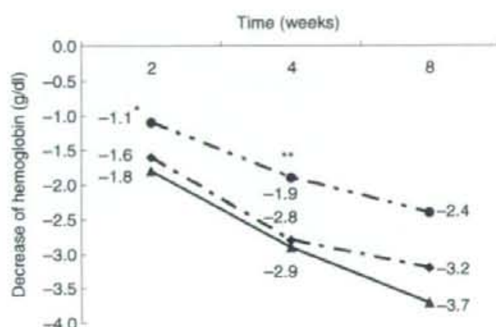

Figure 2 Course of ΔHb in the initial phase. (—···—), No reduction; (—·—·), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).

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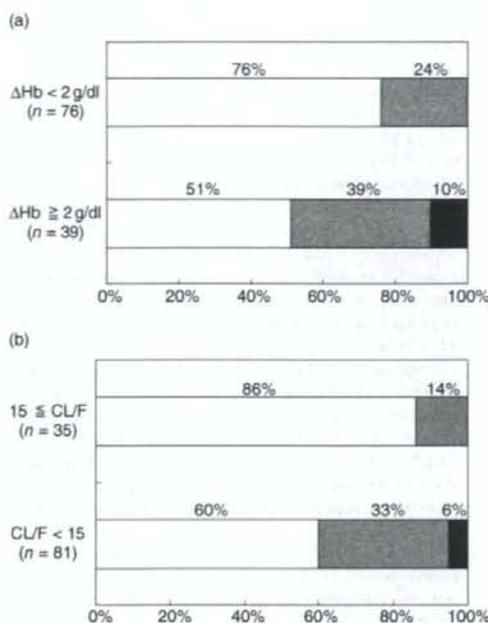

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (■), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dL decrease at two weeks from the baseline). $P = 0.009$ (Mantel–Haenszel χ^2-test); (b) according to CL/F levels. $P = 0.001$ (Mantel–Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL (n = 76)	ΔHb ≥ 2 g/dL (n = 39)
CL/F ≥ 15 (n = 35)	0/25	0/10
CL/F < 15 (n = 80)	0/51	4/29 (14%)

progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients (n = 5) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

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around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

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Original Article

Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet counts

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Aim: We aimed to identify the candidates for antiviral therapy, among patients who are hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT), focused on the inhibition of hepatocellular carcinoma (HCC).

Methods: Four hundred and sixty-four HCV carriers with normal serum ALT and 129 HCV carriers with persistently normal ALT (PNALT) and platelet (PLT) counts ≥150 000/µL who received liver biopsies were enrolled. HCV carriers with normal serum ALT were divided into four groups according to their ALT levels (≤30 U/L or 31–40 U/L) and PLT counts (≥150 000/µL or <150 000/µL).

Results: In 129 HCV carriers with PNALT, the rate of progression of fibrosis stage was 0.05/year and no HCC was detected during the follow up for 10 years. Approximately 20% of patients with ALT ≤40 U/L and PLT counts ≥150 000/µL were at stage F2–3; however, approximately 50% of patients with ALT ≤ 40 U/L and PLT counts <150 000/µL were at stage F2–4. An algorithm for the management of HCV carriers with normal serum ALT was advocated based on ALT and PLT counts.

Conclusion: The combination of ALT and PLT counts is useful for evaluating the fibrosis stage in HCV carriers with normal serum ALT. Most patients with PLT counts <150 000/µL are candidates for antiviral therapy, especially those with ALT levels ≥31 U/L when we focus on the inhibition of the development of HCC.

Key words: antiviral therapy, chronic hepatitis C, hepatitis C virus carriers, normal serum aminotransferase, platelet count

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) caused by hepatitis C virus (HCV) infection usually develops in patients with advanced chronic hepatitis (CH) or liver cirrhosis. The antiviral treatment for chronic hepatitis C (CH-C) is useful for inhibiting hepatic inflammation and progression of hepatic fibrosis, and consequently the development of HCC.[1–6]

Serum aminotransferase (ALT) levels are within the normal ranges in 20–40% of patients with chronic HCV infection,[7–11] defining the upper limit of normal serum ALT as ≤40 U/L. Significant hepatic fibrosis (≥F2 by the METAVIR classification) has been demonstrated in 5–30% of such patients.[9,12–16] We reported previously

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that HCV carriers with persistently normal ALT (PNALT) had histological features ranging from normal to minimal CH[17,18]; they showed slow progression of liver fibrosis and were at very low risk of developing HCC.[18]

The National Institute of Health Consensus Development Conference reported that HCV carriers with normal serum ALT are candidates for antiviral therapy.[19] A controlled study for the treatment of HCV carriers with PNALT with pegylated interferon alpha and ribavirin (PEG-IFN/Riba) for 48 weeks led to the eradication of HCV RNA in 40% of patients with genotype 1 and high viral load,[20] which is similar to the results of CH-C patients with elevated ALT levels.[21,22] However, it remains controversial whether these patients are candidates for antiviral therapy because of the limited efficacy of treatment, post-treatment flare-up, various side-effects, high cost of treatment, and their good prognoses.

In many Western countries, the upper limits of normal serum ALT are below 40 U/L;[23] however, a recent report from Italy demonstrated that the upper limit in healthy individuals was less than 30 U/L for men and 19 U/L for women.[24] We attempted to draft therapeutic guidelines for the treatment of HCV carriers with normal serum ALT. The biochemical and histological analyses were performed in HCV carriers with serum ALT levels below 40 U/L. These patients were divided into two groups based on ALT levels and then further divided into two subgroups according to their platelet (PLT) counts. We proposed an algorithm for the treatment of HCV carriers with normal serum ALT, taking into consideration the risk of progression to cirrhosis and the development of HCC. The present study demonstrated that the ranges of serum ALT and PLT counts are useful for deciding the indication of antiviral therapy for HCV carriers with normal serum ALT.

METHODS

Eligibility and definition

TWELVE HEPATOLOGISTS BELONGING to the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis, supported by the Ministry of Health, Labour and Welfare of Japan, which was settled on April 2004, participated in the study. Hiromitsu Kumada (Toranomon Hospital, Tokyo, Japan) serves as a chief and Takeshi Okanoue served as a researcher responsible for drafting the guidelines for the treatment of HCV carriers with normal serum ALT. In the present study, we tentatively defined the upper limit of the normal serum ALT as ≤40 U/L.

Patients with hepatitis B virus surface antigen, previous IFN treatment, history of heavy alcohol abuse, antinuclear antibody or antismooth muscle antibody, overt diabetes mellitus, or obesity (body mass index; ≥25 kg/m^2) were excluded from the study.

All of the patients underwent liver biopsy (≥2.0 cm in length) within 6 months prior to antiviral therapy, at which time their serum ALT levels were ≤40 U/L. Informed consent was obtained from every patient prior to liver biopsy and antiviral therapy.

Another study was conducted from January 1990 to August 2004 at Kyoto Prefectural University of Medicine (Kyoto, Japan). HCV carriers with PNALT were defined by serum ALT levels ≤30 U/L on at least three different occasions over a 12-month period and PLT counts ≥150 000/μL as reported previously.[18]

Study design

Among the 580 HCV carriers with normal serum ALT (≤40 U/L), 116 patients were excluded from the study because of insufficient data. Thus, 464 patients who received antiviral therapy from 1995 to 2004 were enrolled in this study (Table 1). Formalin-fixed liver specimens were stained with hematoxylin–eosin, and with Masson's trichrome. The liver specimens ($n = 262$) were also stained with Perls' Prussian blue to study hepatic iron loading. The histological findings were scored according to the classification proposed by Desmet *et al.*[25] and Ishak *et al.*[26] Steatosis was defined as fat droplets in >10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0–4+, based on the scoring system of MacSween *et al.*[27]

The serum ALT, blood glucose level, immunoreactive insulin (IRI), serum ferritin, PLT count, serum hyaluronic acid, amount of serum HCV RNA, and the HCV genotype were examined. The homeostasis model assessment–insulin resistance was calculated as follows: plasma fasting glucose (mg/dL) × IRI (ng/mL) ÷ 405. The serum HCV RNA levels were determined using an Amplicor GT HCV monitor (Roche Diagnostic Systems, Tokyo, Japan). HCV genotype 1 (G1) and 2 (G2) were determined by a serologic genotyping assay.[28] G1 and G2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds *et al.*[29]

All the patients received IFN monotherapy or IFN/Riba combination therapy for 12–36 weeks. The average

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