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Value of the Apparent Diffusion Coefficient for Quantification of Low-Grade Hepatic Encephalopathy

Ryosuke Sugimoto,[1] Motoh Iwasa, M.D., Ph.D.,[1] Masayuki Maeda,[2] Naohito Urawa,[1] Hideaki Tanaka,[1] Naoki Fujita,[1] Yoshinao Kobayashi,[1] Kan Takeda,[2] Masahiko Kaito,[1] and Yoshiyuki Takei[1]

[1] Department of Gastroenterology and Hepatology, Division of Clinical Medicine and Biomedical Sciences, Institute of Medical Science, Mie University Graduate School of Medicine, Tsu, Japan; and [2] Department of Radiology, Mie University School of Medicine, Tsu, Japan

BACKGROUND AND AIMS: Minimal hepatic encephalopathy (HE) is associated with poorer quality of life and increased work disability. Recently, low-grade cerebral edema has been implicated in chronic liver disease.

METHODS: We measured the apparent diffusion coefficient (ADC) of water in various regions of the brains of patients with cirrhosis, and elucidated the significance of the evaluation of ADC in quantifying low-grade HE and predicting overt HE and survival. Forty patients with cirrhosis and 24 controls underwent diffusion-weighted imaging, and patients were followed up every month.

RESULTS: The mean ADC values were increased in cirrhotic patients with minimal HE *versus* no HE or controls. Minimal HE patients separated from no HE patients with a sensitivity of 70~90%, and a specificity of 85~90%. ADC values correlated with individual neuropsychological tests. ADC values of white matter, such as the frontal (log-rank test 4.35, $P < 0.05$) and parietal (log-rank test 5.98, $P < 0.05$) white matter, was predictive of further bouts of overt HE.

CONCLUSIONS: ADC is a reliable tool for quantification of low-grade HE, and could predict the development of overt HE.

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INTRODUCTION

Some cirrhotic patients with apparently normal mental status may be found to have abnormalities in cognitive function when they are examined using neuropsychological tests (1, 2). This group of patients is considered to have minimal hepatic encephalopathy (HE) (1, 2). This HE-associated cognitive impairment, sometimes, may be associated with poor quality of life (3, 4), and individuals may become unfit to drive a motor vehicle safely (5). Therefore, early diagnosis and treatment of this condition is important. A combination of neuropsychological tests is generally required to diagnose this minimal HE because the results of neuropsychological tests are influenced by aging, education, and repetition of tests (2, 6). However, there is not yet a consensus on the most appropriate test that should be used in clinical practice.

Recently, low-grade cerebral edema has been implicated in chronic liver disease (7–9). Magnetic resonance (MR) imaging studies, including MR spectroscopy (10, 11), magnetization transfer (12, 13), and diffusion-weighted imaging (7–9), have improved our understanding of the pathophysiological alterations in patients with HE. We reported previously that magnetization transfer imaging is able to detect decreased magnetization transfer ratios in all regions of the brains of pa-

tients with chronic HE (13). The likely explanation proposed is Alzheimer type II change and increased water content in astrocytes (13). In HE, an increase in glutamine and a decrease in myoinositol are observed on MR spectroscopy (10). The changes in the myoinositol are considered suggestive of cellular edema. Use of diffusion-weighted imaging allows assessment of intracellular and extracellular water content in the brain. Lodi et al. studied patients with advanced cirrhosis and HE using diffusion-weighted imaging, and reported increased diffusivity of water in brain parenchyma (7). Recently, Kale et al. performed diffusion-weighted imaging in patients with cirrhosis, with and without HE, and concluded that early HE indicates the presence of interstitial brain edema (8).

The pathogenesis of HE in liver cirrhosis is widely accepted to be due to the failure of the liver to clear toxic products from the gut. The precise toxins involved remain controversial, but ammonia is thought to be an important factor. However, the correlation between plasma ammonia levels and severity of HE is not consistent (14).

Here, we performed diffusion MR imaging in patients with cirrhosis and who did not exhibit overt HE to look for possible microstructural changes in the brain, as suggested by measuring apparent diffusion coefficient (ADC) values. Then, we compared the results with plasma ammonia levels and the

Table 1. Subjects' Clinical Characteristics

Liver cirrhosis	40
Age (yr)	66 ± 9
Sex ratio, m/f	20/20
Etiology of cirrhosis HBV/HCV/alcohol/PBC	3/30/5/2
Previous history of overt HE Chronic/none	3/37
Child-Pugh A/B/C	12/25/3
Laboratory examination	
Plasma ammonia (μmol/L)	43 ± 33
BTR	4.5 ± 2.3
Neuropsychological test	
Trail-making A test (s)	56 ± 33
Digit symbol test (gross point)	36 ± 13
Normal subject	24
Age (yr)	62 ± 9
Sex ratio, m/f	11/13

HBV = hepatitis B virus; HCV = hepatitis C virus; PBC = primary biliary cirrhosis; HE = hepatic encephalopathy; BTR = branched-chain amino acids to tyrosine ratio.

results of neuropsychological tests to elucidate the significance of the evaluation of ADC in quantifying low-grade HE and predicting overt HE and survival.

METHODS

Patients

This study comprised 40 patients with liver cirrhosis (20 men and 20 women, average age 66 ± 9 yr). Cirrhosis was diagnosed by liver biopsy or the presence of biochemical, ultrasonographic, or endoscopic features of portal hypertension and/or liver dysfunction. The etiology of liver cirrhosis was as follows: hepatitis B virus, 3 cases; hepatitis C virus, 30 cases; alcohol, 5 cases; and primary biliary cirrhosis, 2 cases. The severity of liver disease was determined according to the Child-Pugh score. Cirrhosis was graded Child-Pugh A in 12 patients, B in 25, and C in 3. Three cases had a previous history of overt HE. Patients with alcohol-induced liver disease had to be abstinent for at least 3 months prior to the start of the study. The control group consisted of 24 subjects

(11 men and 13 women, average age 62 ± 9 yr), who were examined at our neurological department for minor subjective symptoms: they were free of liver diseases and neurological or psychiatric disorders. Control subjects did not undergo neuropsychological tests. The clinical findings of the patients with liver cirrhosis are summarized in Table 1. Written informed consent was obtained from all subjects, and the study was performed in accordance with the Helsinki Declaration.

Neuropsychological Tests and Laboratory Examinations

Cognitive function was assessed using a combination of trail-making A test (number connection test) and digit symbol test (revised Wechsler adult intelligence scale) that is widely employed as screening examination in hepatology clinics in Japan. Neuropsychological measurements were performed on the same day between 9 am and 5 pm in a quiet room with constant light quality. After explanation of each neuropsychological test, an abbreviated demonstration was carried out to ensure that the patient understood the test correctly. Patients likely to have difficulties performing the neuropsychological tests, such as those with bad vision, were excluded from this study. Peripheral venous blood was collected after overnight fasting. Laboratory examinations included plasma ammonia and the branched-chain amino acids to tyrosine ratio (BTR).

MR Imaging

MR imaging was performed using 1.5-T (Signa, CV/I; GE Medical Systems, Milwaukee, WI). Diffusion-weighted imaging was conducted using single-shot echo-planar imaging (TR/TE, 9999/70 ms; 1 acquisition; 20 sections of 5-mm thickness; 1-mm gap, the matrix of 128 × 128). A neuroradiologist reviewed the MR images of each subject to confirm the absence of major neuropathologies, such as tumors and infarctions. Cases with remarkable brain atrophy and foci of cerebral infarction were excluded from the study. Figure 1 shows the regions of interest used for ADC calculation: left and right putamen, pallidus and thalamus, and posterior cingulate gray matter and frontal and parietal white matter.
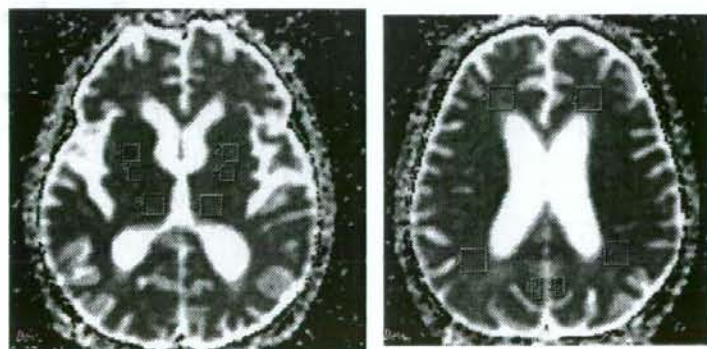

Figure 1. The regions of interest were selected on the diffusion-weighted image. 1, 2 = putamen; 3, 4 = pallidus; 5, 6 = thalamus; 7, 8 = frontal white matter; 9, 10 = parietal white matter; and 11, 12 = cingulate gray matter.

Follow-Up

Each patient was followed up every month at our outpatient clinic until he/she developed an episode of overt HE, had a liver transplant, died, or was observed to be free of any of the events at the end of the study. The patients were assessed for neuropsychiatric symptoms, including alterations in behavior, mood and orientation, and flapping tremor.

Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). The χ^2 test was used to assess differences between qualitative variables. Spearman's or Pearson's coefficients were used to compare quantitative variables. The Mann-Whitney U-test was used to evaluate the statistical difference in clinical or laboratory variables. The analysis of variance was determined with Scheffe's F test for multiple comparisons in the three groups. Kaplan-Meier analysis was used for variables associated with overt HE risk and survival. A P value of < 0.05 was considered as statistically significant.

RESULTS

Assessment of HE Severity

None of the patients showed signs of overt HE; all were alert, without flapping tremor, and were oriented in space, person, and time at the time of their examinations. Liver cirrhosis patients were categorized into two groups based on the result of the neuropsychological tests. Abnormalities in the results of both neuropsychological tests (values of more than 2SD from the mean values for the age-matched control subjects at our hospital, *i.e.*, more than 50 s on the trail-making A test, and less than 30 points on the digit symbol test [gross points]) were considered to be indicative of minimal HE. The patients were considered to have no HE when both tests were normal. Among the 40 cirrhotic patients, 10 showed impairment in both neuropsychological tests, and therefore were included in the minimal HE group. Table 2 summarizes the results of laboratory and neuropsychological tests for both groups. There was no difference in age between the group with minimal HE and that with no HE.

Diagnosis of Minimal HE Using ADC and Factors Associated With ADC

ADC values were similar in corresponding right and left hemispherical regions of interest in the subjects, and therefore are reported as mean values. In cirrhotic patients with minimal HE, mean ADC values were increased significantly in white matter, such as the frontal ($P < 0.01$) and parietal ($P < 0.05$) lobes, compared with patients with no HE (Fig. 2). In patients with minimal HE, the ADC increase did not reach significance in the putamen, pallidus, thalamus, or cingulate. No significant difference was found in brain ADC values between patients with no HE and healthy subjects (Fig. 2). There were no differences in the ADC in patients with prior overt HE compared with that in the others.

Table 2. Assessment of HE Severity

	Minimal HE (N = 10)	No HE (N = 20)
Age (yr)	70 ± 6	63 ± 8
Sex ratio, m/f	7/3	8/12
Etiology of cirrhosis HBV/HCV/alcohol/PBC	1/7/2/0	1/16/2/1
Previous history of overt HE Chronic/none	2/8	1/19
Child-Pugh A/B/C	1/8/1	8/10/2
Laboratory examination		
Plasma ammonia (μmol/L)	57 ± 49	37 ± 23
BTR	3.5 ± 1.2	5.0 ± 2.7
Neuropsychological test		
Trail-making A test (s)	99 ± 40*	37 ± 9
Digit symbol test (gross point	21 ± 7*	44 ± 9

*$P < 0.001$.

HE = hepatic encephalopathy; HBV = hepatitis B virus; HCV = hepatitis C virus; PBC = primary biliary cirrhosis; BTR = branched-chain amino acids to tyrosine ratio.

There is a degree of overlap when classifying patients without overt HE as minimal HE or no HE. Using the cutoff value of 0.841×10^{-3} mm²/s for frontal white matter (+1SD from the mean values for the control subjects), minimal HE patients separated from no HE patients with a sensitivity of 90%, and a specificity of 90%. Using the cutoff value of 0.808×10^{-3} mm²/s for parietal white matter (+1SD from the mean values for the control subjects), minimal HE patients separated from no HE patients with a sensitivity of 70%, and a specificity of 85%. With regard to plasma ammonia levels, using the cutoff level of 40 μmol/L (upper limit at our hospital), minimal HE patients separated from no HE patients with a sensitivity of 50%, and a specificity of 40%.

In the patients, venous ammonia showed a linear relationship with the ADC values in the frontal ($r = 0.413, P < 0.05$) and parietal ($r = 0.537, P < 0.001$) white matter, whereas it failed to reach significance in the putamen, pallidus, thalamus, and cingulate (Table 3). No correlation was found between ADC values for the various brain areas and the Child-Pugh scores or the serum BTR values (Table 3). We found significant correlation of ADC values for frontal and parietal white matter with the results of trail-making A test in the patients ($r = 0.520, P < 0.01; r = 0.483, P < 0.01$, respectively) (Table 4). A significant correlation was found between ADC values for frontal and parietal white matter and the results of the digit symbol test ($r = -0.510, P < 0.01; r = -0.354, P < 0.05$). No correlation was found between ADC values in the various brain areas, except for white matter, and the results of neuropsychological tests (Table 4).

ADC in the Prediction of the Risk of Developing Overt HE and Survival

Time zero was that of the initial neuropsychological and MR assessments, and the end points were overt HE, liver transplantation, or death. The median follow-up period was 11 months. During the period of observation, two patients underwent liver transplantation and three died from liver-related
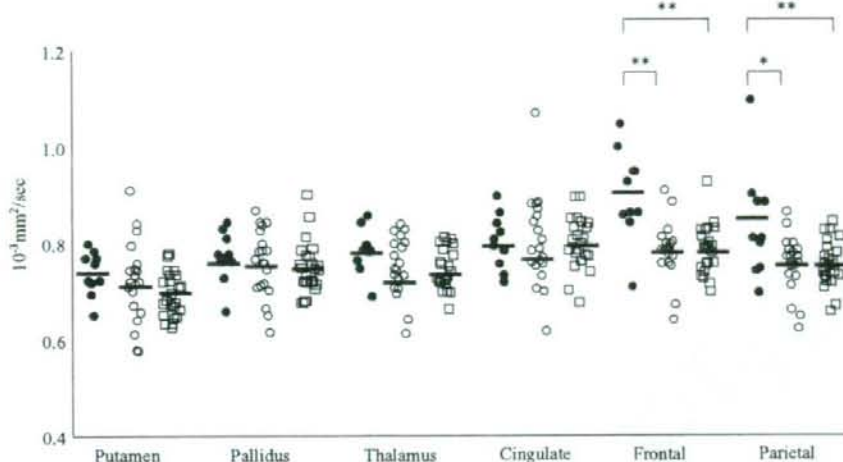
Figure 2. ADCs obtained in 24 control subjects and 40 cirrhotic patients, depending on with and without minimal HE. All patients underwent neuropsychological tests, and were classified to have no HE or minimal HE. ● = minimal HE; ○ = no HE; □ = control subjects. ADC = apparent diffusion coefficient; HE = hepatic encephalopathy.

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complications. In all, 10 patients exhibited an episode of overt HE during follow-up. Seven of the 10 who developed overt HE showed minimal HE defined using ADC values of frontal white matter in the initial assessments, whereas the other three patients showed no HE according to the ADC values. Similar results were obtained using ADC values of parietal white matter. The related factors of those episodes were as

follows: diuretic treatment in three cases, spontaneous bacterial peritonitis in two cases, and digestive hemorrhage in one case. In the nonencephalopathic group (N = 25), 10 subjects developed ascites, and were treated with diuretics, and one developed variceal bleeding. There was no statistically significant difference between groups in the incidence of complications associated with development of overt HE. According to Kaplan-Meier analysis, ADC values of white matter, such as the frontal (log-rank test 4.35, $P < 0.05$) and parietal (log-rank test 5.98, $P < 0.05$) white matter, were associated with a higher risk of overt HE (Fig. 3).

After an episode of overt HE, seven of 10 patients died from liver-related complications. Figure 4 shows the actual

Table 3. Factors Associated With ADC

	r	P
Putamen		
Child-Pugh score	0.06	NS
Ammonia	0.179	NS
BTR	−0.241	NS
Pallidus		
Child-Pugh score	0.181	NS
Ammonia	0.249	NS
BTR	−0.123	NS
Thalamus		
Child-Pugh score	0.024	NS
Ammonia	0.201	NS
BTR	−0.158	NS
Cingulate		
Child-Pugh score	0.003	NS
Ammonia	0.013	NS
BTR	−0.090	NS
Frontal		
Child-Pugh score	0.142	NS
Ammonia	0.413	0.014
BTR	−0.239	NS
Parietal		
Child-Pugh score	0.057	NS
Ammonia	0.537	0.001
BTR	−0.341	NS

ADC = apparent diffusion coefficient; BTR = branched-chain amino acids to tyrosine ratio.

Table 4. Factors Associated With ADC

|	r	P
Putamen		
Trail-making A test	0.027	NS
Digit symbol test	−0.137	NS
Pallidus		
Trail-making A test	0.000	NS
Digit symbol test	−0.125	NS
Thalamus		
Trail-making A test	0.088	NS
Digit symbol test	−0.222	NS
Cingulate		
Trail-making A test	0.154	NS
Digit symbol test	−0.025	NS
Frontal		
Trail-making A test	0.520	0.001
Digit symbol test	−0.510	0.002
Parietal		
Trail-making A test	0.483	0.003
Digit symbol test	−0.354	0.037

ADC = apparent diffusion coefficient.
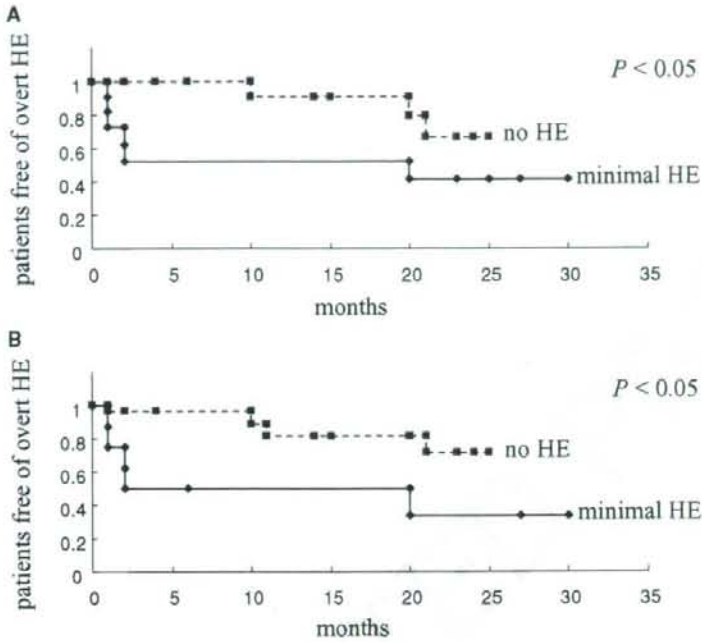
Figure 3. Prediction of overt HE by ADC in patients with cirrhosis. (*A*) frontal and (*B*) parietal. HE = hepatic encephalopathy; ADC = apparent diffusion coefficient.
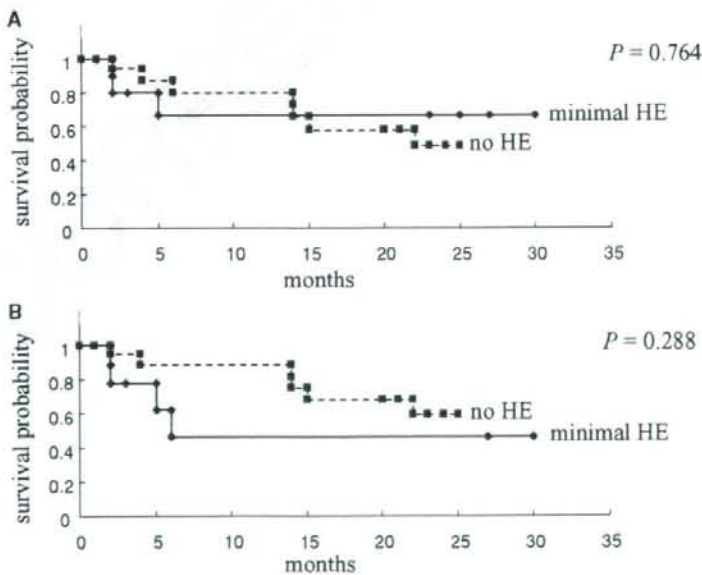

Figure 4. Prediction of survival by ADC in patients with cirrhosis. (*A*) frontal and (*B*) parietal. HE = hepatic encephalopathy; ADC = apparent diffusion coefficient.

survival of patients with minimal HE *versus* patients with no HE; no significant difference was found in frontal (Panel *A*; log-rank test 0.09, $P = 0.764$) and parietal (Panel *B*; log-rank test 1.13, $P = 0.288$) ADC.

DISCUSSION

The present study reveals that ADC was increased in patients with minimal HE compared with patients with no HE. This finding is consistent with the pattern of diffusion-weighted imaging as reported previously (7–9). Lodi *et al.* noted that the highest ADC values were observed in all brain regions of a single patient with grade 2 HE, and were higher than values in grades 0 and 1 HE (7). Kale *et al.* found increased mean diffusivity values in all brain regions in patients with different grades of HE (8). Minimal cellular edema with increases in membrane permeability and intracellular diffusivity, as well as changes in the viscosity of the cytoplasm, may be considered to be factors leading toward an increase in ADC. However, this explanation contradicts the basic understanding of diffusivity. Kale *et al.* reported that increased ADC values with no concomitant changes in fractional anisotropy suggest an increase in the interstitial brain water in patients with HE (8).

One of the mechanisms underlying this low-grade cerebral edema may involve astrocyte function. Ammonia detoxification takes place in astrocytes through glutamine synthesis, and an increase in intracellular glutamine results in intracellular depletion of myoinositol, following an attempt at osmoregulation by the astrocytes (15). However, the precise reason for increased extracellular fluid in HE is unknown. The positive correlation we found between plasma ammonia levels and ADC values in the white matter indicates that ADC changes in patients with minimal HE are, at least in part, attributable to ammonia-mediated low-grade brain edema. However, not only ammonia, but also benzodiazepines, hyponatremia, and cytokines can induce low-grade brain edema (16). Indeed, we found in this study that ADC is a sensitive parameter for diagnosis of minimal HE in cirrhotic patients compared with venous ammonia levels.

Although neuropsychological tests can be used for diagnosis of minimal HE, their value in clinical routine is limited because of methodological problems, age, education, and training effects, as well as lack of standardization (2, 6). We used validated neuropsychological tests with normal values at our hospital. Using this definition, we found a minimal HE prevalence of 25% in our outpatient cirrhotic population, which is in agreement with the prevalence found in other studies using the same methods (6). The neuropsychological HE score includes a battery of five neuropsychological tests that has been found useful for the diagnosis of minimal HE. In a recent consensus meeting, this score was recommended as the gold standard for the diagnosis of minimal HE (2). The weakness of this tool is the need for data on large distribu-

tions of subjects, adjusted for education level and age, and it is not adopted in our unit at present. Neuropsychological tools, such as electroencephalography, exogenously-evoked potentials, and endogenous P300 wave, have been used for the diagnosis of minimal HE (6, 17). However, these tools have lower sensitivity than that of neuropsychological tests. ADC, on the other hand, is a reproducible parameter with no bias for education and training effects.

In this study, we selected cingulate gray matter as the region of interest for analysis, because it has been reported that metabolism and blood flow in the cerebral limbic system, especially in the anterior cingulate gyrus, are closely associated with the findings of neuropsychological tests, as demonstrated by changes in glucose metabolism measured by positron emission tomography or single photon emission tomography (18–20). In the current study, however, we did not evaluate ADC of the anterior cingulated gyrus, but that of the posterior cingulate gyrus, because echo-planar diffusion-weighted imaging does not permit precise evaluation of the anterior cingulate gyrus because of susceptibility artifacts from sinus air. We found that no significant changes in ADC values of the posterior cingulate gray matter were observed in patients with minimal HE. Generally, our observations of altered ADC values were more prominent in white matter than those in gray matter. Lodi *et al.* noted significant elevation in ADC values in all the regions, including the putamen and pallidus, in patients with advanced cirrhosis and HE (7). These findings suggest that, with increasing grade of HE, brain edema increases and progressively affects the gray matter. Another explanation for unaltered ADC values in cingulate gray matter might be the limitation of resolution at 1.5 T. The region of interest in the posterior cingulate gyrus is smaller than that of the other brain areas measured; this suggests that the measurement of the ADC might be affected by the lower resolution at 1.5 T. In addition, the partial volume effect from cerebrospinal fluid might influence the ADC measurement in the posterior cingulate gyrus, although special care was taken to select the region of interest, avoiding the partial volume effect. Those limitations might affect the results of the ADC measurements in the posterior cingulate gyrus.

In this study, we were unable to perform MR imaging repeatedly. Longitudinal MR imaging studies performed after normalization of liver function and improvement in neurological status might provide evidence to support the hypothesis that an increase in the ADC reflects potentially reversible low-grade cerebral edema. Such studies would add to our knowledge of the pathophysiology of HE, and evaluate the potential use of ADC measurements as a surrogate marker for assessing therapy in minimal HE. Further studies should be undertaken in the future.

The relationship between minimal HE diagnosis and development of overt HE is very important because the first episode of HE is associated with a short survival (21). In our study, the survival rate was only 30% after overt HE. However,

the relationship between minimal HE and further overt HE has not been well studied. Romero-Gómez *et al.* found that minimal HE (defined on the basis of number connection test or auditory-evoked potential alteration) could predict a subsequent episode of overt HE (22). We found that ADC values were associated with the development of overt HE in long-term follow-up. Limitations include the small sample size, but despite this limitation, we conclude that diffusion-weighted imaging is well suited to predict the development of overt HE. In addition, ADC measurements may be an objective parameter for assessment of therapeutic regimens. Treatment of minimal HE is not recommended at this time because there are no studies showing a positive influence of treatment on quality of life, driving ability, or prevention of overt HE. Further interventional studies are needed to determine whether treatment could change the natural history of this disease entity.

In summary, ADC is a reliable tool for quantification of low-grade HE. A value of ADC above the cutoff of 0.841×10^{-3} mm^2/s for frontal, or 0.808×10^{-3} mm^2/s for parietal white matter defines a risk factor for overt HE.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Minimal hepatic encephalopathy (HE) is associated with poorer quality of life and increased work disability.
- Although neuropsychological tests can be used for diagnosis of minimal HE, their value in clinical routine is limited.
- Low-grade cerebral edema has been implicated in chronic liver disease.

What Is New Here

- The apparent diffusion coefficient (ADC) values were increased in cirrhotic patients with minimal HE *versus* no HE or controls.
- ADC is a reliable tool for quantification of low-grade cerebral edema, and could predict the development of overt HE.

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CONFLICT OF INTEREST

Guarantor of the article: Motoh Iwasa, M.D., Ph.D.

Patients with chronic hepatitis C achieving a sustained virological response to peginterferon and ribavirin therapy recover from impaired hepcidin secretion ✩

Naoki Fujita[1],*, Ryosuke Sugimoto[1], Satoshi Motonishi[2,3], Naohisa Tomosugi[2], Hideaki Tanaka[1], Masaki Takeo[1], Motoh Iwasa[1], Yoshinao Kobayashi[1], Hisao Hayashi[3], Masahiko Kaito[1], Yoshiyuki Takei[1]

[1]*Department of Gastroenterology and Hepatology, Division of Clinical Medicine and Biomedical Science, Institute of Medical Sciences, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan*
[2]*Division of Clinical Science, Medical Research Institute, Kanazawa Medical University, Kanzawa, Japan*
[3]*School of Pharmacy, Aichi Gakuin University, Aichi, Japan*

(See Editorial, pages 680–685)

Background/Aims: The aim of this study is to determine the clinical relevance of hepatic producing iron regulatory hormone-hepcidin, on iron overload in patients with chronic hepatitis C (CHC).

Methods: Serum hepcidin was measured in 73 CHC patients by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS), and compared to those of healthy controls and anemia of inflammation patients, and analyzed their relationship to hepatic hepcidin mRNA expression levels and clinical, hematological, and histological findings. The sequential changes of hepcidin were investigated in 27 CHC patients treated with a 48 week-course of pegylated-interferon (PEG-IFN) plus ribavirin therapy.

Results: Serum hepcidin was positively correlated with hepatic hepcidin mRNA levels, serum ferritin and the degree of hepatic iron deposition in CHC. Serum hepcidin-to-ferritin ratios were significantly lower in HCV positive patients than in HCV negative controls in both hyper- and normal-ferritinemic conditions. This relative impairment of hepcidin production was fully reversible after successful HCV eradication by PEG-IFN plus ribavirin, concomitantly with the improvement of the iron overload condition.

Conclusions: The impairment of hepatic hepcidin production occurring with chronic HCV infection may enhance iron toxicity and lead to disease progression, and modulation or supplementation of hepcidin may be beneficial for these conditions in CHC.

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1. Introduction

Hepatitis C virus (HCV) infection is a major global health problem, affecting more than 170 million people worldwide [1]. Although the causative factor responsible for the initiation of hepatic disease processes in patients with chronic hepatitis C (CHC) is a single insult, i.e. HCV infection, it has become increasingly evident that the involvement of cofactors is critical in determining disease progression. Importantly, CHC often appears to be associated with disturbances in iron homeostasis, with serum ferritin and hepatic iron stores being elevated in approximately 50% of patients [2–4]. Because iron is a redox-active metal, catalyzing free radical reactions via the Fenton reaction, and because there is substantial evidence to implicate redox active mechanisms in the pathogenesis of CHC [5], iron has been highlighted as an important element affecting the natural history of CHC. However, little is known about the mechanism of excess iron accumulation during chronic HCV infection.

Recently, hepcidin, a 25 amino acid antimicrobial peptide hormone [6,7], has been shown to play a central role in the regulation of iron homeostasis [8]. Hepcidin is mainly secreted by the liver [6,7] and induces internalization and degradation of the iron exporter ferroportin in absorptive enterocytes and reticuloendothelial cells, thereby inhibiting iron absorption from the intestine and iron release from reticuloendothelial cell stores [9]. Hepcidin expression is induced by iron loading, thus providing a feedback mechanism to limit further iron absorption [6,8]. Hepcidin is also recognized as a key mediator of anemia of inflammation (AI) [10]; characterized by impaired intestinal iron absorption, reticuloendothelial cell iron sequestration, and hypo-ferremia and hyper-ferritinemia [11].

Previously, using liver biopsy samples, we demonstrated that hepatic hepcidin mRNA expression levels were relatively low in CHC, compared to chronic hepatitis B [12], suggesting that hepcidin may be involved in the iron overload in chronic HCV infection. But liver biopsy cannot be performed ethically on patients without liver disease, and cannot be carried out repeatedly, even on CHC patients. Thus, comparison of hepatic mRNA expression levels of hepcidin between HCV positive and negative cases, and evaluation of sequential changes after HCV eradication by interferon (IFN)-based therapy, cannot be determined. Recently two reports described the use of surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) for the semiquantitative determination of hepcidin in human serum [13,14]. Therefore, using this assay, we measured the serum hepcidin levels in patients with CHC and compared them to normal subjects and patients with AI, as the normal and positive controls for serum hepcidin. The sequential changes of serum hepcidin also were investigated in patients who cleared HCV infection after a 48-week course of pegylated (PEG)-IFN plus ribavirin combination therapy.

2. Patients and methods

2.1. Patients

A total of 73 consecutive patients with CHC, who were hospitalized at Mie University Hospital between June 2005 and June 2006, were enrolled in this study (Table 1). The diagnosis of chronic HCV infection was based on the consistent detection of serum anti-HCV antibody (the third-generation EIA; Ortho Diagnostic Systems, Raritan, NJ, USA) and HCV RNA (Amplicor-HCV assay; Roche Molecular Diag. Co., Tokyo, Japan). None of the patients complicated with acute inflammatory disorders and received phlebotomy in the year preceding the study. All patients showed absence of the HFE mutations C282Y or H63D. Serum HCV RNA titer was quantified by the Amplicor monitor assay (Roche Molecular Diag. Co.). We also recruited patients with AI, who were hospitalized between August 2005 and June 2006, as a control for high serum hepcidin levels. Ten AI patients attributed to various pathologies (five suffered from rheumatoid arthritis, two from malignant lymphoma, and one from systemic lupus erythematosus, pyelonephritis, and pulmonary tuberculosis, respectively) participated in the study. We also studied a group of age-matched healthy volunteers ($n = 10$) with normal serum iron status, no signs of anemia, liver disease, or inflammation, and without HCV infection as controls for normal serum hepcidin levels between April 2006 and June 2006. Serum ferritin levels were measured by RPHA method (Ohthuka assay Co. Ltd., Tokushima, Japan) in this study. The upper limit of normal-ferritin was defined as 170 ng/mL for males and 120 ng/mL for females by the value of mean ±3 standard deviations (SD) obtained using the screening serum samples of Japanese volunteers ($n = 1562$, 823 males and 739 females) (unpublished data). This normal range of ferritin deviates from the more universally used upper reference values of 300 ng/mL for males and 200 ng/mL for females, and may be a distinctive feature of Japanese population. Blood samples of all these subjects were collected on the morning under fasting conditions, and sera were separated immediately after blood collection and stored at −80 °C until use. Informed consent was obtained from each patient included in the study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the Ethical Committee of Mie University.

2.2. Semi-quantitative assay for serum hepcidin using the SELDI-TOF MS system

We performed serum hepcidin measurement by SELDI-TOF MS system between October 2006 and February 2007 (storage period of serum samples was 5–19 months), as reported previously, with some modification [13]. In brief, 40 μL of the diluted serum samples was applied onto an IMAC 30 chip array (Ciphergen Biosystems, Freemont, CA, USA) and incubated at room temperature for 30 min. The chip arrays were washed and air-dried, and 0.5 μL α-cyano-4-hydroxy-cinnamic acid (CHCA) was added twice to the array surface, and arrays were analyzed with the Chiphergen ProteinChip Reader (model PBS II; Chiphergen Biosystems). The mass–charge ratio (*m/z*) of each of the proteins/peptides captured on the array surface was determined according to the externally calibrated standards: Arg8-vasopressin (1084.25 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), bovine insulin β-chain (3495.95 Da), human insulin (5807.65 Da), bovine ubiquitin (8564.8 Da), and bovine cytochrome C (12,230.9 Da). Serially diluted synthetic hepcidin-25 (Peptide Institute, Osaka, Japan) was used as a quantitative control. The data from the peak intensities were normalized with total ion current using Biomarker Wizard (Ciphergen ProteinChip Software 3.1.1; Chiphergen Biosystems) to compensate for the variations in sample concentrations loaded onto a spot; the data were represented as arbitrary units (AU) calculated using this software.

Table 1
Clinical characteristics of patients in this study

Characteristics	HCV negative		Chronic hepatitis C		Statistics		
	Normal subject ($n = 10$)	AI ($n = 10$)	Normal-ferritinemia ($n = 22$)	Hyper-ferritinemia ($n = 51$)	Normal vs. CH-C(N)	AI vs CH-C(H)	CH-C(N) vs. CH-C(H)
Age (yr)	49.2 ± 11.3	52.3 ± 18.1	51.8 ± 13.3	56.5 ± 9.2	0.5998	0.2783	0.0856
Gender (M/F)	6/4	4/6	14/8	30/21	0.9999	0.4512	0.8977
Laboratory data							
ALT (IU/L)	23.8 ± 9.0	29.1 ± 14.7	62.4 ± 39.7	57.9 ± 36.2	<0.0001	<0.0001	0.6080
AST (IU/L)	20.6 ± 5.3	24.8 ± 12.0	62.1 ± 35.7	56.7 ± 38.3	<0.0001	<0.0001	0.4406
Red blood cell count ($\times 10^4/mm^3$)	473 ± 62	387 ± 80	429 ± 48	377 ± 63	0.0377	0.7843	0.0008
Hemoglobin (g/L)	143 ± 15	112 ± 27	133 ± 15	120 ± 20	0.1000	0.2496	0.0060
Hematocrit (%)	43.7 ± 4.3	36.0 ± 5.3	40.9 ± 3.9	37.3 ± 5.3	0.0739	0.8683	0.0059
Platelet count ($\times 10^4/mm^3$)	20.9 ± 4.9	27.7 ± 10.2	14.9 ± 4.6	12.7 ± 4.4	0.0022	<0.0001	0.0584
Serum iron (µg/dL)	108 ± 42	45 ± 15	119 ± 56	147 ± 55	0.5858	<0.0001	0.0439
Transferrin saturation (%)	31.0 ± 12.5	18.3 ± 6.6	31.5 ± 15.5	46.6 ± 19.9	0.9243	<0.0001	<0.0001
Serum ferritin (ng/mL)	54 ± 30	183 ± 48	78 ± 68	473 ± 346	0.3013	0.0110	<0.0001
Serum HCV RNA (KIU/mL)	-	-	1490 ± 1400	1060 ± 1020			0.1552
Liver histology			($n = 19$)	($n = 40$)			
Inflammatory activity (0/1/2/3)			0/9/7/3	0/22/16/2			0.3781
Fibrosis staging (0/1/2/3/4)	-	-	0/6/6/7/0	0/6/18/9/7			0.0853
Total iron score	-	-	6.8 ± 4.8	15.5 ± 6.0			<0.0001

Note. AI, anemia of inflammation; CH-C(N), chronic hepatitis C with normal-ferritinemia; CH-C(H), chronic hepatitis C with hyper-ferritinemia; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are expressed as means ± SD.
Bold characters indicate these values are ststistically significant ($p < 0.05$).

2.3. Liver biopsy samples

Liver biopsy was performed in 59 CHC patients for clinical histological evaluation. Liver tissue specimens were divided into two groups; one portion was fixed in buffered formalin and embedded in paraffin and the other was immediately frozen and stored at −80 °C for RNA extraction. Hepcidin mRNA quantification was performed using the TaqMan realtime detection–polymerase chain reaction (PCR) assay (Applied Biosystems, Tokyo, Japan), as described previously [12]. Fixed hepatic tissues were stained with Hematoxylin & eosin and Masson's trichrome, and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet et al. [15]. The histological quantification of hepatic iron using liver samples stained with Perls' Prussian blue was carried out according to Total Iron Score (TIS, 0–60) system proposed by Deugnier et al. [16].

2.4. PEG-IFN and ribavirin combination therapy for CHC patients

Among the 73 CHC, 27 completed a 48-week course of PEG-IFN [1.5 µg/kg once per week of PEG-IFNα-2b (Schering-Plough Corporation, Kenilworth, NJ, USA)] and ribavirin [600–1000 mg per day of Rebetol (Schering-Plough Corporation)] combination therapy. Patients with serum HCV RNA negative by reverse transcription-PCR for 24 weeks after the completion of therapy were defined as "sustained virological responders (SVR)" and the remaining patients categorized as "non-SVR subjects".

2.5. Statistical analysis

Data are expressed as means ± SD. The baseline characteristics were compared using the unpaired Student t-test, Mann–Whitney U test, or Oneway Factorial ANOVA and multiple comparison test for continuous variables and χ^2 test for categorical variables. Correlations between two variables were assessed by the Spearman rank correlation test. To analyze the changes of serum hepcidin, ferritin, and their ratio after the PEG-IFN and ribavirin therapy, paired Student t-test was used. Two-sided p values of <0.05 were considered as statistically significant. All statistical analyses were performed using the commercially available software SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical characteristics of the patients

The clinical characteristics of the patients are summarized in Table 1. Patients with AI showed the characteristic clinical profile; mild normocytic or microcytic anemia, low serum iron and transferrin saturation, and elevated serum ferritin levels. Seventy-three patients infected with HCV were included in this study. Fifty-one patients with CHC (69.9%) had increased serum ferritin values (>170 ng/mL for males and >120 ng/mL for females), and remaining 22 patients had normal-ferritinemia. Hyper-ferritinemic CHC patients had low red blood cell counts and low hemoglobin and hematocrit levels compared to normal-ferritinemic CHC, and the anemic levels were comparable to those of the AI. The majority of CHC patients had mild to moderate iron deposition in liver tissue (mean TIS was 12.7 ± 6.9) and hepatic iron deposition was not detected (TIS = 0) only in two cases.

3.2. Serum hepcidin concentrations

Serum hepcidin was measurable in all 10 healthy controls and the 10 AI and 73 CHC patients. The mean serum hepcidin level was 29.4 ± 25.9 AU in CHC with normal-ferritinemia, and this value was not significantly different to healthy controls (34.2 ± 15.6 AU) (Fig. 1). In CHC with hyper-ferritinemia, serum hepcidin levels were significantly higher (81.6 ± 48.8 AU, $p = 0.0002$) than those of the healthy volunteers and also significantly higher than the normal-ferritinemic CHC patients ($p < 0.0001$). As expected, the mean serum hepcidin level was highest (110.5 ± 29.5 AU) in AI patients, but this was not statistically different from the hyper-ferritinemic CHC patients.

Serum hepcidin levels were compared to the hepatic hepcidin mRNA levels in the 59 CHC patients who underwent liver biopsies. Serum hepcidin and hepatic hepcidin mRNA levels appeared to be positively correlated (Fig. 2), demonstrating that the serum hepcidin concentrations can be considered a valid reflection of hepatic hepcidin production.

3.3. Correlation between serum hepcidin concentrations and clinical parameters in CHC patients

The correlations between serum hepcidin concentrations and clinical characteristics in CHC patients were investigated, and the results are summarized in Table 2. Patients' age, gender, and body mass index were not

Fig. 2. Correlation between serum hepcidin concentrations and hepatic hepcidin mRNA levels in patients with chronic hepatitis C ($n = 59$). Hepatic hepcidin mRNA was quantified by TaqMan real-time detection–polymerase chain reaction assay and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantified simultaneously in each liver tissue sample. Regression line is shown.

related to serum hepcidin concentrations. Red blood cell counts and transferrin saturation were weakly correlated with serum hepcidin levels. Serum ferritin levels (Fig. 3A) and TIS score (Fig. 3B) were strongly correlated with serum hepcidin levels, indicating that hepcidin is expressed by the liver in response to iron burden, even in chronic HCV-infected patients. The expression of hepcidin is also known to increase in response to inflammatory stimuli [17]. But, there were no significant correlations between serum hepcidin levels and serum transaminase, CRP levels, and histological inflammatory grading in CHC patients.

3.4. Serum hepcidin concentrations relative to iron burden

Our findings of strong positive correlations between serum hepcidin and serum ferritin and hepatic iron scores in CHC also suggest feedback to hepcidin expression against iron overload, and the ratio of hepcidin levels to iron overload may be constant. To evaluate the relative amounts of hepcidin in relation to iron burden, we calculated the ratio of serum hepcidin/ferritin in each subject. The ratio was significantly lower in HCV positive patients than in HCV negative controls not only for those in a hyper-ferritinemic condition but also for normal-ferritinemic; the ratio of serum hepcidin/ferritin of CHC with hyper-ferritinemia was significantly lower than that of the AI patients (0.25 ± 0.24 vs. 0.65 ± 0.24, $p < 0.0001$) (Fig. 4), and that of CHC patients with normal-ferritinemia was also significantly lower than that of healthy volunteers (0.47 ± 0.46 vs.
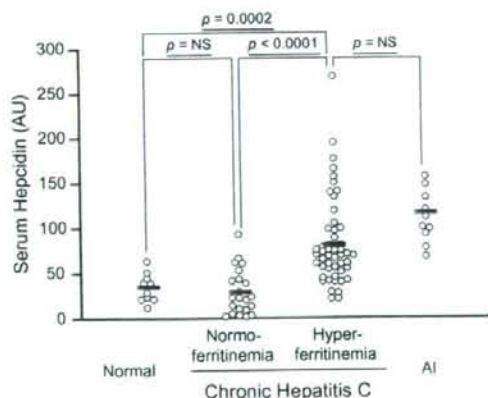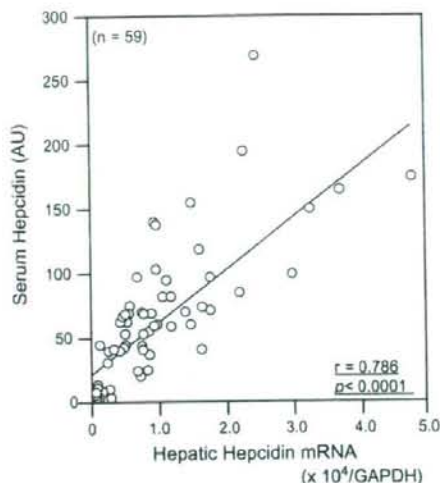
Fig. 1. Serum hepcidin concentrations in normal subjects ($n = 10$) and in patients with chronic hepatitis C (CHC) [with normal-ferritinemia ($n = 22$) and hyper-ferritinemia ($n = 51$)] and anemia of inflammation (AI) ($n = 10$). Serum hepcidin was measured by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) system. Hyper-ferritinemic CHC patients had significantly higher serum hepcidin concentrations than those of normal subjects and normal-ferritinemic CHC patients, but there were not significantly different from AI patients. Serum hepcidin also did not differ significantly between normal subjects and CHC with normal-ferritinemia. The horizontal line shows the mean value of serum hepcidin in each group.

Table 2
Correlations between clinical findings and serum hepcidin levels in patients with chronic hepatitis C (n = 73)

Characteristics	Serum hepcidin (AU)	Statistics	
		r	p Values
Age (yr)		−0.069[a]	0.5699[a]
Gender			
Male (n = 44)	60.8 ± 37.1		
Female (n = 29)	73.6 ± 63.6	0.7780[b]	
Body mass index (kg/m²)		−0.055[a]	0.6386[a]
Laboratory data			
ALT (IU/L)		−0.058[a]	0.6272[a]
AST (IU/L)		−0.093[a]	0.4356[a]
Platelet count (×10⁴/mm³)		−0.218[a]	0.0654[a]
Red blood cell count (×10⁴/mm³)		**−0.237[a]**	**0.0449[a]**
Hemoglobin (g/L)		−0.229[a]	0.0530[a]
Hematocrit (%)		−0.226[a]	0.0554[a]
Serum iron (μg/dL)		0.162[a]	0.1695[a]
Transferrin saturation (%)		**0.285[a]**	**0.0156[a]**
Serum ferritin (ng/mL)		**0.605[a]**	**<0.0001[a]**
Serum HCV RNA (KIU/mL)		−0.078[a]	0.5081[a]
CRP (mg/dL)		0.061[a]	0.6071[a]
Liver histology (n = 59)			
Inflammatory activity			
A1 (n = 31)	73.5 ± 56.4		
A2 (n = 23)	66.7 ± 41.9	0.4051[c]	
A3 (n = 5)	40.8 ± 69.8		
Fibrosis staging			
F1 (n = 12)	48.3 ± 31.1		
F2 (n = 24)	79.5 ± 58.0		0.2359[c]
F3 (n = 16)	65.0 ± 41.8		
F4 (n = 7)	94.9 ± 38.3		
Total iron score		**0.797[a]**	**<0.0001[a]**
Hepcidin mRNA levels		**0.786[a]**	**<0.0001[a]**

Note. ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are expressed as means ± SD.
Bold characters indicate these values are statistically significant (p < 0.05).
  [a] Spearman rank correlation test.
  [b] Unpaired Student t-test.
  [c] Oneway Factorial ANOVA and multiple comparison test.

0.73 ± 0.36, p = 0.0253). Conversely, the ratio was not significantly different between healthy volunteers and AI patients. Serum ferritin shows a skewed distribution. Therefore, hepcidin/log scale ferritin levels were also calculated, and the ratio was again lower in CHC patients than in HCV negative controls (30.9 ± 18.0 vs. 49.2 ± 13.9 in hyper-ferritinemic group, p = 0.0010; 15.3 ± 11.3 vs. 20.2 ± 8.4 in normal-ferritinemic group, p = 0.2226). These results indicate that hepcidin expression in the liver may be relatively impaired in chronic HCV-infected patients, when adjusted for serum concentrations of ferritin.

3.5. Changes of serum hepcidin concentrations after PEG-IFN plus ribavirin therapy for CHC patients

To evaluate the direct involvement of HCV infection in impairing hepatic hepcidin production, serum hepcidin was determined before and after the completion of 48-week course of PFG-IFN plus ribavirin therapy in 27 CHC patients. Twelve patients were assigned to SVR and the remaining 15 to non-SVR, and serum hepcidin was measured again 24 weeks after completing therapy (i.e., after the 72 weeks of pretreatment measurement of serum hepcidin). After the PEG-IFN plus ribavirin therapy, serum hepcidin levels were significantly increased in SVR patients (from 69.8 ± 24.5 to 117.0 ± 28.3 AU, p = 0.0059), but on the contrary, they were decreased significantly in non-SVR patients (from 66.3 ± 39.7 to 47.1 ± 20.5 AU, p = 0.0350) (Table 3) (Fig. 5A). Serum ferritin levels were significantly decreased in both SVR and non-SVR patients after the therapy (Fig. 5B). Remarkably, when HCV was eradicated from the patients by PEG-IFN plus ribavirin, their hepcidin/ferritin ratios were significantly elevated (from 0.21 ± 0.12 to 0.70 ± 0.30, p < 0.0001) (Fig. 5C) and reached the same levels as healthy subjects (0.73 ± 0.36, p = 0.8951) (Table 4), but they were constantly low in non-SVR patients (from 0.20 ± 0.14 to 0.18 ± 0.12)
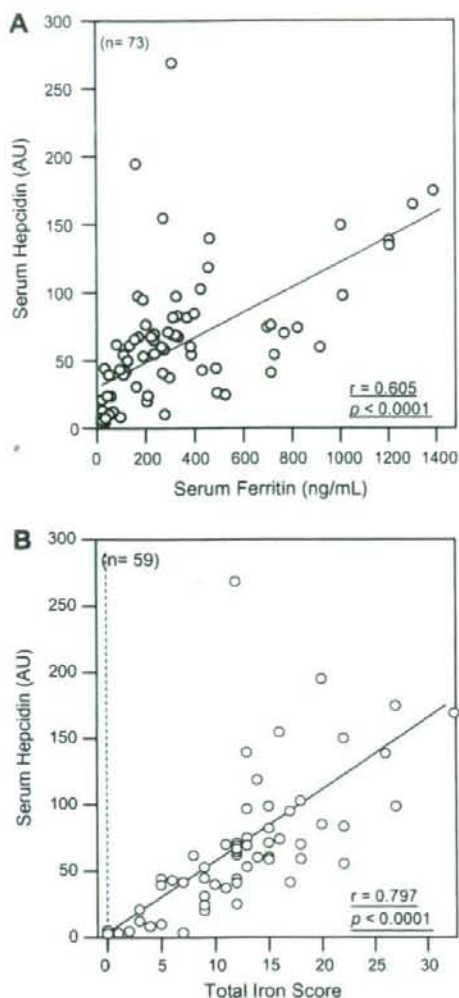
Fig. 3. Correlations between serum hepcidin concentrations and serum ferritin (A) ($n = 73$) and total iron score in hepatic tissues (B) ($n = 59$) in patients with chronic hepatitis C. Regression lines are shown.
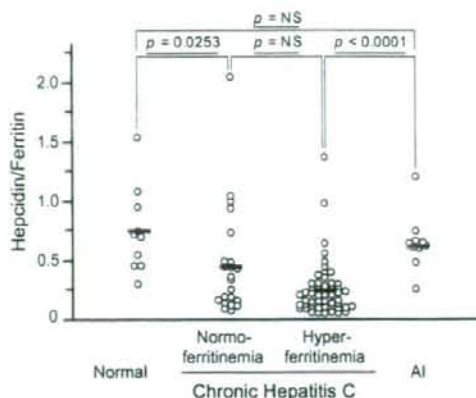

Fig. 4. Serum hepcidin-to-ferritin ratio in normal subjects ($n = 10$) and in patients with chronic hepatitis C (CHC) [with normal-ferritinemia ($n = 22$) and hyper-ferritinemia ($n = 51$)] and anemia of inflammation (AI) ($n = 10$). The ratio was significantly lower in normal-ferritinemic CHC patients than in normal subjects and in hyper-ferritinemic CHC patients than in AI patients. The horizontal line shows the mean value of hepcidin-to-ferrtin ratio in each group.

(Fig. 5C). After the PEG-IFN plus ribavirin therapy, the slopes of regression line of serum hepcidin and ferritin correlations were considerably different between SVR and non-SVR groups, although they did not reach the statistical significance, perhaps due to the limited number of patients and the narrow range of post-treatment ferritin levels in SVR patients (Fig. 5D).

4. Discussion

Recently, hepcidin is emerging as a central player in the regulation of iron homeostasis and measurement of serum hepcidin seems to be useful for understanding the mechanism of iron dysregulation in patients suffering from iron regulatory disorders. Previously, due to the lack of availability of a serum hepcidin assay, some investigators have employed an ELISA method which measures prohepcidin, the 64 amino acid precursor [18,19]. Utilizing this assay, no significant change was observed in human serum prohepcidin in response to a lipopolysaccharide (LPS) injection, whereas the urinary hepcidin increased significantly [20]. Thus, this casts doubt on the value of measurement of prohepcidin as a diagnostic surrogate for iron regulatory disorders or as a correlative determinate of circulating hepcidin concentrations. In this study, we used a SELDI-TOF MS analysis and evaluated serum hepcidin concentrations in CHC patients, and compared them to healthy subjects and AI patients. Serum hepcidin concentrations were correlated significantly with hepatic hepcidin mRNA levels in patients with CHC, indicating that serum hepcidin semi-quantification by this assay is a valid approach to evaluate hepatic hepcidin production. In many CHC patients with hyper-ferritinemia, serum hepcidin was increased and its mean concentrations reached the level of AI. But in patients with normal-ferritinemic CHC, serum hepcidin concentrations were relatively low and did not differ significantly from those of normal subjects. In addition, serum hepcidin was strongly correlated with serum ferritin and hepatic iron deposition score in CHC. A strongly positive relationship between serum ferritin and hepcidin expression levels already has been reported in congenital chronic anemic patients [21] and normal volunteers [22], all were free of HCV infection. Thus, these results suggest that hepatic expression of hepcidin also is tightly regulated

Table 3
Clinical characteristics and changes of laboratory data during the PEG-IFN plus ribavirin therapy in patients with chronic hepatitis C

Characteristics	PEG-IFN + RBV				
	SVR (n = 12)			Non-SVR(n = 15)	
	Before	After		Before	After
Age (yr)	52.7 ± 10.2			59.8 ± 7.6	
Gender (M/F)	9/3			7/8	
Body mass index (kg/m^2)	24.2 ± 3.2			23.1 ± 2.3	
Laboratory data					
ALT (IU/L)	66.0 ± 29.8	30.3 ± 16.2		58.0 ± 54.5	44.8 ± 23.4
AST (IU/L)	64.5 ± 24.9	29.8 ± 14.1		62.0 ± 44.0	42.4 ± 22.2
Platelet count ($\times 10^4$/mm^3)	16.0 ± 4.0	17.4 ± 3.7		13.4 ± 2.9	13.5 ± 3.4
Red blood cell count ($\times 10^4$/mm^3)	457 ± 47	453 ± 52		381 ± 34	386 ± 22
Hemoglobin (g/L)	147 ± 13	146 ± 12		120 ± 11	126 ± 8
Hematocrit (%)	44.0 ± 3.6	40.4 ± 10.2		37.3 ± 2.8	37.2 ± 3.7
Serum iron (μg/dL)	144 ± 40	115 ± 43		148 ± 60	124 ± 61
Transferrin saturation (%)	43.0 ± 18.5	33.9 ± 8.1		45.7 ± 19.2	33.8 ± 14.6
Serum ferritin (ng/mL)	491 ± 376	190 ± 79		488 ± 385	332 ± 190
Serum HCV RNA (KIU/mL)	1160 ± 1200	–		1510 ± 1230	1600 ± 1480
Serum hepcidin (AU)	69.8 ± 24.5	117.0 ± 28.3		66.3 ± 39.7	47.1 ± 20.5
Liver histology	(n = 9)			(n = 9)	
Inflammatory activity (0/1/2/3)	0/3/6/0			0/4/3/2	
Fibrosis staging (0/1/2/3/4)	0/2/4/1/2			0/0/6/3/0	
Total iron score	16.3 ± 4.9			15.3 ± 7.9	

Note. PEG-IFN, pegylated-interferon; RBV, ribavirin; SVR, sustained virological responder;
ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are expressed as means ± SD.

in response to iron burden, even in the liver of chronically infected with HCV.

It is surprising that the serum hepcidin/ferritin ratio was significantly lower in HCV positive patients than in HCV negative controls, in both in hyper- and normal-ferritinemic conditions, indicating that the hepcidin induction per iron burden was relatively impaired in CHC patients. This result was contrary to our expectations, because it has been reported that the expression of hepcidin can be upregulated by inflammation caused by several bacterial [20] and viral infections [23]. Because, as with the results of Aoki's reports [24], the serum transaminase levels and histological grading were not significantly correlated with serum hepcidin in CHC, mild and local inflammation which occurred in the liver of CHC may not induce hepcidin expression. Interleukin (IL)-6 and IL-1β appear to be the major cytokines responsible for the induction of hepcidin in inflammation [25], but these cytokines were not evaluated in this study. This relative impairment of hepcidin production in CHC was fully reversible after successful eradication of HCV following PEG-IFN and ribavirin therapy. When the patients were assigned to SVR, serum hepcidin levels were significantly increased after PEG-IFN plus ribavirin treatment, and iron overload condition was also significantly improved in SVR patients, judging from serum ferritin, transferrin saturation, and iron levels (Table 3). Consequently, the relative hepcidin per iron burden recovered to the levels of HCV negative

healthy controls. On the contrary, in non-SVR patients, serum hepcidin, consistent with serum ferritin, was significantly decreased after PEG-IFN and ribavirin, and the hepcidin/ferritin ratio was not significantly changed during the treatment. From these results, relatively low hepcidin expression in CHC seems to be directly related to HCV replication in the liver.

The molecular mechanism by which HCV induces the impairment of hepcidin production is currently unknown. Even the regulatory mechanisms of hepcidin have just begun to be investigated very recently. During preparation of this manuscript, Nishina et al. [26] clearly demonstrated that transgenic mice expressing HCV polyprotein had decreased hepcidin expression and increased ferritin expression in the liver. They also showed the down-regulation of hepcidin promoter activity and increased hepatic reactive oxygen species. Because it is well known that hepatic oxidative stress is elevated in patients with CHC [5], HCV-induced oxidative stress formation may involve the down-regulation of hepcidin, causing iron accumulation during chronic HCV infection.

In this study, mild anemic state was a complication in CHC patients with hyper-ferritinemia (Table 1), and this anemia may also influence the relatively diminished hepatic hepcidin production in these patients. Hyper-ferritinemic CHC patients had lower platelet counts and more advanced hepatic fibrosis compared to normal-ferritinemic CHC (not reach the significance), suggesting that hypersplenism may com-
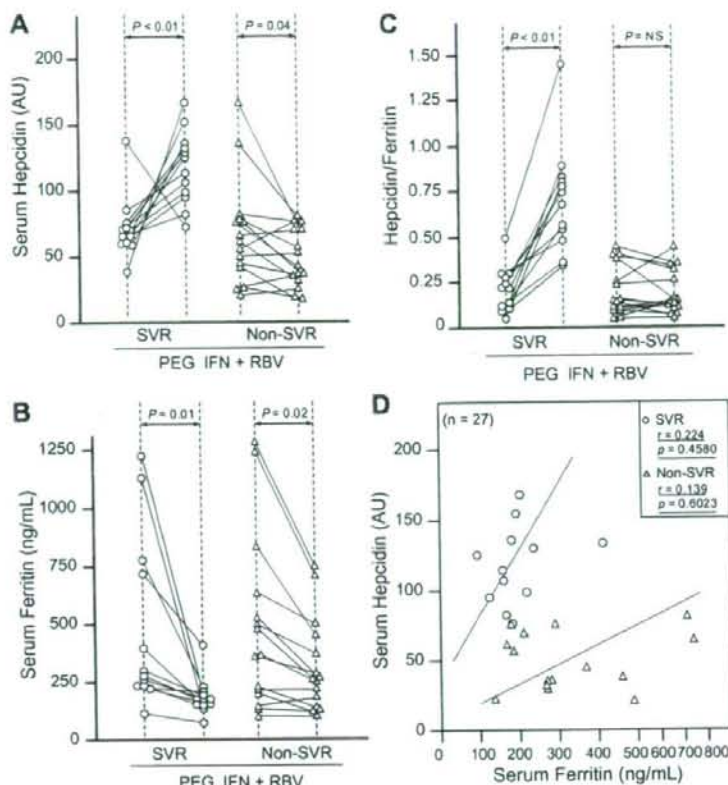
Fig. 5. Changes of serum hepcidin (A), serum ferritin (B), and their ratio (C) after a 48-week course of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) therapy in patients with chronic hepatitis C (CHC). (A) Serum hepcidin concentrations increased significantly after the PEG-IFN + RBV in sustained virological responders (SVR). On the contrary, serum hepcidin levels decreased significantly in non-SVR patients. (B) Serum ferritin concentrations decreased significantly after the PEG-IFN + RBV therapy in both SVR and non-SVR groups. (C) The serum hepcidin/ferritin ratio was significantly increased after the therapy in SVR, but the ratio was not significantly changed in non-SVR patients. (D) Correlation between serum hepcidin concentrations and serum ferritin after PEG-IFN plus RBV therapy in patients with CHC. Open circles depicted SVR patients and open triangles depicted non-SVR patients. Regression lines are shown.

plicate in some of hyper-ferritinemic CHC. Additional study is necessary to determine the influence of hypersplenism on hepatic hepcidin production in chronic liver disease patients.

In conclusion, we measured serum hepcidin concentrations in patients with CHC and relatively low levels were observed compared to HCV negative individuals. This effect of HCV on hepcidin expression was fully reversible after successful eradication of HCV. The consequences of this dysregulation of the hepcidin expression by HCV may be an important mechanism underlying the iron overload seen in CHC and may have significant implications for the management of chronic HCV infection. Improvement for its regulation or supplementation of hepcidin may be beneficial for CHC patients with iron overload.

Table 4
Serum hepcidin, ferritin, and its ratio after PEG-IFN + RBV therapy in patients with chronic hepatitis C and comparison to normal subject

Characteristics	After PEG-IFN + RBV		Normal subject	Statistics		
	SVR($n = 12$)	Non-SVR($n = 15$)	($n = 10$)	SVR vs. Non-SVR	SVR vs. Normal	Non-SVR vs. Normal
Serum hepcidin (AU)	117 ± 28.3	47.1 ± 20.5	34.2 ± 15.6	<0.0001	<0.0001	0.1492
Serum ferritin (ng/mL)	190 ± 79.2	332 ± 190	54.4 ± 30.4	0.0157	0.0002	<0.0001
Hepcidin/ferritin ratio	0.70 ± 0.30	0.18 ± 0.12	0.73 ± 0.36	<0.0001	0.8951	<0.0001

Note. PEG-IFN, pegylated-interferon; RBV, ribavirin; SVR, sustained virological responder.
Data are expressed as means ± SD.

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Iron Overload Is Associated with Hepatic Oxidative Damage to DNA in Nonalcoholic Steatohepatitis

Naoki Fujita,[1] Hirohide Miyachi,[1] Hideaki Tanaka,[1] Masaki Takeo,[1] Naoki Nakagawa,[1] Yoshinao Kobayashi,[1] Motoh Iwasa,[1] Shozo Watanabe,[2] and Yoshiyuki Takei[1]

[1]Department of Gastroenterology and Hepatology, Division of Clinical Medicine and Biomedical Science, Institute of Medical Sciences, Mie University Graduate School of Medicine; [2]Center for Physical and Mental Health, Mie University, Tsu, Japan

Abstract

Several lines of evidence have suggested that oxidative stress plays an important role for the pathogenesis of nonalcoholic steatohepatitis (NASH). Therefore, by using immunohistochemical staining of liver biopsy samples, we measured hepatic 7,8-dihydro-8-oxo-2′ deoxyguanosine (8-oxodG), a DNA base-modified product generated by hydroxyl radicals, of 38 NASH patients and compared with 24 simple steatosis and 10 healthy subjects. Relation of hepatic 8-oxodG with clinical, biochemical, and histologic variables and changes after iron reduction therapy (phlebotomy plus iron–restricted diet) were also examined. Hepatic 8-oxodG levels were significantly higher in NASH compared with simple steatosis (17.5 versus 2.0 8-oxodG–positive cells/10^5 μm^2; $P < 0.0001$). 8-oxodG was significantly related to iron overload condition, glucose-insulin metabolic abnormality, and severities of hepatic steatosis in NASH patients. Logistic regression analysis also showed that hepatic iron deposit and insulin resistance were independent variables associated with elevated hepatic 8-oxodG. After the iron reduction therapy, hepatic 8-oxodG levels were significantly decreased (from 20.7 to 13.8 positive cells/10^5 μm^2; $P < 0.01$) with concomitant reductions of serum transaminase levels in NASH patients. In conclusion, iron overload may play an important role in the pathogenesis of NASH by generating oxidative DNA damage and iron reduction therapy may reduce hepatocellular carcinoma incidence in patients with NASH. (Cancer Epidemiol Biomarkers Prev 2009;18(2):424–32)

Introduction

Nonalcoholic fatty liver disease, the leading cause of liver disease in Western countries, includes a spectrum of clinical entities ranging from pure fatty liver to nonalcoholic steatohepatitis (NASH; ref. 1). Simple steatosis is usually considered benign, but the development of NASH is recognized as a precursor to more severe liver disease and sometimes evolves into cryptogenic cirrhosis and hepatocellular carcinoma (2). A commonly accepted model for the pathogenesis of NASH is the so-called "two-hit" hypothesis, wherein the "first hit" leads to accumulation of hepatic free fatty acids resulting in a histologic picture of macrovesicular steatosis (3). Several lines of evidence have suggested that oxidative stress may play an important role for the pathogenesis of NASH as the "second hit" (4-6), but little is understood about the molecular mechanisms of its formation in the liver of NASH and involvement of hepatocarcinogenesis. One convincing candidate for the source of oxidative stress is excessive accumulated iron in the liver of patients with NASH because mild to moderate iron overload in the liver is common in NASH (7-9). It is known that ferrous iron in the presence of hydrogen peroxide generates hydroxyl radical through the Fenton reaction (10). In the representative iron-related liver injury disorder, genetic hemochromatosis, it is clearly shown that hepatic iron is responsible for liver damage through reactive oxygen species formation leading to lipid peroxidation and accumulated oxidative stress, which causes hepatic cancer (11). It is therefore plausible that hepatic iron overload may contribute to oxidative stress formation among patients with NASH.

7,8-Dihydro-8-oxo-2′ deoxyguanosine (8-oxodG) is a modification of guanine that induces a point mutation in the daughter DNA strands (12) and it is used as a marker of oxidatively generated DNA damage in several diseases (13). Therefore, we examined 8-oxodG levels in the liver of NASH patients, compared with those of simple steatosis, and evaluated its relation with clinical, biochemical, and histologic findings. Changes of hepatic 8-oxodG levels after iron reduction therapy were also investigated in NASH patients with hyperferritinemia.

Materials and Methods

Patients. A total of 38 NASH and 24 simple steatosis patients who underwent needle liver biopsy at Mie University Hospital between March 2003 and December 2006 were enrolled in the study (Table 1). In addition, 10 liver specimens from HBV/HCV-negative and normal liver function patients (6 males and 4 females; median age, 59 y; range, 41-70 y) were obtained during hepatobiliary surgery for either resection of hemangioma or benign tumors, and their histologically

Table 1. Clinical characteristics of patients with NASH and simple steatosis

Characteristics	NASH ($n = 38$)	Simple steatosis ($n = 24$)	P
Age (y)	59.0 (29-78)	51.0 (19-73)	NS
Gender (M/F)	22/16	11/13	NS
BMI (kg/m^2)	25.6 (22.5-36.7)	24.7 (16.3-35.1)	NS
Obesity	23 (60.5%)	11 (45.8%)	NS
Type II diabetes, n (%)	18 (47.4%)	8 (33.3%)	NS
Hypertension, n (%)	14 (36.8%)	9 (37.5%)	NS
Hyperlipidemia, n (%)	25 (65.8%)	15 (62.5%)	NS
Laboratory data			
ALT (IU/L)	63.0 (23-171)	59.0 (12-863)	NS
AST (IU/L)	58.0 (27-134)	37.0 (17-443)	0.0047
Total cholesterol (mg/dL)	201 (151-358)	216 (155-276)	NS
Triglyceride (mg/dL)	155 (63-443)	125 (73-261)	NS
Glucose (mg/dL)	102 (71-241)	99 (73-427)	NS
Serum insulin (microunits/mL)	12.1 (2.4-34)	9.2 (1.0-18)	0.0083
HOMA-IR	3.48 (1.51-9.56)	2.21 (1.05-9.24)	0.0010
Hyaluronic acid (ng/mL)	66.5 (5-365)	19.6 (6-258)	0.0004
Platelet count ($\times 10^4$/mm^3)	18.0 (4.9-37.0)	23.0 (13.1-45.2)	0.0146
RBC count ($\times 10^4$/mm^3)	448 (274-633)	461 (367-558)	NS
Hemoglobin (g/dL)	14.3 (8.3-18.9)	14.7 (11.5-18.9)	NS
Serum iron (μg/dL)	126 (88-220)	93 (25-188)	0.0059
Transferrin saturation (%)	38.0 (22.3-87.6)	32.4 (9.4-43.8)	0.0152
Serum ferritin (ng/mL)	283 (69-847)	139 (18-640)	<0.0001
Liver histology			
Inflammatory activity (1/2/3)*	14/21/3	—	—
Fibrosis staging (1/2/3/4)*	8/17/11/2	—	—
Steatosis (%)	43 (15-86)	51 (28-90)	NS
TIS [‡]	3 (0-8)	0 (0-7)	<0.0001

NOTE: Results are presented as numbers (percentages) for qualitative data and as medians (ranges) for quantitative data.
Abbreviation: NS, not significant.
*Inflammatory activity and fibrosis staging in NASH was scored according to Brunt classification (16).
[†] Hepatic steatosis degree was assessed based on the percentage of affected hepatocytes.
[‡] The histologic quantification of iron was assessed by TIS proposed by Deugnier et al. (17).

normal liver tissue surrounding the resected lesion was used as a control. A diagnosis of NASH was established if a combination of the following clinical and histopathologic features was present: (a) a persistent abnormal liver biochemistry for >3 mo; (b) a liver biopsy showing steatosis (>10%) in the presence of lobular and/or portal inflammation, with or without Mallory bodies or fibrosis; and (c) the exclusion of other liver diseases, such as viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin deficiency–associated liver disease. Patients consuming >20 g of alcohol per day were excluded from the study. None of the patients had ingested drugs known to produce hepatic steatosis (including corticosteroids, high-dose estrogens, methotrexate, tetracycline, calcium channel blockers, or amiodarone) or those capable of interfering with free radical production (nonsteroidal anti-inflammatory drugs, vitamins, and iron-containing drugs) in the previous 6 mo. One patient with NASH had a history of gastrointestinal surgery. Simple steatosis was also diagnosed by liver biopsy. Obesity was defined as a body mass index (BMI) of >25 kg/m^2, according to the criteria of the Japan Society for the Study of Obesity (14). Patients were assigned a diagnosis of diabetes mellitus if a documented use of oral hypoglycemic medication or insulin, a random glucose level in excess of 200 mg/dL, or a fasting glucose of >126 mg/dL on at least two occasions was present (15). Hyperlipidemia was diagnosed if the cholesterol level was higher than

220 mg/dL and/or the triglyceride level was over 160 mg/dL. Hypertension was diagnosed if the patients were on antihypertensive medication and/or had a resting recumbent blood pressure of ≥140/90 mmHg on at least two occasions. Serum biochemical, hematologic, and iron-related markers were obtained from medical and laboratory records closest to the dates of liver biopsies. Informed consent was obtained from each patient and the study was approved by the Ethical Committee of Mie University. The study was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki.

Histologic Evaluation. Biopsy specimens were fixed in buffered formalin and embedded in paraffin. Sections were stained with H&E for morphologic evaluation, Masson's trichrome for assessment of fibrosis, and Perls' Prussian blue stain for assessment of iron loading. The histologic findings of NASH were interpreted and scored according to the classification proposed by Brunt et al. (16). The activity of hepatitis (necroinflammatory grade) was determined by the presence of hepatocellular steatosis, ballooning, and inflammation (acinar and portal) features as follows: grade 1, mild; grade 2, moderate; and grade 3, severe. The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis. Macrovesicular steatosis was quantified as the percentage of hepatocytes that