Table 6 Clinical characteristics of patients with normal distribution of body water and those with altered distribution

	ECW ratio < 0.398 (n = 25)	ECW ratio ≥ 0.398 (n = 19)
Age (years)	64 ± 12	73 ± 10*
Male/Female	15/10	61/48
HBV/HCV/Alcohol/other	4/15/5/1	0/12/2/5
Laboratory test		
Platelet count (× 10^3/μL)	97 ± 57	123 ± 68
Albumin (g/dL)	3.6 ± 0.5	3.2 ± 0.4**
Total Bilirubin (mg/dL)	0.9 ± 0.4	1.1 ± 0.6
Prothrombin time (%)	76 ± 15	73 ± 15
ALT (IU/L)	52 ± 37	50 ± 56
Sodium (mEq/L)	141 ± 3	140 ± 3
Child-Pugh A/B/C	16/9/0	8/11/0
HCC, number (%)	5 (20)	3 (16)

*P < 0.01 vs. < 0.398; **P < 0.05 vs. < 0.398.
ALT, alanine aminotransferase; ECW, extracellular water; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

of ECW > 0.398 (log-rank 6.94; P < 0.01) (Fig. 2) and low albumin < 3.5 g/dL (log-rank 5.45; P < 0.05). There were no statistically significant associations with age, platelets, bilirubin, prothrombin time, ALT, sodium, or BMI. In a Cox multivariate regression analysis, the independent predictor of developing ascites was ECW ratio (ECW ratio, hazard ratio 4.04, 95 % CI 0.04–4.82, P < 0.05; albumin, hazard ratio 2.66, 95% CI −3.83–0.32, P = 0.10).

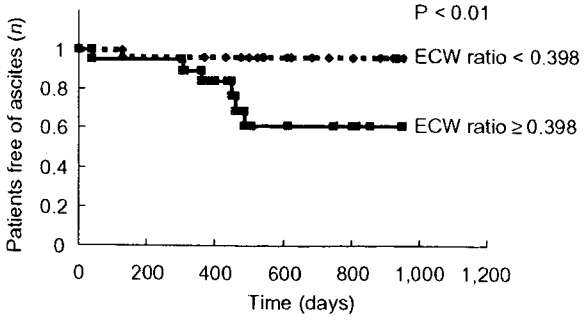

Figure 2 Extracellular water (ECW) ratio in the prediction of the risk of developing ascites. According to Kaplan–Meier analysis, a relative expansion of ECW (i.e. more than 0.398 in the initial assessment) was associated with a higher risk of developing ascites (long-rank test 6.94, P < 0.01).

DISCUSSION

ESTIMATION OF BODY water compartments is important for the assessment and monitoring of cirrhotic patients.[23] Previous studies of animals and healthy humans indicated that measurements of body water using multiple-frequency BIA are reliable.[10] However, reports of the use of multiple-frequency BIA for the assessment of body water compartments in cirrhotic patients with ascites are limited.[24] Lehnert *et al.* found that ECW and TBW (as determined by isotope methods) were predicted accurately by multiple-frequency BIA (r = 0.73 and 0.89, respectively).[13] However, they noted that the 95% confidence interval for the limits of agreement between the multiple-frequency BIA and isotope methods was acceptable for the control group (± 5%) but was slightly higher for the cirrhotic group (± 9%).[13] The reasons why multiple-frequency BIA does not measure water compartments accurately in patients with cirrhosis, and particularly those with ascites, are unknown. The body water compartments are determined with BIA by tissue cellularity, tissue hydration and membrane potential. The known altered distribution of water in cirrhotic patients may alter the capacitant effect of all membranes on conductance through the fluid compartments. A limitation of this study is that we did not validate body water compartments, because other methods for the determination of the body water were not available for our patients.

In this study, the changes in ICW, ECW, or TBW did not correlate with the severity of liver disease. Similarly, Müller *et al.* reported no differences in the sizes of the TBW compartments between groups of patients with Child–Pugh A, B, or C.[25] Conversely, Figueiredo *et al.* showed that ECW was increased, and ICW decreased, in patients with cirrhosis.[23] This discrepancy may be explained by whether or not adjustments were made for physical characteristics. In our study, height was used for normalization because it is not affected by fluid retention. However, it has not been established fully whether height is suitable for normalization, and further study is needed.

One of the important conclusions of our study is that the ECW ratio was the most sensitive indicator, detecting redistribution of body water in patients with liver cirrhosis with and without ascites. In addition, the ECW ratios of our patients were also related to development of ascites. Recently, Planas *et al.* followed up 263 cirrhotic patients after their first significant episode of ascites and found that the overall survival rate at 5 years

for their cirrhotic patients with ascites was 56.5%.[26,27] However, the probability of survival decreased markedly in those patients who developed refractory ascites during follow-up.[26] The natural history of cirrhotic patients with ascites may improve with the implementation of strict management if body water measurements could be made in a simple manner.

Medical treatment based on sodium-restricted diet, spironolactone, and furosemide achieves a response rate in up to 90% of patients without renal failure in controlled clinical trials.[28,29] Indeed, we found that there were no differences in body water compartments, including the ECW ratio, between patients on diuretic treatment and those who were not. This finding suggests that diuretics strongly affect body water compartments and it is therefore necessary to consider whether diuretics have been administered when performing body water measurements.

Our study demonstrated that ECW ratios of the trunk and leg are correlated with the grade of ascites. In patients with grade 1 ascites, the ECW ratio of the trunk seems to be reliable to assess body water compartments. An explanation of this finding may be the presence of leg edema in a relevant number of patients with ascites, whilst none of the patients had edema of the arms. We conclude that segmental body water measurements may be superior to commonly used whole body information for the detailed assessment of cirrhotic patients with ascites.

In conclusion, the present study shows that the ECW ratio is a reliable indicator for assessing body water compartments in patients with cirrhosis, even in those with an advanced disease and ascites. Inclusion of segmental ECW ratios should be considered for the detailed assessment of cirrhotic patients. In addition, ECW ratio measurements may be an objective parameter for assessment of therapeutic regimens (i.e. sodium restriction or oral supplementation with branched-chain amino acids).[30,31] Early management before ascites and edema are obvious is not recommended at this time because there are no studies showing a positive influence of treatment on quality of life, or survival. Further interventional studies may be needed to determine whether treatment could change the natural history of this disease entity.

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

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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 patients 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 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 who did not exhibit overt HE to look for possible microstructural changes in the brain, as suggested by

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 score, 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 points)	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.

measuring apparent diffusion coefficient (ADC) values. Then, we compared the results with plasma ammonia levels and the 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 the trail-making A test (number connection test) and digit symbol test (revised Wechsler adult intelligence scale) that is widely employed as a screening examination in hepatology clinics in Japan. Neuropsychological measurements were performed on the same day between 9 a.m. and 5 p.m. 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 9,999/70 ms, 1 acquisition, 20 sections of 5-mm thickness, 1-mm gap, 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, posterior cingulate gray matter, and frontal and parietal white matter.

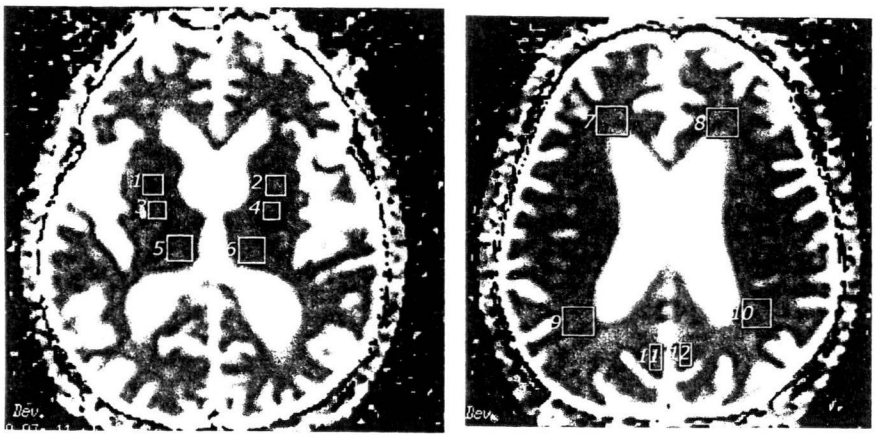

Figure 1. The regions of interest were selected on the diffusion-weighted image. 1, 2 = putamen; 3, 4 = pallidus; 5, 6 = thalamus; 7, 8 = frontal white matter; 9, 10 = parietal white matter; and 11, 12 = cingulate gray matter.

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 2 SD 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.

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 points)	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.

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 the others.

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} mm^2/s for frontal white matter (+1 SD from the mean value 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} mm^2/s for parietal white matter (+1 SD from the mean value 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 the 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

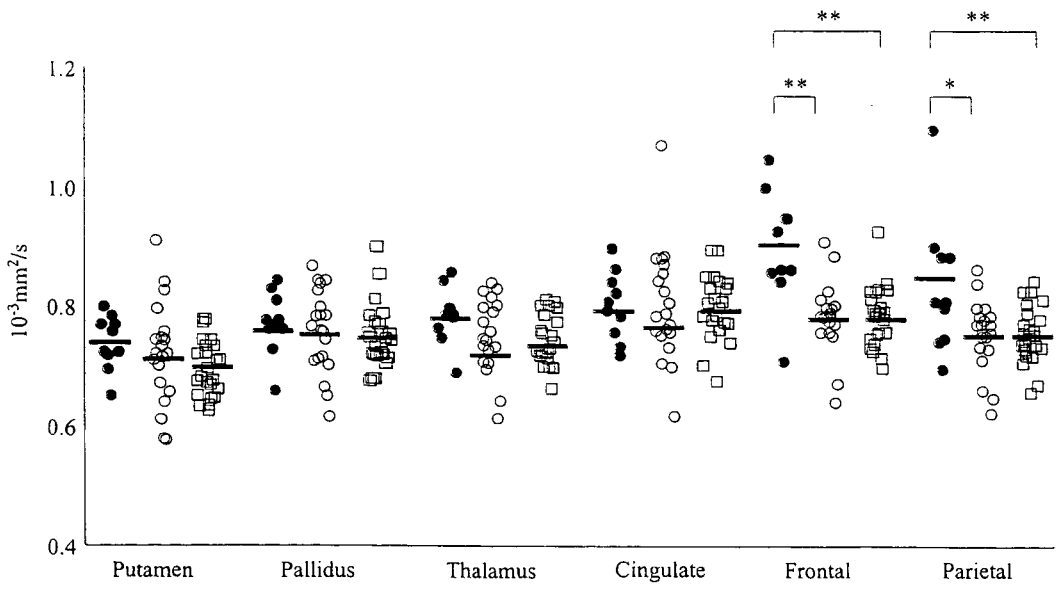

Figure 2. ADCs obtained in 24 control subjects and 40 cirrhotic patients, with and without minimal HE. All patients underwent neuropsychological tests and were classified to have no HE or minimal HE. • = minimal HE; ○ = no HE; □ = control subjects. ADC = apparent diffusion coefficient; HE = hepatic encephalopathy. $^*P < 0.05$, $^{**}P < 0.01$.

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:

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.

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 the 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 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.

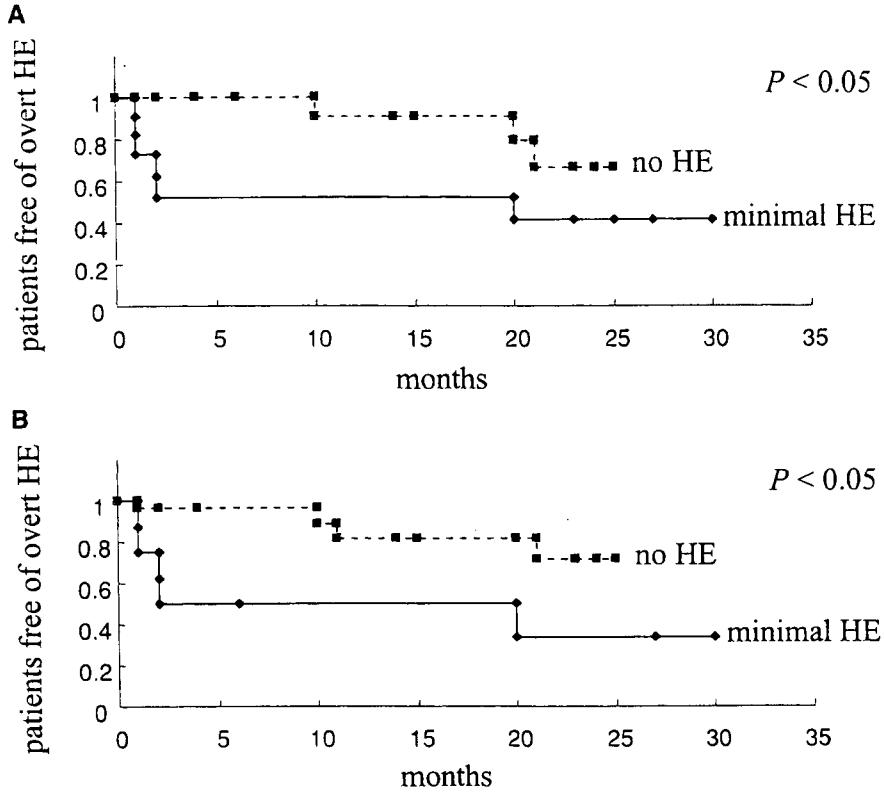

Figure 3. Prediction of overt HE by ADC in patients with cirrhosis: (*A*) frontal and (*B*) parietal. HE = hepatic encephalopathy; ADC = apparent diffusion coefficient.

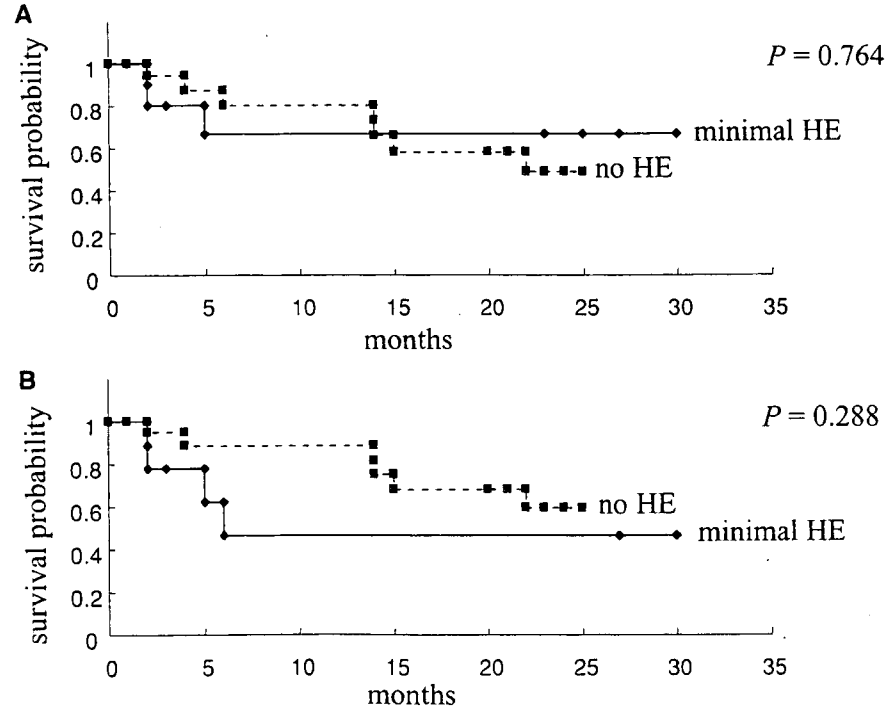

Figure 4. Prediction of survival by ADC in patients with cirrhosis: (*A*) frontal and (*B*) parietal. HE = hepatic encephalopathy; ADC = apparent diffusion coefficient.

survival of patients with minimal HE *versus* patients with no HE; no significant difference was found in frontal (Fig. 4A; log-rank test 0.09, $P = 0.764$) and parietal (Fig. 4B; 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 distributions 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 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 cingulate 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 in gray matter. Lodi *et al.* noted significant elevation in ADC values in all 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 ADC measurement in the posterior cingulate gyrus, although special care was taken to select the region of interest, avoiding the partial volume effect. These 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

- 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.

Specific author contributions: Ryosuke Sugimoto: planning of the study, data collection, data analyses, and writing of the manuscript; Motoh Iwasa: planning of the study, study design, data interpretation, and writing of the manuscript; Masayuki Maeda: planning of the study, imaging analyses, and writing of the manuscript; Naohito Urawa, Hideaki Tanaka, Naoki Fujita, and Yoshinao Kobayashi: data collection; Kan Takeda and Masahiko Kaito: planning of the study; and Yoshiyuki Takei: planning of the study and final review of the manuscript.

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

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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

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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 (×10^4/mm^3)	18.0 (4.9-37.0)	23.0 (13.1-45.2)	0.0146
RBC count (×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

contained fat droplets. The histologic quantification of hepatic iron was done according to Deugnier et al. (17) by scoring iron separately within hepatocytes (hepatic iron score, 0-36), sinusoidal cells (sinusoidal iron score, 0-12), and portal tracts or fibrotic tissue (portal iron score, 0-12). The total iron score (TIS, 0-60) was defined by the sum of these scores. This score has been shown to highly correlate with the biochemical hepatic iron index and hepatic iron concentration as measured by the atomic absorption spectrophotometry in patients with chronic liver diseases (18-20). All histologic grading and staging were done by a single pathologist without knowledge of the patients' clinical and laboratory data.

Immunohistochemical Detection of 8-oxodG Adducts in Liver Biopsy Samples. Immunohistochemical staining of 8-oxodG was done as previously described (21). Mouse monoclonal antibody against 8-oxodG (Japan Institute for the Control of Aging, Shizuoka, Japan) and Alexa Fluor 488–labeled goat antibody against mouse IgG (Molecular Probes) were used. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (version 1.62, NIH, Image program; ref. 21).

The specificity of the anti-8-oxodG antibody used in this study was confirmed by several parallel experiments. Sections in which the primary antibody was omitted or those treated with normal control serum instead of the primary 8-oxodG antibody consistently yielded negative staining. Localization of 8-oxodG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/mL of 8-oxodG but not by over a thousand-fold greater concentration of guanosine. When the primary antibody was preincubated with graded 8-oxodG competitively, a similar blocking of immunolabeling was obtained. Further, enzymatic treatment with RNase did not affect the immunoreactivity toward oxidized DNA.

Iron Reduction Therapy for NASH. To evaluate the clinical effects of iron reduction for NASH, 11 NASH patients with iron overload [serum ferritin levels were elevated above the reference range (>300 ng/mL for male and >200 ng/mL for female)] underwent iron reduction therapy and the changes of serum and histologic features were analyzed. We selected patients that fulfilled the following criteria for iron reduction: no complication with hypertension and/or cardiovascular disorder, <70 y, and their histology showed without cirrhosis and TIS is not score 0. Iron depletion was accomplished by doing intermittent phlebotomies in combination with regulation of dietary iron intake as described previously (22). In brief, at the initial phase of iron depletion, all patients underwent weekly or biweekly phlebotomy of 200 g until a state of mild iron deficiency was achieved (defined by a serum ferritin levels <50 ng/mL and/or a blood hemoglobin concentration of 12 g/dL). The mild iron deficiency state was maintained by additional phlebotomies during the study period: patients were followed up every 1 to 2 mo for the duration, and a phlebotomy was done if the serum ferritin level exceeded 80 ng/mL. In addition, those subjects were instructed both orally and in writing by a registered dietitian to reduce their intake of iron-rich foods during the intervention. The subjects were not required to alter their total caloric intake but were expected to replace iron-rich foods with appropriate substitutes.

Statistical Analysis. Results are presented as the medians and ranges for quantitative data or as numbers with percentages in parentheses for qualitative data. Demographic and baseline data were compared

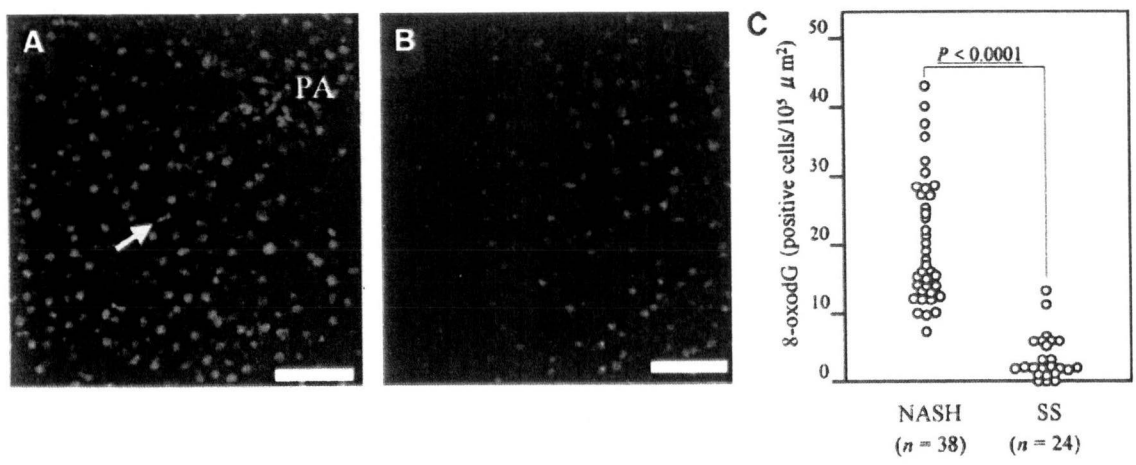

Figure 1. A and **B.** Representative 8-oxodG immunohistochemical staining in liver tissues from patients with NASH (**A**) and simple steatosis (**B**). In the liver of NASH, 8-oxodG immunoreactivity was strongly observed at the nuclei of many hepatocytes and several Kupffer cells (*arrow*) throughout the whole acinus. *PA,* portal area. In the liver of simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and rarely in the cytoplasm. *Scale bar,* 100 μm. **C.** Comparison between 8-oxodG–positive hepatocytic nuclear counts of patients with NASH and those of simple steatosis (*SS*). Positive cells were significantly higher in NASH patients than in simple steatosis. O, individual data of patients.

Table 2. Correlations between clinical findings and 8-oxodG levels in the liver of patients with NASH (n = 38)

Characteristics	8-oxodG (positive cells/10^5 μm^2)	Statistics	
		r	P
Age (y)		0.048*	NS*
Gender			
Male (n = 22)	20.7 (10.0-43.3) †		NS‡
Female (n = 16)	15.4 (7.3-35.7) †		
BMI (kg/m^2)		−0.057*	NS*
Laboratory data			
ALT (IU/L)		−0.012*	NS*
AST (IU/L)		0.068*	NS*
Total cholesterol (mg/dL)		−0.258*	NS*
Triglyceride (mg/dL)		−0.050*	NS*
Glucose (mg/dL)		0.628*	0.0001*
Serum insulin (microunits/mL)		0.359*	0.0294*
HOMA-IR		0.683*	<0.0001*
Hyaluronic acid (ng/mL)		0.307*	NS*
Platelet count ($\times 10^4/mm^3$)		−0.491*	0.0028*
RBC count ($\times 10^4/mm^3$)		−0.119*	NS*
Hemoglobin (g/dL)		0.009*	NS*
Serum iron (μg/dL)		0.587*	0.0004*
Transferrin saturation (%)		0.364*	0.0267*
Serum ferritin (ng/mL)		0.325*	0.0481*
Liver histology			
Inflammatory activity§			
A1 (n = 14)	18.9 (10.0-40.0) †		
A2 (n = 21)	19.0 (7.3-43.3) †		NS‖
A3 (n = 3)	14.7 (12.0-17.6) †		
Fibrosis staging§			
F1 (n = 8)	14.9 (10.0-43.3) †		
F2 (n = 17)	15.0 (7.3-37.7) †		NS‖
F3/4 (n = 13)	21.0 (12.0-40.0) †		
Steatosis¶		0.392*	0.0172*
TIS**		0.455*	0.0056*

*Spearman rank correlation test.
† Data are expressed as median (range).
‡ Unpaired Student's t test.
§ Inflammatory activity and fibrosis staging in NASH was scored according to Brunt classification (16).
‖ One-way factorial ANOVA and multiple comparison test.
¶ 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).

by use of Kruskal-Wallis ANOVA, which is independent of the distribution of the data. Distribution of variables was first evaluated to determine the most appropriate statistical method across group comparisons. Normally distributed data were compared using one-way ANOVA. Data that were not normally distributed were analyzed using Kruskal-Wallis ANOVA. The mean values of two groups of normally distributed data were compared by a t test, and the median values of two groups of data that were not normally distributed were compared using the Mann-Whitney U test. Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables. To analyze the changes of BMI, serum, and histologic variables after the iron reduction therapy, paired Student's t test was used. Logistic regression analysis was used to identify significant factors that influence elevated hepatic 8-oxodG expression in NASH and simple steatosis patients. Categorical variables with more than two levels were coded as dummy variables. All tests were two tailed, and P values <0.05 were considered as statistically significant. Statistical analysis was done using the commercially available software Statistical Package for the Social Sciences 11.5 (SPSS, Inc.).

Results

Clinical Characteristics of the Patients with NASH and Simple Steatosis. The main demographic and clinical laboratory features of the patients with NASH and simple steatosis are compared in Table 1. Patients with NASH were older, and more male and obese subjects than in simple steatosis, but they did not reach the statistical significance. The prevalence of type II diabetes, hypertension, and hyperlipidemia, and serum total cholesterol, triglyceride, and glucose levels were not significantly different between the two groups. Serum aspartate aminotransferase (AST), fasting insulin levels, insulin resistance [assessed by homeostasis model assessment of insulin resistance (HOMA-IR)], and hyaluronic acid were significantly higher in NASH than in simple steatosis. Iron-related serum markers (i.e., serum iron, transferrin saturation, and ferritin) were found to be significantly elevated in NASH compared with those of simple steatosis. Although liver histology showed no significant difference in steatosis degree between the NASH and simple steatosis, hepatic iron deposition was more prominent in NASH; TIS was significantly higher in NASH compared with simple steatosis [3 (0-8) versus 0 (0-5); P < 0.0001].

Hepatic 8-oxodG Levels in NASH and Simple Steatosis Patients. Figure 1A and B showed the 8-oxodG immunohistochemical staining in liver biopsy samples in patients with NASH and simple steatosis, as representative. 8-oxodG immunoreactivity was strongly observed in the nuclei (and weakly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated inflammatory cells in NASH patients' liver biopsy specimen (Fig. 1A). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses at the point of nuclear shape and size. 8-oxodG–immunoreactive hepatocytes were distributed throughout the whole acinus in liver of patients. Using the liver samples of patients with simple steatosis, relatively faint immunoreactivity of 8-oxodG was observed in the nuclei of hepatocytes and was rarely in the cytoplasm (Fig. 1B). As a whole, 8-oxodG–positive hepatocyte counts were significantly higher in NASH patients than in simple steatosis [17.5 (range, 7.3-43.3) versus 2.0 (range, 0.0-13.3) cells/10^5 μm^2; $P < 0.0001$; Fig. 1C]. In the liver of 10 healthy controls, immunoreactivities of 8-oxodG were rarely detected in the nuclei of hepatocytes.

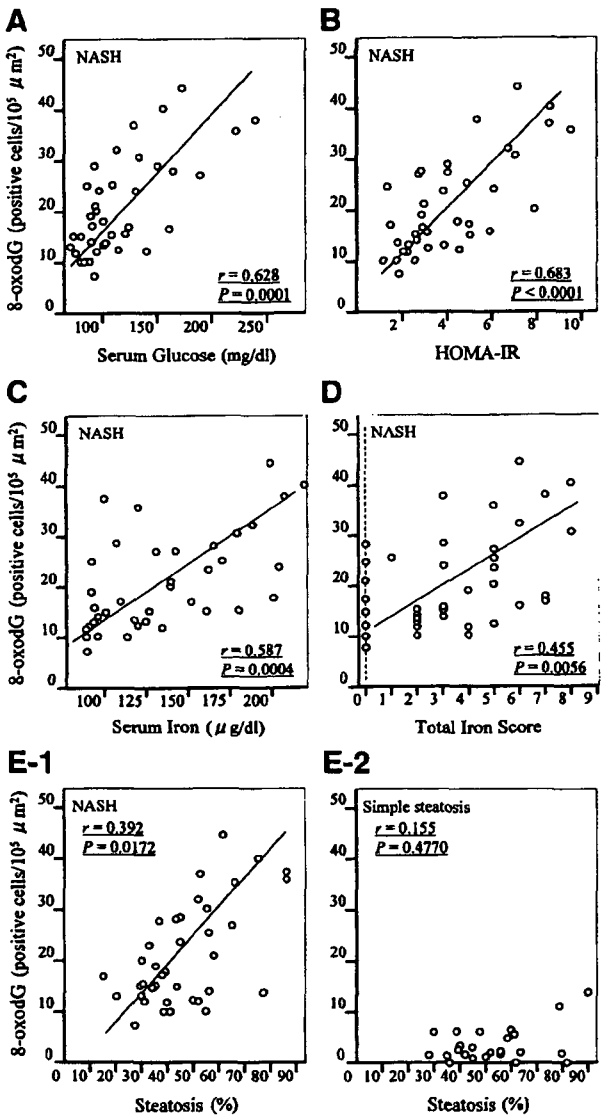

Figure 2. Correlations between 8-oxodG–positive hepatocytic nuclear counts and clinical variables in 38 NASH or 24 simple steatosis patients. **A.** 8-oxodG counts and serum glucose levels in NASH. **B.** 8-oxodG counts and HOMA-IR in NASH. **C.** 8-oxodG counts and serum iron levels in NASH. **D.** 8-oxodG counts and TIS in hepatic tissues in NASH. Dotted vertical line indicates that the TIS is 0. **E-1.** 8-oxodG counts and extent of hepatic steatosis in NASH. **E-2.** 8-oxodG counts and extent of hepatic steatosis in simple steatosis.

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in NASH Patients. To estimate the source of oxidant-generated DNA damage that frequently occurred in the livers of patients with NASH, the correlations of clinical and histologic findings with the degree of hepatic damaged DNA were evaluated, and the results are summarized in Table 2. Patients' age, gender, and BMI were not related to hepatic 8-oxodG counts in NASH patients. Although the 8-oxodG–positive hepatocytic counts were not correlated with serum transaminases, cholesterol, and triglyceride levels, hepatic 8-oxodG levels were elevated in parallel with increase of fasting glucose, serum insulin, and HOMA-IR in patients with NASH [8-oxodG versus glucose ($r = 0.628$, $P = 0.0001$) versus serum insulin ($r = 0.359$, $P = 0.0294$) versus HOMA-IR ($r = 0.683$, $P < 0.0001$); Table 2; Fig. 2A and B]. It is noteworthy that the hepatic 8-oxodG levels were also positively correlated with body and hepatic iron deposition markers; serum iron, transferrin saturation, ferritin, and the hepatic iron deposit grade (i.e., TIS) were significantly correlated with 8-oxodG–positive hepatocyte nucleus counts [8-oxodG versus iron ($r = 0.587$, $P = 0.0004$) versus transferrin saturation ($r = 0.364$, $P = 0.0267$) versus ferritin ($r = 0.325$, $P = 0.0481$) versus TIS ($r = 0.455$, $P = 0.0056$); Table 2; Fig. 2C and D]. Platelet count was also correlated with hepatic 8-oxodG levels, but histologic features (inflammatory activity and fibrosis staging) were not related to hepatic oxidative damage to DNA in patients with NASH. Moreover, elevated hepatocytic 8-oxodG levels were significantly correlated with the extent of hepatic steatosis in patients with NASH (8-oxodG versus steatosis, $r = 0.392$, $P = 0.0172$; Fig. 2E-1), but these two variables were not related in patients with simple steatosis (Fig. 2E-2). The degree of hepatic iron deposition (TIS) and insulin resistance (HOMA-IR) was also correlated mutually in patients with NASH (Fig. 3).

Clinical Variables That Correlate with Hepatic 8-oxodG Levels in Simple Steatosis Patients. The correlations of clinical and histologic findings with the hepatic 8-oxodG levels were also investigated in simple steatosis patients (Table 3). Patients' age and serum ferritin levels were significantly related to hepatic 8-oxodG levels in simple steatosis, but other variables, including HOMA-IR, serum iron levels, and TIS, were not correlated.

Factors Independently Associated with Elevated Hepatic 8-oxodG Levels. To identify the variables independently associated with elevated hepatic 8-oxodG

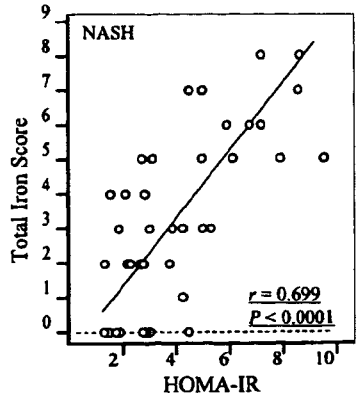

Figure 3. Correlation between TIS in hepatic tissues and HOMA-IR in NASH patients. Dotted horizontal line indicates that the TIS is 0.

levels in NASH and simple steatosis patients, logistic regression analysis was done using the variables recorded in Tables 2 and 3. When the analysis was done in combination NASH and simple steatosis, positive for hepatic iron deposit (i.e., TIS > 0) and insulin resistance (i.e., HOMA-IR > 2) were independent variables contributing to elevated (>10 positive cells/10^5 μm^2) hepatic 8-oxodG (Table 4).

Changes of Serum and Hepatic Histologic Features by Iron Reduction in NASH Patients. To directly evaluate the effect of iron overload to oxidatively generated damage to DNA in the liver of patients with NASH, iron reduction therapy (phlebotomy plus iron-restricted diet) was done in 11 hyperferritinemic NASH patients (7 males and 4 females; range, 39-67 years) and changes of serum and histologic variables were examined (Table 5). A mean blood volume of 1,700 ± 630 mL was removed by 8.5 ± 3.1 venesection times done over a period of 10.8 ± 1.9 months. Serum hemoglobin, iron, and ferritin levels were decreased in all treated patients at the end of iron reduction. Serum alanine aminotransferase (ALT), TIS score, and hepatic 8-oxodG levels were also decreased in most treated patients, and mean values were significantly decreased after the treatment. Serum cholesterol, triglyceride, fasting glucose, and insulin levels were not significantly changed by iron reduction therapy.

Discussion

In this study, we used immunohistochemical approaches using a monoclonal antibody against 8-oxodG in formalin-fixed, paraffin-embedded liver sections for assessment of oxidatively generated damage to DNA in the liver of nonalcoholic fatty liver disease. Using this approach, 8-oxodG-positive signals in liver tissue were detected in all patients with NASH, suggesting that oxidative stress is a frequent event in the liver of NASH patients. At present, a commonly accepted model for the pathogenesis of NASH is the so-called two-hit hypothesis; first hit leads to accumulation of hepatic free fatty acids resulting in a histologic picture of macrovesicular steatosis, and a subsequent second hit may result in liver

Table 3. Correlations between clinical findings and 8-oxodG levels in the liver of patients with simple steatosis (*n* = 24)

Characteristics	8-oxodG (positive cells/10^5 μm^2)	Statistics	
		r	P
Age (y)		0.485*	0.0251*
Gender			
Male (*n* = 11)	2.0 (0.7-13.3)†		NS‡
Female (*n* = 13)	2.0 (0.0-6.3)†		
BMI (kg/m^2)		0.221*	NS*
Laboratory data			
ALT (IU/L)		0.276*	NS*
AST (IU/L)		0.310*	NS*
Total cholesterol (mg/dL)		0.009*	NS*
Triglyceride (mg/dL)		−0.070*	NS*
Glucose (mg/dL)		0.321*	NS*
Serum insulin (microunits/mL)		−0.225*	NS*
HOMA-IR		0.001*	NS*
Hyaluronic acid (ng/mL)		0.360*	NS*
Platelet count (×10^4/mm^3)		−0.265*	NS*
RBC count (×10^4/mm^3)		−0.265*	NS*
Hemoglobin (g/dL)		−0.237*	NS*
Serum iron (μg/dL)		0.094*	NS*
Transferrin saturation (%)		0.141*	NS*
Serum ferritin (ng/mL)		0.577*	0.0082*
Liver histology			
Steatosis§		0.155*	NS*
TIS‖		0.282*	NS*

*Spearman rank correlation test.
† Data are expressed as median (range).
‡ Unpaired Student's *t* test.
§ 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).

Table 4. Factors associated with the elevated hepatic 8-oxodG in NASH and simple steatosis patients by regression analysis

Factors	RR (95% CI)	*P*
TIS > 0	3.69 (2.18-13.97)	0.0088
HOMA-IR > 2	2.61 (1.50-6.46)	0.0273

Abbreviations: RR, relative risk; 95% CI, confidence interval.

injury (3). Although the precise mechanism of how the second hit occurs and concerns in liver disease progression remains unclear, oxidative stress is recognized as the most convincing mediator of second hit in NASH (4-6). Significantly elevated hepatic 8-oxodG in NASH compared with simple steatosis supports the hypothesis that oxidative stress may contribute to the pathogenesis of NASH. Because the hepatocytic 8-oxodG counts were significantly correlated with platelet count, oxidative stress may be related to disease progression in NASH, especially fibrogenesis. Seki et al. (4) also reported that hepatic oxidative stress formation as assessed by the level of 4-hydroxy-2'-2 nonenal was significantly increased with the progression of histologic fibrosis staging in NASH. The degree of hepatic fat deposit seems to be relevant to hepatic oxidative stress formation in NASH because hepatic 8-oxodG levels were positively correlated to the extent of steatosis in NASH. But steatosis alone could not cause the hepatic oxidative stress because the degree of hepatic steatosis was not significantly different between the NASH and simple steatosis, and steatosis and 8-oxodG levels were not correlated in simple steatosis patients. These results clearly indicate that second hit is necessary for the development from simple steatosis to NASH.

Some authors believe that iron may be the substrate of oxidative stress and could be responsible for the second hit in patients with NASH (23, 24). In steatotic livers, the saturation of β-oxidation by excess free fatty acids will ultimately lead to the generation of hydrogen peroxide, which in turn can be converted to highly reactive hydroxyl radicals in the presence of free iron via Fenton reaction (10). Indeed, there is strong evidence, from *in vitro* and *in vivo* studies, that iron overload enhances oxidative stress (25-27). Consistent with several previous findings (7-9), the present data showing that serum iron, transferrin saturation, and ferritin levels and the grade of hepatic iron staining (TIS) are significantly higher in NASH compared with simple steatosis also suggest that iron overload may be responsible for the second hit and pathogenesis of NASH. Quantitative analysis revealed that hepatocytic 8-oxodG levels were significantly correlated with these iron-related markers in NASH, strongly indicating that the increase in the body stored iron is specifically related to increased hepatocytic oxidatively generated damage to DNA in NASH patients.

Because serum insulin and HOMA-IR were significantly higher in NASH than in simple steatosis, and fasting glucose levels and HOMA-IR were significantly correlated with hepatic oxidative damage to DNA in NASH patients, another important factor for hepatic oxidative stress formation in NASH may be insulin resistance, as same as the iron overload. A strong association between iron overload and insulin resistance has been proposed. In fact, Mendler et al. (28) defined a syndrome of "insulin resistance-associated iron overload" in the presence of unexplained hepatic iron overload and at least one component of the insulin resistance. Insulin resistance also seemed to be closely linked to total body iron stores in the general population. Body iron stores are positively associated with the development of glucose intolerance and type 2 diabetes (29, 30). Iron overload and insulin resistance relationship also confirms the fact that iron depletion can improve insulin sensitivity (31-33). Iron overload can interfere with insulin signaling through the induction of reactive oxygen species, the latter impairing insulin uptake through a direct effect on insulin receptor function, by inhibiting the translocation of glucose

Table 5. Profile, phlebotomy, and changes in individual data after iron reduction therapy in patients with NASH

Patient no.	Age/ gender	Phlebotomy period (mo)/volume (mL)	BMI (kg/m^2)		ALT (IU/L)		Hemoglobin (g/dL)		Serum iron (μg/dL)		Ferritin (ng/mL)		TIS		8-oxodG ($/10^5$ μm^2)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	47/M	9/2,200	31.0	29.7	59	32	15.6	14.7	220	172	718	77	8	4	40.0	21.7
2	66/M	9/1,000	26.2	25.9	171	110	14.5	14.2	188	155	431	382	6	8	32.0	39.0
3	67/M	12/1,800	27.3	26.5	98	56	14.2	13.6	141	115	539	123	5	4	27.0	22.3
4	41/F	14/2,400	29.0	27.8	136	97	14.5	13.2	170	122	481	53	5	2	25.3	12.3
5	59/F	13/1,400	35.0	32.2	46	38	14.9	13.6	138	101	223	75	5	1	20.0	12.7
6	41/M	12/2,800	25.1	25.3	122	89	15.3	13.9	92	72	374	68	4	4	19.0	5.7
7	59/F	10/800	23.5	22.2	133	72	12.2	11.5	202	154	847	272	7	5	17.6	6.7
8	54/F	8/1,200	25.2	25.3	82	49	15.7	15.2	124	107	300	109	2	2	13.3	12.0
9	42/M	12/1,800	28.1	28.3	94	42	16.4	14.7	120	77	537	39	5	2	12.3	5.0
10	39/M	9/2,000	28.1	24.5	118	77	16.2	14.3	134	100	306	34	2	0	11.7	3.7
11	64/M	11/1,200	30.4	30.1	37	49	15.0	14.2	96	94	339	46	4	2	10.0	10.7
Mean			28.1*	27.1*	99.6†	64.6†	15.0‡	13.9‡	148§	115§	463‖	116‖	4.8¶	3.1¶	20.7**	13.8**

*Statistically significant difference at $P = 0.0222$ (paired t test).
†Statistically significant difference at $P = 0.0003$ (paired t test).
‡Statistically significant difference at $P < 0.0001$ (paired t test).
§Statistically significant difference at $P < 0.0001$ (paired t test).
‖Statistically significant difference at $P < 0.0001$ (paired t test).
¶Statistically significant difference at $P = 0.0113$ (paired t test).
**Statistically significant difference at $P = 0.0092$ (paired t test).

transporter GLUT4 to the plasma membrane (34, 35). The relation of insulin resistance and iron overload is also important in reverse, as insulin stimulates cellular iron uptake through increased transferrin receptor externalization (36, 37). It is also known that the glycation of transferrin decreases its ability to bind ferrous iron (38) and, by increasing the pool of free iron, stimulates ferritin synthesis. Glycated holotransferrin is additionally known to facilitate the production of free oxygen radicals, which further amplify the oxidative effects of iron (38). Reciprocally, the oxidative stress also induces both insulin resistance [by decreasing internalization of insulin (34)] and increased ferritin synthesis. Therefore, iron overload, insulin resistance, and oxidative stress may amplify each other and may compose the vicious cycle to progress liver injury in NASH.

The above-mentioned results prompted us to investigate the possibility of iron reduction for improvement of hepatic oxidative damage to DNA in NASH. Iron reduction (phlebotomy plus iron-restricted diet) therapy for NASH significantly reduced the serum ALT and hepatic 8-oxodG levels, suggesting the possibility of iron reduction for treatment option for NASH. Recently, Valenti et al. (33) reported that iron reduction also improved insulin resistance in 64 phlebotomized nonalcoholic fatty liver disease patients with hyperferritinemia. A randomized study also suggests that iron reduction may recover insulin action in type 2 diabetic patients (39). But in our treated NASH patients, iron reduction did not significantly affect insulin resistance state. Large randomized controlled studies, considering histology as final outcomes, are nonetheless required to determine the clinical effect of iron reduction therapy in patients with NASH before this therapy can be proposed.

In conclusion, iron overload, insulin resistance, and hepatic oxidatively generated damage to DNA tightly correlate each other in NASH patients, suggesting that these three factors may play an important role in the pathogenesis of NASH. Simple and inexpensive therapies, such as phlebotomy and iron-restricted diet, may be emerging as effective treatment options, which may lead to reduction of hepatocellular carcinoma incidence in NASH patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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