Specificity and quantification of immunohistochemistrical assay

The specificity of the anti-8-OHdG 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 antibody consistently yielded negative staining. Localization of 8-OHdG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/ml of 8-OHdG, but not by over a 1000-fold greater concentration of guanosine (data not shown). When the primary antibody was preincubated with graded 8-OHdG competitively, a similar blocking of immunolabeling was obtained. Further, enzymatic treatment with RNase did not affect the immunoreaction of oxidized DNA. To confirm the quantifiability of this assay, counts of immunohistochemically positive hepatocytes and 8-OHdG levels in the same liver DNA measured by HPLC-ECD were compared in several samples. Significantly positive correlations were observed between these two parameters ($r = 0.654$, $p = 0.0143$) (Fig. 2).

Correlation between hepatic 8-OHdG count and clinical characteristics in CH-C patients

The correlation of clinical findings with the degree of hepatic oxidatively generated DNA damage (the number of 8-OHdG-positive hepatocytes) was evaluated in CH-C patients; and the results are summarized in Table 2. Patients' age, gender, and body mass index were not related with hepatic 8-OHdG counts. Serum transaminase levels were significantly correlated with hepatic 8-OHdG levels (8-OHdG vs ALT, $r = 0.560$, $p = 0.0005$; vs AST, $r = 0.461$, $p = 0.0040$) (Table 2; Figs. 3A and 3B). The histological inflammatory grade was also correlated with hepatic 8-OHdG levels; 8-OHdG count in patients with histological grade 3 (severe hepatitis) was significantly higher than in grade 1 (median = 97.7 vs 46.7 cells/10^5 μm^2, $p = 0.0021$; ANOVA) and grade 2 (vs 35.3 cells/10^5 μm^2, $p = 0.0004$; ANOVA) (Fig. 3C). Hepatic 8-OHdG levels were also significantly related to body and hepatic iron deposition markers. Serum ferritin levels and the hepatic iron deposit grade, i.e., TIS, were strongly correlated with hepatic 8-OHdG counts (8-OHdG vs ferritin, $r = 0.565$, $p = 0.0004$; vs TIS, $r = 0.403$, $p = 0.0119$) (Table 2; Figs. 3D and 3E). Hepcidin, which is exclusively synthesized in the liver, was recently identified as a key regulatory hormone of iron homeostasis, and it was reported to be up-regulated in response to iron overload [27,28]. Therefore, we evaluated the relation of 8-OHdG levels with hepatic hepcidin expression. A statistically significant positive correlation was observed between 8-OHdG levels and hepcidin mRNA levels in the liver of patients with CH-C ($r = 0.516$, $p = 0.0013$) (Table 2; Fig. 3F).

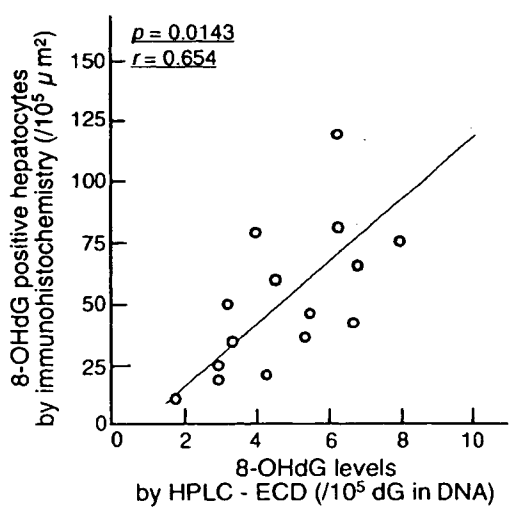

Fig. 2. Correlation of 8-hydroxy-2′-deoxyguanosine (8-OHdG)-positive hepatocyte counts by immunohistochemical staining and 8-OHdG levels quantified by an electrochemical detector coupled to high-pressure liquid chromatography in liver tissue from chronic hepatitis C patients.

Table 2
Correlations between clinical findings and 8-OHdG expression levels in the liver of patients with chronic hepatitis C ($n = 40$)

Characteristics	8-OHdG expression (positive cells/10^5 μm^2)	Statistics r	p values
Age (years)		0.215[a]	0.1791[a]
Gender			
Male ($n = 27$)	55.6 ± 34.8 (15–142)		0.4183[b]
Female ($n = 13$)	47.1 ± 29.9 (11–120)		
Body mass index (kg/m^2)		0.278[a]	0.0821[a]
Laboratory data			
ALT (IU/liter)		**0.560[a]**	**0.0005[a]**
AST (IU/liter)		**0.461[a]**	**0.0040[a]**
Bilirubin (mg/dl)		0.123[a]	0.4437[a]
Total cholesterol (mg/dl)		−0.151[a]	0.3469[a]
Hyaluronic acid (ng/ml)		0.307[a]	0.0550[a]
Platelet count (× 10^4/mm^3)		−0.166[a]	0.3013[a]
Serum iron (μg/dl)		0.161[a]	0.3147[a]
Transferrin saturation (%)		0.147[a]	0.3597[a]
Serum ferritin (ng/ml)		**0.565[a]**	**0.0004[a]**
Serum HCV-RNA (KIU/ml)		0.255[a]	0.1307[a]
Liver histology			
Inflammatory activity			**0.0013[c]**
Fibrosis staging			0.5148[c]
Total iron score		**0.403[a]**	**0.0119[a]**
Hepcidin mRNA levels		**0.516[a]**	**0.0013[a]**

ALT, alanine aminotransferase; AST, aspartate aminotransferase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.
Data are expressed as median (range).
[a] Spearman rank correlation test.
[b] Mann-Whitney U test.
[c] One-way factorial ANOVA and multiple-comparison test.

Relation of hepatic oxidativly generated DNA damage with IFN/ribavirin treatment response in CH-C patients

Among 40 CH-C patients treated with a 24-week course of IFN-α and ribavirin combination therapy, 12 patients (30.0%) were assigned to SVR and the remaining 28 to non-SVR. To examine the effect of hepatic oxidatively damaged DNA on treatment response, clinical variables including hepatic 8-OHdG levels at the start of treatment were compared between SVR and non-SVR patients (Table 3). Patients' age and gender were not

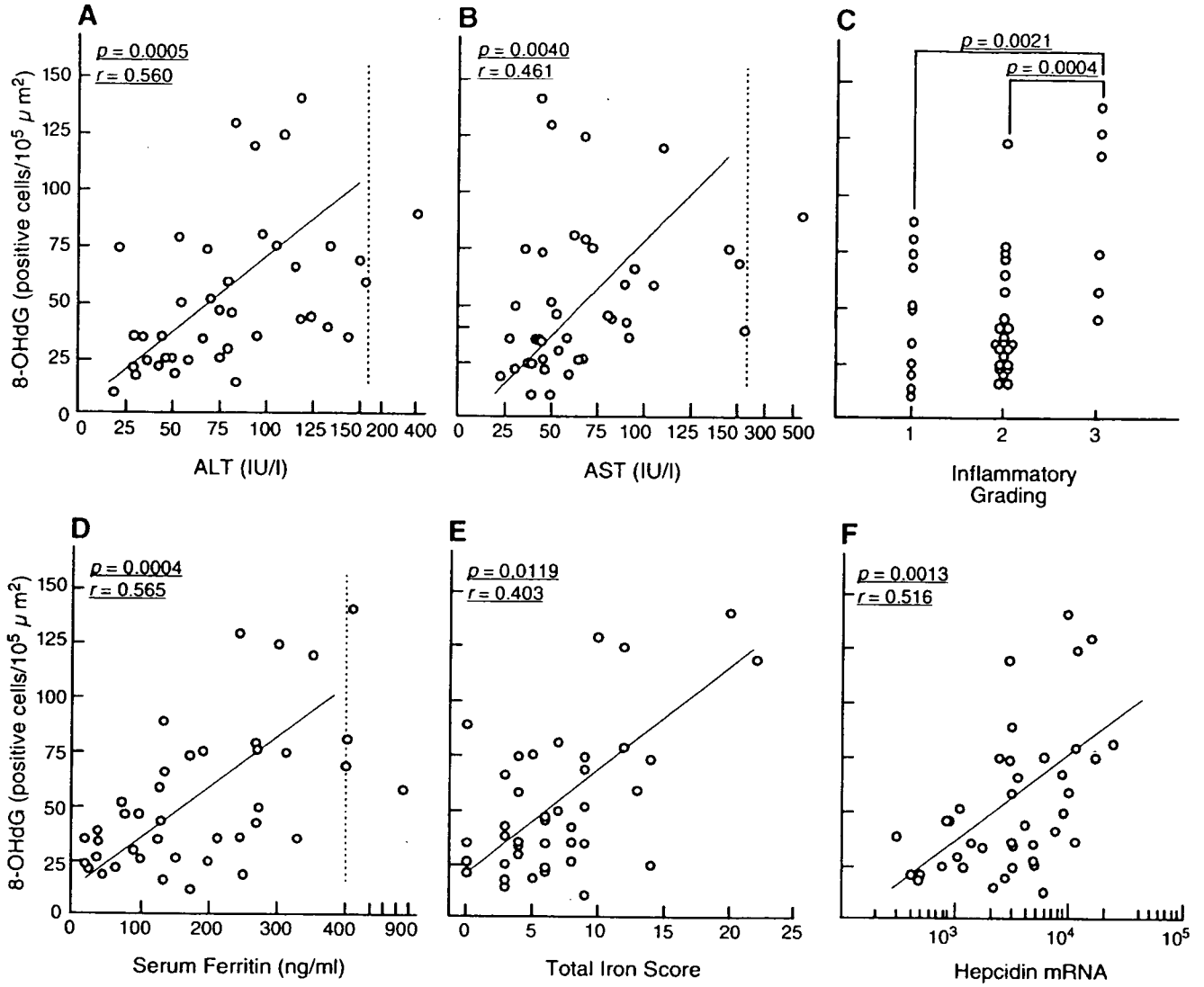

Fig. 3. Correlations between hepatocytic 8-hydroxy-2′-deoxyguanosine (8-OHdG) staining and clinical variables in 40 patients with chronic hepatitis C. (A) 8-OHdG count and serum alanine aminotransferase levels. (B) 8-OHdG count and serum aspartate aminotransferase levels. (C) 8-OHdG count and histological inflammatory activity. (D) 8-OHdG count and serum ferritin levels. (E) 8-OHdG count and total iron score in hepatic tissue. (F) 8-OHdG count and hepcidin messenger RNA levels extracted from hepatic tissue.

related to treatment response. Baseline laboratory data, except red blood cell count, hemoglobin, and hematocrit, were not statistically different between SVR and non-SVR patients. Red blood cell count, hemoglobin, and hematocrit levels were significantly higher in SVR than in non-SVR, but the serum levels of iron and ferritin were not significantly different between the two groups. Histological characteristics (grading, staging, and TIS) were comparable between the SVR and the non-SVR groups. The proportion of patients with a history of previous IFN monotherapy (with no response) was not significantly different between the two groups. Eight patients discontinued the combination therapy before 24 weeks because of side effects, and the therapeutic responses of these patients were all non-SVR except one case, but these differences were not statistically significant (p = 0.3955; Fisher's exact test). Hepatic oxidatively generated DNA damage was more prominent in patients resistant to IFN/ribavirin treatment; hepatic 8-OHdG levels were statistically significant higher in non-SVR patients than in SVR (median = 50.8 vs 32.7 cells/10^5 μm^2, p = 0.0086; Mann-Whitney U) (Fig. 4).

Reduction of hepatic oxidatively generated DNA damage by phlebotomy

Five of 28 CH-C patients that did not respond to IFN/ribavirin therapy underwent phlebotomy after the 6 months of completion of IFN/ribavirin. The age of the 5 patients (three males and two females) ranged from 28 to 67 years (Table 4). A mean blood volume of 2000 ± 810 ml was removed by 6.4 ± 0.9 venesection times performed over a period of 4.3 ± 1.3 months. Compared to baseline (i.e., before the IFN/ribavirin treatment), serum ALT, hemoglobin, iron, and ferritin levels decreased in all patients after phlebotomy. Notably, hepatic 8-OHdG count was also reduced in all patients after phlebotomy, the mean changes

Table 3
Comparison between SVR and non-SVR patients with chronic hepatitis C treated with IFN plus ribavirin ($n = 40$)

Characteristics	SVR ($n = 12$)	Non-SVR ($n = 28$)	p values
Age (years)	51.5 (25–69)	57.0 (28–70)	0.3832[a]
Gender (male/female)	10/2	17/11	0.2714[b]
Body mass index (kg/m^2)	22.7 (17.9–26.1)	23.6 (19.3–28.6)	0.3084[a]
Laboratory data			
ALT (IU/liter)	89.5 (19–166)	73.5 (22–411)	0.2263[a]
AST (IU/liter)	59.5 (23–172)	52.0 (28–565)	0.4428[a]
Bilirubin (mg/dl)	0.6 (0.4–0.7)	0.6 (0.2–5.3)	0.5564[a]
Total cholesterol (mg/dl)	167.5 (99–226)	161.0 (99–226)	0.4970[a]
Hyaluronic acid (ng/ml)	42.8 (9.0–450)	80.1 (9.2–338)	0.1566[a]
Platelet count ($\times 10^4$/mm^3)	17.0 (6.0–26.3)	15.2 (4.9–24.6)	0.3449[a]
Red blood cell count ($\times 10^4$/mm^3)	471.5 (404–516)	422.0 (344–511)	**0.0116**[a]
Hemoglobin (g/liter)	152 (126–160)	137 (114–168)	**0.0086**[a]
Hematocrit (%)	44.3 (38.8–47.6)	41.0 (34.8–47.4)	**0.0090**[a]
Serum iron (μg/dl)	134.0 (42.2–334)	134.5 (68.0–285)	0.6686[a]
Transferrin saturation (%)	39.7 (10.7–80.9)	37.5 (19.9–68.3)	0.5952[a]
Serum ferritin (ng/ml)	221.8 (39.0–884)	134.0 (17.5–444)	0.2813[a]
Serum HCV-RNA (KIU/ml)	710.0 (192–1310)	892.5 (28.1–2100)	0.2875[a]
Liver histology			
Inflammatory activity (0/1/2/3)	0/3/8/1	0/8/15/5	0.6712[c]
Fibrosis staging (0/1/2/3/4)	0/2/6/4/0	1/7/8/8/4	0.4677[c]
Total iron score	5.5 (3–13)	6.5 (0–22)	0.5729[a]
Previous IFN monotherapy (+/−)	3/9	12/16	0.4774[b]
Dropout (+/−)	1/11	7/21	0.3955[b]

SVR, sustained virological response; IFN, interferon; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are expressed as median (range).
[a] Mann-Whitney U test.
[b] Fisher's exact probability test.
[c] Chi-square test.

reaching statistical significance (from 53.4 to 21.1 cells/10^5 μm^2, $p = 0.0125$; paired Student's t test).

Discussion

In the present study, we employed immunohistochemical approaches using a monoclonal antibody against 8-OHdG in formalin-fixed, paraffin-embedded liver sections for assessment of oxidatively damaged DNA in the liver of CH-C patients. The characteristics and the specificity of the monoclonal antibody used in this study (N45.1) have been reported elsewhere [29], and the antibody has been widely used to evaluate oxidatively generated DNA damage in animal and human tissues [29,30]. The specificity of this anti-8-OHdG antibody was confirmed for immunohistochemistry based on the following observations: (i) procedures using nonimmune mouse IgG of the same isotype or PBS instead of N45.1 showed no positivity; (ii) preincubation of N45.1 with 8-OHdG dose dependently suppressed the nuclear staining, and (iii) pretreatment with DNase-free RNase did not reduce nuclear staining. Toyokuni et al. [29] verified that this anti-8-OHdG antibody (N45.1) did not cross-react with the original of deoxyribonucleosides, guanine, other DNA base-modified products such as 8-hydroxy-2′-deoxyadenosine and O^6-methyl-2′-deoxyguanosine, or urine components such as uric acid, creatine, and creatinine by using competitive ELISA assay. The epitope of N45.1 spans and restricts from hydroxyl function (C8) of guanine to the 2-deoxyribose backbone [29]. We applied immunohistochemistry to quantitative evaluation of 8-OHdG amounts by the use of NIH images, because of the risk of artificial production of 8-OHdG during the DNA extraction and hydrolytic processes in HPLC-ECD and gas chromatography/mass spectrometry methods [31].

The mechanisms by which HCV causes liver cell injury remains obscure, but clinical evidence suggests a role for oxidative stress. Patients with CH-C show an increase in the serum or liver content of oxidative stress markers, such as malondialdehyde, 8-isoprostane, and protein carbonyls [7–9,32]. 8-OHdG, a DNA base-modified product generated by hydroxyl radicals, induces G-C to T-A transversion at DNA replication [33], and it is a good marker of oxidatively generated DNA damage in several diseases [5]. In this study, using a highly sensitive immunohistochemical staining with specific antibodies, 8-OHdG-positive signals in the liver tissue were detected in all patients with CH-C, suggesting that oxidative stress is frequent in the liver of chronic HCV-infected patients. A link between oxidative stress and pathogenesis in CH-C is also supported by a pilot study with antioxidant therapy showing improvement in liver cell injury [10]. Despite this evidence, little is known about the mechanisms by which oxidative stress is induced by chronic HCV infection.

To determine which involve the occurrence of hepatic oxidative stress and to evaluate its clinical implications, numerous demographic, laboratory, and histological variables were examined for association with hepatocytic 8-OHdG staining counts in CH-C patients. Quantitative analysis revealed that hepatocytic 8-OHdG expression levels were significantly correlated with serum aminotransferase levels and with the histological grading of necro-inflammation, suggesting a

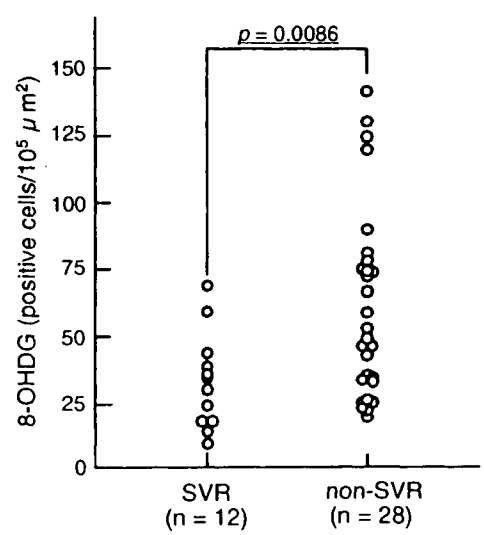

Fig. 4. Comparison between hepatocytic 8-hydroxy-2′-deoxyguanosine (8-OHdG) count in sustained virological responders (SVR) and those in non-SVR patients treated with interferon plus ribavirin. Baseline 8-OHdG count was significantly higher in non-SVR than in SVR in chronic hepatitis C.

Table 4
Profile, phlebotomy, and changes in individual data after phlebotomy in patients with chronic hepatitis C

Patient no.	Age/ gender	Phlebotomy period/volume	ALT (IU/liter)		Hemoglobin (g/liter)		Serum iron (μg/dl)		Ferritin (ng/ml)		8-OHdG (/10^5 μm^2)	
			Before	After	Before	After	Before	After	Before	After	Before	After
1	48/M	3M/2800 ml	54	33	149	135	137	82	279	32.0	78.7	32.7
2	67/F	4.5M/1400 ml	56	27	136	123	100	70	277	17.8	49.7	18.7
3	62/M	6M/2000 ml	106	49	140	132	162	120	172	43.2	75.7	25.3
4	28/M	5M/2800 ml	73	58	145	134	120	66	73.8	21.7	45.0	12.7
5	62/F	3M/1000 ml	42	33	127	120	68	42	64.0	7.9	22.3	16.0
Mean			66[a]	40[a]	139[b]	129[b]	117[c]	56[c]	173[d]	24.5[d]	54.3[e]	21.1[e]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; SVR, sustained virological response; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.
[a] Statistically significant different at $p = 0.0353$ (paired t test).
[b] Statistically significant different at $p = 0.0015$ (paired t test).
[c] Statistically significant different at $p = 0.0432$ (paired t test).
[d] Statistically significant different at $p = 0.0295$ (paired t test).
[e] Statistically significant different at $p = 0.0125$ (paired t test).

possible link between hepatic oxidatively generated DNA damage and necro-inflammation in CH-C. These results are in accord with reports showing that plasma levels of lipid peroxidation products are correlated with aminotransferase levels in CH-C [32]. It is unclear whether oxidative stress is the cause or the consequence of liver injury, but it has been demonstrated that oxidative stress can directly activate Kupffer cells, causing the release of inflammatory and profibrogenic cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) [34], that can cause hepatic injury. Thus, oxidatively generated DNA damage may be partly responsible for the pathological findings of CH-C. Sumida et al. [35] reported that oxidative stress as assessed by the serum thioredoxin levels in CH-C were significantly correlated with the serum levels of fibrosis markers (hyaluronic acid, type IV collagen-7S domain, procollagen III peptide). In this study, the hepatocytic 8-OHdG counts were not significantly correlated with hepatic fibrosis as assessed by the serum hyaluronic acid, platelet count, and histological staging; it may be due to the relatively small number of patients ($n = 40$).

It is possible that proteins expressed by HCV itself may be the source of hepatic oxidative stress in CH-C patients. Indeed, it has been demonstrated that expression of the HCV core, NS3, or NS5A proteins induce ROS formation and altered the liver antioxidant status in several transgenic mouse models and in human hepatoma cells lines [11–15]. However, in humans, it is not clear whether ROS production is mediated directly by the action of HCV proteins. In this study, no difference in hepatocytic 8-OHdG counts was observed among patients with different HCV-RNA levels, and thus it can be concluded that the production of 8-OHdG in the liver is not influenced by the amount of HCV proteins.

It is plausible that ROS production during chronic HCV infection is an effect of high iron levels in hepatic tissue, because patients with CH-C commonly have elevated serum and liver iron levels [16,17], and free iron is known to be a potent catalyzer of oxygen free radicals. In the present study, hepatic 8-OHdG counts were significantly correlated with the serum ferritin levels, which reflect the total body iron store [36]. The liver is the major storage site for iron and is particularly susceptible to iron overload; hepatic iron amount as assessed by the TIS values was significantly correlated with hepatic 8-OHdG levels. Our data therefore suggest that the increase in the body (especially in the liver) store iron is specifically related to increased hepatocytic oxidatively damaged DNA in HCV-positive patients. Although our findings do not establish any cause and effect relationship among hepatic iron deposition, accumulation of hepatic oxidative stress, and liver injury, these findings suggest that HCV-mediated liver damage is at least in part a consequence of abnormally high free iron overload in the liver. The mechanisms involved in such a modification of iron metabolism associated with chronic HCV infection remain unclear. Increased intestinal iron absorption in CH-C has been previously suggested [37]. Recently, hepcidin, which is exclusively synthesized in hepatocytes, was identified as an important regulator of iron release into the system by duodenal enterocytes and reticuloendothelial macrophages [38,39]. It was clearly demonstrated that change in hepcidin expression is inversely associated with intestinal iron absorption [28]. In this study, significant correlation was demonstrated between hepatic 8-OHdG count and hepatic hepcidin expression in CH-C patients, suggesting a direct involvement of iron overload in hepatic oxidative stress in CH-C.

Factors such as sex, age, serum HCV-RNA titer, HCV genotype, and staging of hepatic fibrosis have been suggested as an independent predictor of IFN monotherapy response [40]; however, these factors are not predictors of therapeutic response to IFN/ribavirin combination therapy, except for HCV genotype. In this study, red blood cell count, hemoglobin, and hematocrit levels were significantly higher in SVR than in non-SVR patients at the start of therapy, and there were no patients, except one case, achieving SVR among the patients dropping out because of the side effects. Patients that receive combination therapy frequently develop moderate-to-severe hemolytic anemia, which may lead to ribavirin dose reduction or treatment discontinuation [41]. Therefore, high hemoglobin levels at the start of combination therapy may be required for treatment completion. The data shown in Fig. 4 demonstrate that the hepatocytic 8-OHdG counts are significantly higher in non-SVR than in SVR to IFN/ribavirin therapy. Oxidative stress, therefore, may be

another important factor responsible for resistance to IFN/ribavirin therapy. The mechanism responsible for treatment resistance induced by hepatic oxidative DNA stress remains unclear. Serum ferritin levels and TIS were not significantly different between SVR and non-SVR groups, suggesting that treatment resistance is not just the result of iron overload in CH-C. Further study is required to determine the relationship between the oxidative stress and the IFN-based treatment response in CH-C patients.

Removal of hepatic excess iron by phlebotomy has been recently accepted as a useful treatment for improving serum transaminase levels [42,43] and histological findings [44] without reducing serum HCV-RNA titers in CH-C patients. Our study clearly demonstrated that iron reduction by phlebotomy, sequentially performed after 6 months of IFN/ribavirin therapy, is indeed effective for decreasing 8-OHdG in hepatocytes, with concomitant suppression of body iron overload. Furthermore, we demonstrated that not only the mean values but also the values in each patient show a significant decrease. This result also suggests the direct involvement of an excessive iron store in the production of hepatocytic DNA oxidative stress in CH-C. Because the hepatocytic 8-OHdG counts were compared before and after the IFN/ribavirin and phlebotomy therapy was performed, whether the reduction of hepatocytic DNA damage was due to IFN/ribavirin therapy, phlebotomy, or its combination is not known. However, this effect was most probably due to phlebotomy, because we have previously demonstrated that hepatocytic 8-OHdG count is not reduced after 6 months of IFN monotherapy if patients were not assigned to SVR to the IFN [25], and Kato et al. [45] also demonstrated that iron reduction therapy alone (phlebotomy and low iron diet therapy) significantly reduces hepatic 8-OHdG levels in patients with CH-C. If hepatic 8-OHdG levels were depleted by phlebotomy, patients phlebotomized before IFN plus ribavirin therapy might have a better response to IFN/ribavirin. Phlebotomy in combination with IFN monotherapy was reported to improve antiviral efficacy compared with IFN alone [46]. However, a combination of IFN/ribavirin and phlebotomy seems to be more difficult to carry out without dropout, because phlebotomy frequently decreases serum hemoglobin levels [44]. Desferroxamine or dietary iron restriction, which is also effective for serum ALT reduction in CH-C patients [47], may be effective for reduction of hepatocytic oxidative stress and may improve response to IFN/ribavirin therapy. Additional studies are warranted to determine whether these combination therapies are effective in CH-C patients.

In conclusion, this study showed that iron accumulation in HCV-related chronic hepatitis is clinically relevant in hepatocytic oxidatively generated DNA damage, which was correlated with hepatic inflammation, and response to IFN/ribavirin therapy. Iron reduction therapy is a safe and potentially promising therapeutic modality for patients with CH-C and is associated with the prevention of oxidatively generated damage to DNA in hepatocytes. Although the ultimate therapeutic goal is the eradication of HCV from the body, long-term iron reduction may be an adjunctive therapy in CH-C patients.

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Hepcidin Expression in the Liver: Relatively Low Level in Patients with Chronic Hepatitis C

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Patients with chronic hepatitis C frequently have serum and hepatic iron overload, but the mechanism is unknown. Recently identified hepcidin, exclusively synthesized in the liver, is thought to be a key regulator for iron homeostasis and is induced by infection and inflammation. This study was conducted to determine the hepatic hepcidin expression levels in patients with various liver diseases. We investigated hepcidin mRNA levels of liver samples by real-time detection-polymerase chain reaction; 56 were hepatitis C virus (HCV) positive, 34 were hepatitis B virus (HBV) positive, and 42 were negative for HCV and HBV (3 cases of autoimmune hepatitis, 7 alcoholic liver disease, 13 primary biliary cirrhosis, 9 nonalcoholic fatty liver disease, and 10 normal liver). We analyzed the relation of hepcidin to clinical, hematological, histological, and etiological findings. Hepcidin expression levels were strongly correlated with serum ferritin ($P < 0.0001$) and the degree of iron deposit in liver tissues ($P < 0.0001$). Hepcidin was also correlated with hematological parameters (vs. hemoglobin, $P = 0.0073$; vs. serum iron, $P = 0.0012$; vs. transferrin saturation, $P < 0.0001$) and transaminase levels ($P = 0.0013$). The hepcidin-to-ferritin ratio was significantly lower in HCV$^+$ patients than in HBV$^+$ patients ($P = 0.0129$) or control subjects ($P = 0.0080$). In conclusion, hepcidin expression levels in chronic liver diseases were strongly correlated with either the serum ferritin concentration or degree of iron deposits in the liver. When adjusted by either serum ferritin values or hepatic iron scores, hepcidin indices were significantly lower in HCV$^+$ patients than in HBV$^+$ patients, suggesting that hepcidin may play a pivotal role in the pathogenesis of iron overload in patients with chronic hepatitis C.

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INTRODUCTION

Iron is an essential element for all living organisms, being a requirement in a wide range of metabolic processes including DNA synthesis, oxygen transport, and energy production, but excess iron can be harmful to the organism, in part through the generation of oxygen radicals, and is potentially lethal (1). Therefore, iron homeostasis must be tightly regulated in all organisms. Recent work has established the importance of the peptide hormone hepcidin in iron homeostasis as a negative regulator of iron release into the system by duodenal enterocytes and reticuloendothelial macrophages (2,3). Hepcidin binds to the iron exporter ferroportin, which results in ferroportin internalization and degradation (4). In addition to its response to iron homeostasis, hepcidin is induced by inflammation (5), an effect believed to be dependent on cytokine production (6); how hepcidin levels are kept in balance through upstream signaling pathways is still under investigation.

Iron accumulation in the liver, where hepcidin is exclusively synthesized, is common in patients with chronic liver diseases (7), especially in patients with chronic hepatitis C virus (HCV) infection (8,9). Increased hepatic iron concentration is present in 10% to 36% of patients with chronic hepatitis C (8,9), and hepatic iron overload is even more common among patients with end-stage liver disease due to hepatitis C (10,11). Excess iron deposition in the liver is known to be hepatotoxic and may exacerbate liver injury (12) and be resistant to interferon-based therapy in patients with chronic hepatitis C (13,14); however, little is known about the mechanism of iron accumulation in the liver. We previously reported that transferrin receptor 2 (TfR2), which was recently identified as the second receptor for transferrin (15), was higher in the liver of patients with chronic hepatitis C compared than those with chronic hepatitis B (16). TfR2 function was thought at first to be consistent with its homology to classical transferrin receptor-TfR1 (15,17); iron uptake by the hepatocytes [TfR2 has limited tissue distribution, with prominent expression in the liver, especially in hepatocytes

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(15,18)]. But a disabling mutation in the *TfR2* gene in humans (19,20) and mice (18) leads to significant hepatic iron accumulation despite an absence of expression of TfR2, suggesting that iron uptake by the hepatocytes is not a principal role of TfR2, and that TfR2 has a important but unknown role for maintenance of iron homeostasis. Recently, Kawabata et al. (21) demonstrated that hepcidin is downregulated in *TfR2* mutant mice, suggesting that TfR2 may be the upstream sensor for hepcidin production in the pathway of iron homeostasis.

In view of these considerations, we decided to examine the gene expression of hepcidin in liver samples from patients with various liver conditions. In addition, we assessed the relationship of hepcidin gene expression with clinical, hematological, histological, and etiological findings.

MATERIALS AND METHODS

Liver Samples

We used 132 liver samples in this study. Fifty-six samples were obtained from patients with chronic HCV infection (HCV^+ group; positive serum HCV RNA and negative serum hepatitis B surface antigen, or HBsAg), and 34 were from patients with chronic hepatitis B virus (HBV) infection [HBV^+ group; positive serum HBsAg and negative serum HCV antibody]. Forty-two samples were obtained from patients without HCV or HBV infections (HCV^-HBV^- group; 3 cases of autoimmune hepatitis, 7 alcoholic liver disease with ethanol intake > 80 g/day [range, 95-155 g/day] in the 10 years that preceded the hospital admission, 13 primary biliary cirrhosis, 9 nonalcoholic fatty liver disease, and 10 normal liver). In cases of normal liver, surgically resected liver specimens were obtained during operation for metastatic liver cancer, while the other liver specimens were obtained by needle biopsy for diagnosis of chronic liver diseases. Liver biopsy was performed under stable conditions, without fever of unknown etiology, and before any interferon therapy. Social drinkers were included in HCV^+ and HBV^+ groups. Serum iron parameters were determined by routine automated laboratory methods on the day of liver tissue sampling. History of blood transfusions, use of iron-containing medications, and daily consumption of alcohol were investigated in all patients. Informed consent was obtained from each patient, and the study was approved by the Mie University Ethics Committee and carried out according to the guidelines of the 1975 Declaration of Helsinki.

Histological Evaluation

Liver biopsy specimens were divided in two parts. One portion was fixed in buffered formalin and embedded in paraffin for histological examination, and the other was immediately frozen and stored at –80°C for RNA extraction. Hematoxylin & eosin, Masson's trichrome, and Perls' Prussian blue staining for iron were performed. Liver histology was evaluated by two pathologists who were blinded to clinical conditions or hepcidin expression levels. Liver specimens were scored for stage of liver fibrosis and grade of inflammatory activity according to the classification of Desmet et al. (22). The histological quantification of iron was done according to Deugnier et al. (23) by scoring iron separately within hepatocytes (hepatic iron score, or HIS, 0 to 36), sinusoidal cells (sinusoidal iron score, or SIS, 0 to 12), and portal tracts or fibrotic tissue (portal iron score, or PIS, 0 to 12). The total iron score (TIS), 0 to 60, was the sum of these scores. This score is highly correlated with the biochemical hepatic iron index and hepatic iron concentration measured by atomic absorption spectrophotometry in patients with chronic liver diseases (24,25).

mRNA Preparation and Reverse Transcription

mRNA was extracted from liver tissue using the SV RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, liver tissue samples were homogenized in a Dounce glass homogenizer with lysis buffer (4 M guanidine thiocyanate, 10 mM Tris-HCl, pH 7.5, 0.97% β-mercaptoethanol). Dilution buffer (350 µL) was added to the homogenized solution and incubated at 70°C for 3 min. After separating by centrifugation, the supernatant was precipitated using ethanol. RNA was treated with DNase (Boehringer Mannheim, Mannheim, Germany) to remove any contaminating genomic DNA. Precipitated RNA was purified by spin column assembly and dissolved in 100 µL DEPC-treated water containing 10 mM DTT and 200 U/mL RNase inhibitor using a siliconized tube. The amount of mRNA was determined by spectrophotometry. The cDNA was generated by reverse-transcription of 2 µg adjusted RNA, with random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA, USA) for 30 min at 48°C.

Quantification of Hepatic Hepcidin mRNA Expression Levels by RTD-PCR

The hepatic mRNA levels of hepcidin were determined by TaqMan real-time detection-polymerase chain reaction (RTD-PCR). RTD-PCR primers and probes were designed using Primer Express software (Applied Biosystems, Tokyo, Japan), which spanned introns to avoid coamplification of genomic DNA. The sequences were forward primer 5'-TTCCCCATCTGCATTTTCTG-3', reverse primer 5'-TCTACGTCTTGCAGC ACATCC-3', and FAM/TAMURA probe 5'-TGCGGCTGCTGTCATCGATCAA-3'. cDNA of liver tissue (5 µL) was incubated with 20.75 µL TaqMan Master Mix (Perkin Elmer, Yokohama. Japan) (8% glycerol; 10× TaqMan buffer; 10 mM each dCTP, dATP, and dGTP; 20 mM dUTP; 0.01 U/µL AmpErase uracil *N*-glycosylase; 25 mM $MgCl_2$; and 0.025 U/µL AmpliTaq Gold DNA polymerase), 10 µM forward primer, 10 µM reverse primer, and 5 µM probe. The reaction mixture was brought up to a final volume of 50 µL with RNase-free distilled water. The amplification was performed using the ABIPRISM 7700 sequence detection system (Applied Biosystems). Amplification conditions were 2 min at

50°C, 10 min at 95°C, 53 cycles of 15 s at 95°C, and then 1 min at 60°C. Data were analyzed using Sequence Detector 1.6 software (Applied Biosystems). The results for hepcidin mRNA are expressed as the amount relative to that of GAPDH mRNA quantified simultaneously in each liver sample. Experiments were performed in triplicate, and the amount of RNA was calculated from a standard curve drawn using serial dilutions of total RNA extracted from a wedge-resected liver specimen. Standard samples were run in parallel during each analysis. This method is able to measure hepcidin mRNA linearly from 10^2 to more than 10^5/GAPDH mRNA.

Statistical Analysis

Data are expressed as the median and range or mean ± standard deviation. Categorical variables were compared using the chi-squared test or Fisher exact test. Continuous variables were compared using the Student *t* test, one-way factorial ANOVA test, Wilcoxon rank-sum test, and Kruskal-Wallis test. Correlation was assessed by Spearman rank correlation. Two-sided *P* values < 0.05 were considered statistically significant. Calculations were performed using StatView of JMP software (SAS Institute).

RESULTS

Clinical Characteristics of the Patients

Clinical characteristics of patients in this study according to viral infection (HCV^+, HBV^+, and HCV^-HBV^-) are shown in Table 1. There was no statistical difference in age and sex distribution in the three groups. Serum hemoglobin, iron levels, transferrin saturation, and ferritin levels were significantly higher in HCV^+ group than in the HCV^-HBV^- non–iron overload subgroup, but there was no statistical difference between the HCV^+ group and the HCV^-HBV^- iron overload subgroup (elevated serum ferritin levels, > 220 ng/mL for men and > 100 ng/mL for women). Regarding liver histological findings, patients with chronic viral infection (HCV^+ and HBV^+) had more progressive grading and staging scores than patients without viral infection (HCV^-HBV^-). Iron deposition in the liver was prominent in chronic HCV-infected patients, and TIS scores were significantly higher in HCV^+ than in HBV^+ or HCV^-HBV^- non–iron overload patients.

Correlation between Hepatic Hepcidin mRNA Expression Levels and Clinical Findings

Hepatic mRNA expression levels of hepcidin were measurable in all 132 patients. Correlation of clinical findings with hepatic hepcidin expression levels, normalized by GAPDH mRNA, was evaluated, and the results summarized in Table 2. Patients' age was not related to hepcidin expression levels. Hepcidin levels were significantly higher in men than in women (10,900 ± 13,100, median 6590, vs. 5820 ± 10,700, median 2600, $P = 0.0012$, Wilcoxon rank-sum test). Hepcidin levels were not significantly different between the HCV^+, HBV^+, and

Table 1. Baseline characteristics.

			HCV^-HBV^- ($n = 42$)		*P*		
	HCV^+	HBV^+	Non–iron overload	Iron overload	HCV^+ vs. HBV^+	HCV^+ vs. HCV^-HBV^- non–iron overload	HCV^+ vs. HCV^-HBV^- iron overload
n	56	34	24	18			
Age, y	56.0 ± 13.7	50.4 ± 15.2	55.0 ± 17.5	53.4 ± 16.9	NS	NS	NS
Sex, M/F	33/23	23/11	9/15	8/10	NS	NS	NS
ALT, IU/L	76.9 ± 67.7	104 ± 131	63.4 ± 67.7	149 ± 157	NS	NS	0.0472
AST, IU/L	80.6 ± 85.0	110 ± 141	81.5 ± 126	183 ± 238	NS	NS	NS
Bilirubin, mg/dL	1.50 ± 3.60	1.26 ± 1.59	1.64 ± 3.88	5.90 ± 11.7	NS	NS	NS
RBC, $\times 10^4/mm^3$	418 ± 71	430 ± 56	401 ± 54	407 ± 77	NS	NS	NS
Hemoglobin, g/L	133 ± 19	137 ± 17	118 ± 19	131 ± 21	NS	0.0014	NS
Hematocrit, %	40.0 ± 5.2	41.6 ± 4.9	37.7 ± 5.3	38.8 ± 6.6	NS	NS	NS
Serum iron, µg/dL	131 ± 48	123 ± 50	60 ± 37	99 ± 53	NS	<0.0001	NS
Transferrin saturation, %	39.3 ± 15.7	40.7 ± 17.0	19.5 ± 13.5	38.7 ± 22.8	NS	<0.0001	NS
Serum ferritin, ng/mL	244 ± 162	140 ± 101	91 ± 76	585 ± 784	NS	<0.0001	NS
Liver histology							
Inflammatory activity, 0/1/2/3[a]	1/23/28/4	1/12/14/7	9/13/2/0	2/13/1/2	NS	<0.0001	0.0052
Fibrosis staging, 0/1/2/3/4[b]	2/19/14/10/11	0/6/10/9/9	9/10/4/1/0	6/9/0/1/2	NS	0.0002	0.0016
TIS[c]	7.36 ± 5.35	4.41 ± 4.49	1.21 ± 2.00	7.72 ± 8.50	0.0163	<0.0001	NS

Data are means ± SD. ALT, alanine aminotransferase; AST, apsartate aminotransferase; NS, not significant; RBC, red blood cell count. [a]Intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [b]0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [c]Total iron score proposed by Deugnier et al. (23).

Table 2. Correlations between clinical findings and hepcidin mRNA expression levels in liver of patients with various liver conditions (n = 132).

Characteristic	Hepcidin mRNA level			Statistics	
	n	Mean ± SD	Median	r	P
Age, y				0.003	0.9764
Sex					0.0012[a]
Male	73	10,900 ± 13,100	6590		
Female	59	5820 ± 10,700	2600		
Virus infection					
Male					
HCV^+	33	9120 ± 7660	6650		
HBV^+	23	11,800 ± 16,100	2600		
HCV^-HBV^-					0.0611[b]
Non-iron overload	9	7750 ± 5320	6590		
Iron overload	8	19,600 ± 23,000	15,800		
Female					
HCV^+	23	5890 ± 7900	3690		
HBV^+	11	3000 ± 2190	2360		
HCV^-HBV^-					0.0724[b]
Non-iron overload	15	1300 ± 1030	814.5		
Iron overload	10	15,500 ± 20,800	6485		
Total					
HCV^+	56	7790 ± 7850	5730		
HBV^+	34	8950 ± 13,900	3290		
HCV^-HBV^-					0.7733[b]
Non-iron overload	24	3720 ± 4550	1650		
Iron overload	18	17,300 ± 21,200	6770		
ALT, IU/L				0.281	0.0013
AST, IU/L				0.249	0.0043
Serum albumin, g/dL				-0.036	0.7844
Bilirubin, mg/dL				0.239	0.0065
Hyaluronic acid, ng/mL				0.145	0.0968
RBC, $\times 10^4/mm^3$				0.171	0.0510
Hemoglobin, g/L				0.235	0.0073
Hematocrit, %				0.160	0.0675
Platelets, $\times 10^4/mm^3$				-0.174	0.0465
Serum iron, µg/dL				0.283	0.0012
Transferrin saturation, %				0.360	<0.0001
Serum ferritin, ng/mL				0.832	<0.0001
Liver histology					
Inflammatory activity, 0/1/2/3[c]				0.073	0.4011
Fibrosis staging, 0/1/2/3/4[d]				0.031	0.7209
TIS[e]				0.457	<0.0001

ALT, alanine aminotransferase; AST, apsartate aminotransferase; RBC, red blood cell count. Statistics are Spearman rank correlation test unless otherwise noted. [a]Wilcoxon rank-sum test. [b]Kruskal-Wallis test. [c]Intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [d]0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [e]Total iron score proposed by Deugnier et al. (23).

HCV^-HBV^- groups (HCV^+, 7790 ± 7850, median 5730; HBV^+, 8950 ± 13,900, median 3290; HCV^-HBV^-, 9560 ± 15,700, median 3150) (Figure 1). Serum alanine aminotransferase (r = 0.281, P = 0.0013) (Figure 2a) and aspartate aminotransferase (r = 0.249, P = 0.0043) levels were marginally correlated with hepcidin mRNA levels when all patients were included in the statistical evaluation.

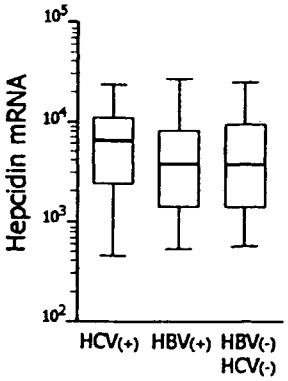

Figure 1. Hepatic mRNA expression levels of hepcidin in various liver diseases. Hepatic hepcidin mRNA expression levels were compared between HCV^+, HBV^+, and HCV^-HBV^- groups. Graphs depict the median (line within the box), 25th to 75th percentiles (upper and lower border of the box), and 10th and 90th percentiles (whiskers).

Serum bilirubin concentrations were marginally correlated with hepcidin levels (r = 0.239, P = 0.0065), but serum albumin and hyaluronic acid were not correlated (Table 2). Hepcidin expression levels were correlated with hemoglobin concentrations (r = 0.235, P = 0.0073), serum iron levels (r = 0.283, P = 0.0012) (Figure 2b), and serum transferrin saturation (r = 0.360, P < 0.0001) (Figure 2c). Serum ferritin levels were strongly and positively correlated with hepatic hepcidin mRNA expression levels in various patients with liver disease (r = 0.832, P < 0.0001) (Figure 2d). In this study, 4 patients (3 HCV^-HBV^- and 1 HCV^+; enclosed by open square in Figure 2d), had relatively low hepcidin expression levels with severe hyperferritinemia, suggesting the possibility of hepcidin dysregulation in these patients. Clinical features of these cases are summarized in Table 3. When the exceptional cases were excluded from analysis because of possible hemochromatosis traits, the correlation statistic was improved to r = 0.916 and P < 0.0001 (n = 128). There were no significant correlations between hepatic hepcidin mRNA levels and inflammatory activity score or fibrosis staging in liver biopsy specimens. A significant

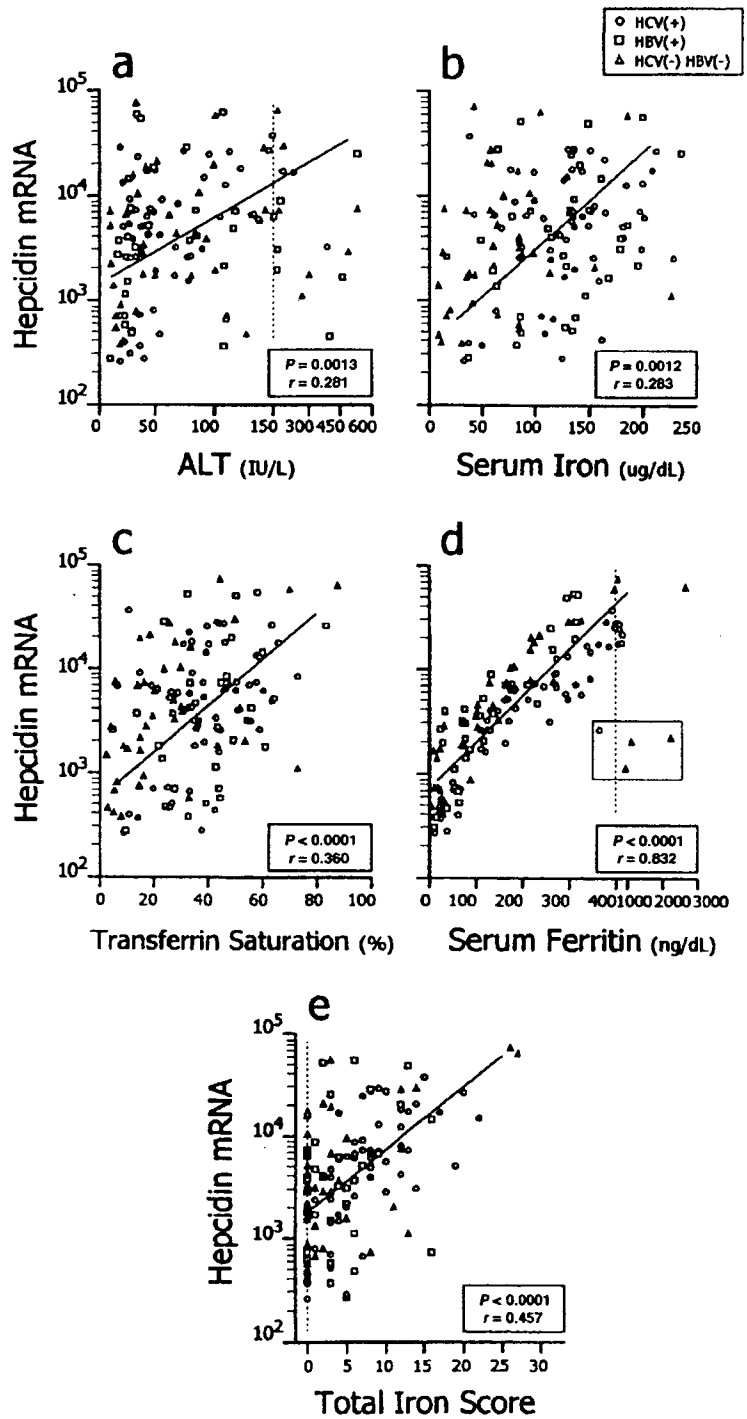

Figure 2. Correlations between hepatic hepcidin mRNA expression levels and clinical laboratory and histological data in patients with various liver diseases. Relationship between hepcidin mRNA expression levels and serum alanine aminotransferase levels (a), serum iron levels (b), transferrin saturation (c), serum ferritin levels (d), and total iron score of liver tissues (e) in 132 patients. ○, HCV^+; □, HBV^+; △, HCV^-HBV^-.

positive correlation was found between hepatic hepcidin mRNA expression levels and the degree of iron deposition in the liver as evaluated by TIS score (r = 0.457, $P < 0.0001$) (Figure 2e).

Comparison of mRNA Expression Levels of Hepcidin in Patients with Various Liver Diseases

Hepcidin expression has been reported to increase in response to iron overload in an experimental setting (2,26). Our findings of strong positive correlations between hepatic hepcidin mRNA expression levels and serum ferritin and hepatic iron levels also suggest feedback to hepcidin expression against iron overload in humans, and the ratio of hepcidin levels per iron overload may be constant in various liver conditions. To evaluate the relative amounts of hepcidin in relation to iron overload, we calculated the ratio of liver hepcidin mRNA/serum ferritin levels in each patient. The ratio of hepcidin/serum ferritin was significantly lower in the HCV^+ group (26.8 ± 15.4, median 23.3) than in the HBV^+ group (46.5 ± 41.0, median 31.6; P = 0.0129) or the HCV^-HBV^- group (44.7 ± 33.7, median 34.0; P = 0.0080) (Figure 3a). The relative amount of hepatic hepcidin per hepatic iron deposition (the ratio of hepcidin mRNA/TIS) was also significantly lower in the HCV^+ group (1280 ± 2160, median 773) than in the HBV^+ group (2920 ± 4600, median 1130; P = 0.0392) or the HCV^-HBV^- group (3320 ± 4210, median 1870; P = 0.0098) (Figure 3b). The ratios of hepcidin/serum ferritin and hepcidin/TIS were not statistically different between the HBV^+ and HCV^-HBV^- groups. These results indicate that hepatic hepcidin expression levels in relation to body iron store are lower in HCV-infected patients compared with HBV-infected or uninfected patients.

DISCUSSION

Hepcidin, exclusively synthesized in the liver, was originally isolated from human serum and urine as having an antimicrobial activity (27,28). The lack of hepcidin expression in knockout mice leads to iron overload (29), and

Table 3. Patients with relatively low hepcidin expression levels in hyperferritinemia.

Patient no.	Age, y	Sex	Group	Ethanol intake, g/day	ALT, IU/L	Hemoglobin, g/L	Platelets, $\times 10^4/mm^3$	Serum iron, μg/dL	Ferritin, ng/mL	TIS[a]
1	52	M	HCV^+	64	78	149	17.7	228	371	6
2	72	M	HCV^-HBV^-: ALD	140	102	135	9.7	155	1110	11
3	56	M	HCV^-HBV^-: ALD	140	287	143	20.7	227	978	13
4	62	M	HCV^-HBV^-: normal	0	11	103	26.4	61	2335	0

ALT, alanine aminotransferase; ALD alcoholic liver disease. [a]Total iron score proposed by Deugnier et al. (23).

conversely, overexpression of hepcidin in transgenic mice causes severe iron deficiency (30). Moreover, hepcidin mutations are associated with a new type of severe juvenile hemochromatosis not related to *HFE* mutations (31). In normal mice, iron overload increases and iron deficiency decreases hepatic mRNA expression of hepcidin; change in hepcidin expression is associated with inverse changes in intestinal iron absorption (26,32). Recently, it was demonstrated that hepcidin can bind ferroportin, the major cellular iron exporter protein, inducing ferroportin internalization and degradation and resulting in reduced iron efflux from enterocytes (4). Thus, hepcidin is thought to be a major negative regulatory hormone for iron homeostasis.

In the present study, we investigated hepcidin expression in the liver of patients with chronic liver diseases and normal subjects and analyzed correlations between clinical parameters and hepatic hepcidin expression levels. Hepcidin mRNA levels were strongly and positively correlated with serum ferritin levels and the degree of hepatic iron accumulation as assessed by TIS. These results are consistent with presently elucidated hepcidin functions; when iron storage increases, serum ferritin elevates, and hepatic hepcidin is upregulated, leading to decreased intestinal iron absorption for maintenance of iron homeostasis. Our study demonstrated a relationship between serum hemoglobin, iron, transferrin saturation levels, and hepcidin mRNA levels, supporting the hypothesis of an impact of anemia and/or hypoxia on hepcidin expression, as reported in mice (5,33). However, we did not find a significant correlation between red blood cell count or hematocrit levels and hepcidin levels in our study, suggesting additional regulatory mechanisms of hepcidin may exist. Serum alanine aminotransferase and aspartate aminotransferase levels were positively correlated with hepatic hepcidin expression levels, indicating that hepatic inflammatory status also may influence the expression levels of hepcidin in patients with chronic liver disease, although the histological inflammatory activity score was not related to hepcidin levels. We examined the relationship between parameters reflecting hepatic function and hepcidin expression. Although serum albumin levels and hepatic fibrosis status (histological staging and hyaluronic acid) were not correlated with hepcidin levels, serum bilirubin was positively correlated with hepcidin. This correlation may be caused by the positive relationship of serum bilirubin levels and accumulation of hepatic iron in our patients (bilirubin vs. TIS, $r = 0.305$, $P = 0.0005$; Spearman). Further study is necessary to determine the participation of hepcidin in liver function.

From these results, that several clinical factors were associated with hepatic hepcidin expression, it is suggested that multiregulatory mechanisms act to alter hepatic expression of hepcidin. First, our results of a strong positive relationship between hepatic hepcidin expression and serum ferritin and liver TIS leads to the idea that hepcidin is regulated by body-stored iron, most strongly. Second, hep-

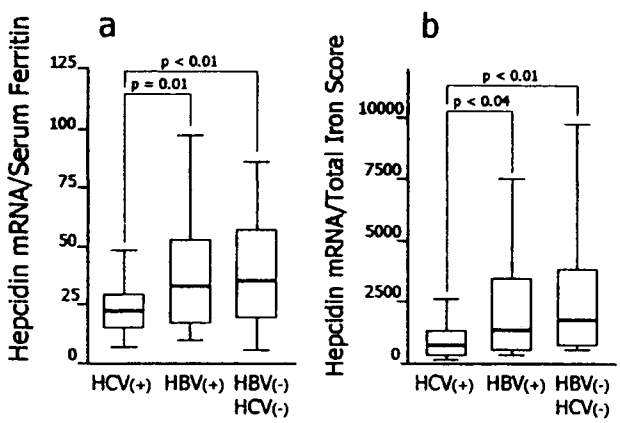

Figure 3. Relative hepatic mRNA expression levels of hepcidin in various liver diseases. (a) The ratio of hepatic hepcidin mRNA/serum ferritin was calculated in each patient and compared between HCV^+, HBV^+, and HCV^-HBV^- groups. (b) The ratio of hepatic hepcidin mRNA/hepatic total iron score was calculated in each patient and compared between HCV^+, HBV^+, and HCV^-HBV^- groups. Graphs depict the median (line within the box), 25th to 75th percentiles (upper and lower border of the box), and 10th and 90th percentiles (whiskers).

cidin expression is regulated by sensing the iron amount in circulation, because hepcidin levels were also correlated with serum iron and transferrin saturation levels. It is reported that hepcidin expression is greatly diminished in *TfR2*-mutated hemochromatosis patients (34) and mice (21) despite elevated iron stores. Thus, TfR2 appears to be an upstream regulator of hepcidin and is required for hepcidin to respond appropriately to changes in serum transferrin saturation. Therefore, TfR2 may act as a communicator between iron status in serum and hepcidin production.

Third, inflammatory status in the liver may influence hepatic hepcidin expression. Hepcidin is the key mediator of anemia due to chronic inflammation (3,5), and its mRNA expression is increased in response to inflammatory stimuli such as lipopolysaccharide or interleukin-6 (IL-6) (6,33). Recently, it was demonstrated that Kuppfer cells, which release IL-6, are required for the activation of hepcidin synthesis during inflammation in the liver, but not for regulatory activity by iron conditions (35). The positive correlation between hepatic hepcidin levels and serum aminotransferase levels in our study also supports the existence of this regulatory pathway, although serum IL-6 levels were not evaluated in our study. Thus, there are at least three major, distinct mechanisms for regulation of hepcidin: by body-stored iron condition, serum iron status, and inflammatory condition.

Previous studies demonstrated that chronic HCV infection is frequently associated with elevated serum and liver iron storage markers (8-11). In our study, serum iron levels and transferrin saturation in HCV^+ patients were significantly higher than those without viral infection, and TIS was significantly higher in the HCV^+ group than in the HBV^+ or HCV^-HBV^- groups. Hepatocellular iron uptake may be upregulated during chronic inflammation. It has been previously demonstrated that inflammatory cytokines enhance TfR1-mediated iron uptake by hepatocytes (36). However, considering that the serum aminotransferase levels and hepatic inflammatory score are not statistically different between HCV^+ and HBV^+ groups and that TIS is more prominent in HCV^+ than in HBV^+ patients, chronic hepatic inflammation alone does not seem to be responsible for hepatic iron accumulation in patients with chronic hepatitis C. HCV infection itself seems to have a direct influence on hepatic iron accumulation. In this study, hepcidin expression in relation to serum ferritin and the hepatic TIS were significantly lower in the HCV^+ group than in the HBV^+ or HCV^-HBV^- groups, suggesting that upregulation of hepatic hepcidin expression by increased body-stored iron may be relatively diminished in the HCV-infected liver. We previously reported that TfR2 expression in the liver was significantly higher in the HCV^+ patients than in the HBV^+ patients (16), although its clinical implication for iron accumulation in the liver of patients with chronic hepatitis C was unknown. It was reported that *TfR2* disabling mutations in humans (34) and mice (21) decrease hepcidin expression. Therefore, at first we expected that hepcidin expression would be higher in HCV patients (higher TfR2 expression group) than the other patients; the result was opposite. Because hepcidin and TfR2 expression in the liver were inversely correlated in our study (data not shown), upregulation of TfR2 may involve the downregulation of hepcidin in the liver of patients with chronic hepatitis C. Further in vitro study is required to clarify the distinct interaction between TfR2, hepcidin, and the degree of intracellular iron content in hepatocytes during chronic HCV infection.

Patients with known HFE genetic hemochromatosis expected to exhibit abnormal hepcidin regulation (37,38) were excluded in this study, because no patient had HFE mutations C282Y or H63D. We could not role out the other types of hereditary hemochromatosis completely (caused by mutations of hepcidin, hemojuvelin, TfR2, and ferroportin) (39). In this study, four patients had relatively low hepatic hepcidin expression levels with severe hyperferritinemia, suggesting hemochromatosis traits (Table 3). In Japan, one patient with HFE-hemochromatosis and a few patients with non–HFE-hemochromatosis and ferroportin disease have been reported during the last 10 years (40-43). Therefore, further investigation should be necessary in these patients with hepcidin dysregulation.

Aoki et al. (44) also reported that hepatic hepcidin expression is increased in response to iron overload in patients with chronic hepatitis C. We have extended the measurement of hepcidin expression in HBV^+ and noninfected patients and compared it to data from HCV^+ patients, showing the relatively low levels of hepcidin in patients with chronic hepatitis C.

In conclusion, we evaluated hepcidin mRNA expression in the liver of patients with various liver conditions. Despite the heterogeneity of our patients, hepcidin levels were related to hepatic and body iron stores, hematological parameters, and serum transaminase levels, suggesting that multiregulatory mechanisms act in hepcidin production. Relatively low levels of hepatic hepcidin expression for the degree of iron burden may be involved in the pathophysiologic mechanism of increased iron overload in patients with chronic hepatitis C, and supplementation of hepcidin may be beneficial for these conditions.

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Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C

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Although the oxidative stress frequently occurs in patients with chronic hepatitis C, its role in future hepatocellular carcinoma (HCC) development is unknown. Hepatic 8-hydroxydeoxyguanosine (8-OHdG) was quantified using liver biopsy samples from 118 naïve patients who underwent liver biopsy from 1995 to 2001. The predictability of 8-OHdG for future HCC development and its relations to epidemiologic, biochemical and histological baseline characteristics were evaluated. During the follow-up period (mean was 6.7 ± 3.3 years), HCC was identified in 36 patients (30.5%). Univariate analysis revealed that 16 variables, including 8-OHdG counts (65.2 ± 20.2 *vs* 40.0 ± 23.5 cells per 10^5 μm^2, $P < 0.0001$), were significantly different between patients with and without HCC. Cox proportional hazard analysis showed that the hepatic 8-OHdG ($P = 0.0058$) and fibrosis ($P = 0.0181$) were independent predicting factors of HCC. Remarkably, 8-OHdG levels were positively correlated with body and hepatic iron storage markers (*vs* ferritin, $P < 0.0001$ *vs* hepatic iron score, $P < 0.0001$). This study showed that oxidative DNA damage is associated with increased risk for HCC and hepatic 8-OHdG levels are useful as markers to identify the extreme high-risk subgroup. The strong correlation between hepatic DNA damage and iron overload suggests that the iron content may be a strong mediator of oxidative stress and iron reduction may reduce HCC incidence in patients with chronic hepatitis C.
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Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and the death rate due to this tumour has been increasing over the past 20–30 years in the Unites States (El-Serag, 2002) and in Japan (Nishioka *et al*, 1991). Chronic infection with hepatitis C virus (HCV) has been recognised as an increased risk of HCC; approximately 20% of HCV-infected individuals have diseases that progress to cirrhosis, and about 40% of these patients develop HCC after a mean of 10–15 years (Seeff, 2002). Consequently, surveillance programmes based on periodic ultrasound examination and serum α-fetoprotein determination are recommended for patients with chronic hepatitis C. However, the effectiveness of these protocols has not been fully assessed and they afford no contribution to improvement of clinical outcomes in patients with chronic hepatitis C (Gebo *et al*, 2002). Therefore, it is desirable to establish a useful marker that could identify cases at high risk of developing HCC among chronic HCV-infected patients.

Although the mechanisms underlying HCC development during chronic HCV infection have been widely investigated, they are still unclear. It has been reported that structural and nonstructural proteins of HCV, such as Core and NS3, play a role in cell transformation, using *in vitro* cell culture systems (Sakamuro *et al*, 1995; Ray *et al*, 1996) and transgenic animals (Moriya *et al*, 1998). But whether expression of HCV protein(s) directly induces HCC in chronic HCV-infected patients is unknown. Recently, it has been assumed that oxidative stress may be relevant to this process of HCV-induced carcinogenesis, as has been suggested in several other malignancies (Kasai, 1997). Considerable data suggest that reactive oxygen species (ROS) may play a pathogenic role in carcinogenesis (Crawford and Cerutti, 1985). The most damaging species among the many ROS is the hydroxyl radical. The hydroxyl radical has been shown to be responsible for a number of base modifications that include thymine glycol, thymidine glycol (Cathcart *et al*, 1984), 5-(hydroxylmethyl)uracil (Hollstein *et al*, 1984), and also 8-hydroxydeoxyguanosine (8-OHdG) (Shigenaga *et al*, 1989; Kasai, 1997). 8-Hydroxydeoxyguanosine is a modification of guanine that induces a point mutation in the daughter DNA strands (Kuchino *et al*, 1987; Shibutani *et al*, 1991) and it is therefore used as a marker of oxidatively generated DNA damage in several diseases (Shigenaga *et al*, 1989; Kasai, 1997). In patients with chronic hepatitis C, increased 8-OHdG in DNA extracted from liver tissue was also reported (Shimoda *et al*, 1994; Mahmood *et al*, 2004; Fujita *et al*, 2007). These reports suggest that oxidative stress may be involved in the progression of liver disease, but they showed no direct participation of oxidative stress in hepato-

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carcinogenesis in the liver of HCV-infected patients. Also, no information is available on whether ROS-mediated damage to DNA is useful for prediction of HCC development in chronic hepatitis C patients.

In view of these considerations, we have examined the influence of the degree of ROS-mediated hepatic DNA damage as measured by counts of 8-OHdG immunohistochemically positive hepatocyte nuclei on the prevalence of future HCC development in chronic HCV-infected patients. Moreover, we evaluated the relation of the degree of ROS-mediated DNA damage with the epidemiologic, biochemical, and histological findings in chronic hepatitis C.

PATIENTS AND METHODS

Patients with chronic hepatitis C

This study comprised 118 consecutive patients (66 males and 52 females; mean age 55.8 ± 10.8 years) recruited between January 1995 and October 2001 with HCV-related chronic hepatitis (Table 1). All patients fulfilled the following inclusion criteria: (1) liver injury caused by chronic HCV infection. All patients had persistently elevated serum alanine aminotransferase (ALT) levels and were seropositive for both anti-HCV antibody (the third-generation enzyme-linked immunosorbent assay; Ortho Diagnostic Systems, Raritan, NJ, USA) and HCV-RNA (Amplicor-HCV assay; Roche Molecular Diag. Co., Tokyo, Japan). (2) Liver biopsy. Liver tissue was obtained by percutaneous needle biopsy in all patients for diagnostic purposes. (3) Follow-up without interferon (IFN)-based therapy. The follow-up consisted of monthly blood tests and monitoring of tumour markers at the outpatients clinic of our department, and ultrasonography and dynamic computed tomography were performed at regular intervals. As it is known that IFN treatment reduces the incidence of HCC in patients with chronic hepatitis C (Nishiguchi *et al*, 1995), patients with a history of previous IFN-based treatment were excluded from the study. None of them had received any antiviral therapy during the follow-up period.

Exclusion criteria were as follows: a family history of haemochromatosis; haemolytic disease; serological markers for hepatitis B virus (HBV) (hepatitis B surface antigen and hepatitis B core antibody); or human immunodeficiency virus infection. Patients with concurrent diseases or those taking medications that may interfere with free radical production, such as nonsteroidal anti-inflammatory drugs, vitamins and iron-containing drugs, were excluded from the study. Patients with chronic alcohol consumption of ethanol in excess of 40 g day^{-1} for male and 20 g day^{-1} for female for at least 5 years were also excluded. All patients had no HCC or other cancers, by an initial screening examination. 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.

Clinical parameters were obtained for each patient at the time of liver biopsy: age; sex; body mass index; duration of HCV infection (when contamination was very probable and a precise data; transfusion or drug addiction in the past year); alcohol intake; biochemical, haematological, iron-related, and virological serum markers; and liver histological findings and 8-OHdG immunoreactivity.

The diagnosis of HCC was made by several imaging methods (ultrasonography, dynamic computed tomography, arteriography, or magnetic resonance imaging) and confirmed histologically in 22 cases. Time to HCC occurrence was defined as the interval between the date of liver biopsy and the detection of tumour, death without HCC occurrence, or the last examination until 31 October 2006. All patient deaths were considered end points irrespective of cause of death. The mean follow-up period was 6.7 ± 3.3 (range, 0.4–11.8) years.

Table 1 Baseline characteristics of patients with chronic hepatitis C

Characteristics	Chronic hepatitis C ($N = 118$)
Age (years)	55.8 ± 10.8 (57.5)
Gender (M/F)	66/52
Laboratory data	
ALT (IU l^{-1})	73.5 ± 53.4 (58.0)
AST (IU l^{-1})	70.1 ± 41.8 (61.5)
Platelet count ($\times 10^4$ mm^{-3})	14.9 ± 5.9 (14.6)
Serum HCV-RNA (kIU ml^{-1}) ($N = 89$)	1570 ± 1240 (1420)
HCV genotype (1a/1b/2a/2b) ($N = 60$)	0/53/5/2
Liver histology	
Inflammatory activity (0/1/2/3)[a]	1/41/49/27
Fibrosis staging (0/1/2/3/4)[b]	1/29/26/27/35
Total iron score[c]	7.75 ± 5.80 (7.00)
8-OHdG-positive hepatocytes (per 10^5 μm^2)	48.4 ± 26.2 (42.5)

Data are expressed as mean ± s.d. (median). ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; 8-OHdG = 8-hydroxydeoxyguanosine. [a]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [b]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [c]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

Histological evaluation

All of the liver biopsy samples were stained with haematoxylin–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* (1994). The histological quantification of hepatic iron was carried out according to Deugnier *et al* (1992) 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) using liver samples stained with Perls' Prussian blue. 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 (Piperno *et al*, 1998; Silvia *et al*, 2005).

Immunohistochemical study

Immunohistochemical staining of 8-OHdG was performed as previously described (Fujita *et al*, 2007). Mouse monoclonal antibody against 8-OHdG (Japanese Aging Control Institute, Shizuoka, Japan) and Alexa 488-labelled goat antibody against mouse IgG (Molecular Probes, Eugene, OR, USA) 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 (Vers. 1.62, National Institute of Health, image program).

Statistical analysis

Results were expressed as mean ± s.d. or median. Comparisons between groups were performed using the Mann–Whitney *U*-test or Kruskal–Wallis test for continuous variables and the χ^2 or Fisher's exact test for categorical data. Correlation coefficients between numerical variables were calculated as Spearman's rank test. Cumulative HCC incidence curves were determined using the Kaplan–Meier method and the differences between groups were

assessed with the log-rank test. Cox proportional hazard regression analysis was used to identify significant factors that influence future HCC development. All tests were two-tailed, and *P*-values less than 0.05 were considered as statistically significant. Statistical analyses were performed using the SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

RESULTS

In situ detection of 8-OHdG-positive hepatocytes using biopsy samples

In the liver of patients with chronic hepatitis C, 8-OHdG immunoreactivity was strongly observed in the nuclei (weakly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated lymphocytes (Figure 1A). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses at the point of nuclear shape and size. The number of 8-OHdG-positive hepatocytes in patients with chronic hepatitis C was counted from 7 to 123 cells per $10^5\,\mu m^2$, the median being 42.5 cells per $10^5\,\mu m^2$. Using the liver samples of patients with simple fatty liver as controls, immunoreactivity of 8-OHdG was faintly observed in the nuclei of hepatocytes in this experimental setting (Figure 1B). The specificity of the anti-8-OHdG 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-OHdG antibody consistently yielded negative staining. Localisation of 8-OHdG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with $25\,ng\,ml^{-1}$ of 8-OHdG, but not by over a thousand-fold greater concentration of guanosine. Further, enzymatic treatment with RNase did not affect the immunoreaction of oxidised DNA.

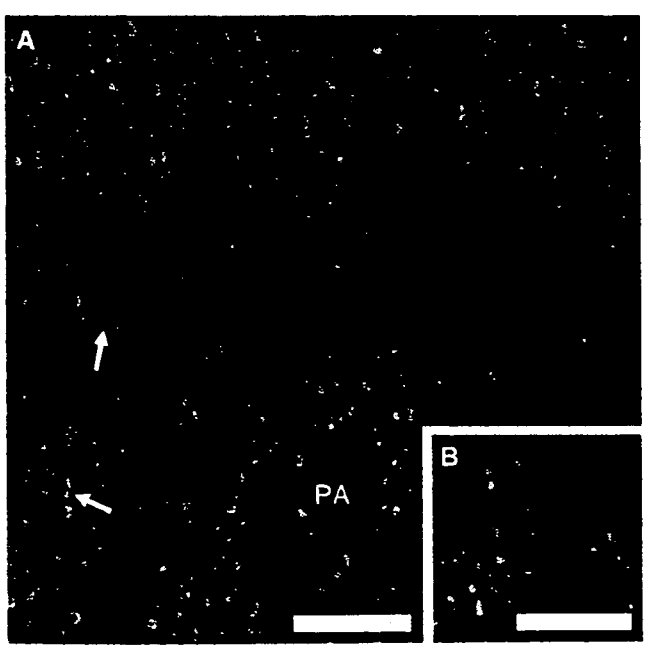

Figure 1 8-Hydroxydeoxyguanosine immunohistochemical staining in liver tissue from chronic hepatitis C and control (simple fatty liver) patients. (**A**) In the liver of chronic hepatitis C patient, 8-OHdG immunoreactivity was strongly observed throughout the whole acinus (PA = portal area) and mainly in the nuclei of hepatocytes and Kupffer cells (arrows in (**A**)). (**B**) In the liver of control (simple fatty liver), immunoreactivity of 8-OHdG was weak in the nuclei of hepatocytes. Scale bar, 100 μm in (**A**) and (**B**).

Analysis of factors associated with the occurrence of HCC in patients with chronic hepatitis C

Until the end of follow-up (mean was 6.7 ± 3.3 years), HCC occurrence was identified in 36 patients (30.5%) in this study. Seven patients died with no sign of HCC. The overall cumulative incidence of HCC was 3.4, 12.0, 17.2, and 38.9% at 1, 3, 5, and 10 years, respectively. To examine the effect of degree of liver oxidative DNA damage on HCC development during chronic HCV infection, clinical variables, including hepatic 8-OHdG quantification, were compared between patients who developed HCC and those who did not develop (non-HCC group) during the follow-up (Table 2). No significant difference was found in the patient age, body mass index, alcohol consumption, serum albumin levels, red blood cell count, and HCV genotype distribution between patients with and without HCC. In the group of patients with HCC, the proportion of male subjects, duration of infection, serum ALT, aspartate aminotransferase (AST), total bilirubin, hyaluronic acid, haemoglobin, iron, transferrin saturation, and ferritin levels at liver biopsy were significantly higher, and HCV-RNA titres and platelet count were significantly lower, than in the group of

Table 2 Comparison of epidemiologic and clinical variables of patients who developed HCC and patients who remained free of HCC during the follow-up period

Characteristics	HCC group (*N* = 36)	Non-HCC group (*N* = 82)	*P*-value
Age (years)	57.3 ± 8.2	54.7 ± 11.4	0.3718[a]
Gender (M/F)	26/10	40/42	**0.0182**[b]
Body mass index ($kg\,m^{-2}$)	23.6 ± 3.5	24.1 ± 3.2	0.6657[a]
Duration of HCV infection (years) (*N* = 58)	31.7 ± 10.5	26.9 ± 9.8	**0.0463**[a]
Alcohol intake ($g\,day^{-1}$)	21.0 ± 37.0	21.2 ± 38.9	0.6221[a]
Laboratory data			
ALT ($IU\,l^{-1}$)	91.9 ± 50.4	65.6 ± 52.9	**0.0021**[a]
AST ($IU\,l^{-1}$)	91.4 ± 42.7	60.5 ± 38.3	**0.0003**[a]
Serum albumin ($g\,dl^{-1}$)	3.65 ± 0.40	3.75 ± 0.45	0.1235[a]
Total bilirubin ($mg\,dl^{-1}$)	0.96 ± 0.29	0.75 ± 0.88	**<0.0001**[a]
Hyaluronic acid ($ng\,ml^{-1}$)	206 ± 138	132 ± 151	**0.0003**[a]
Platelet count ($\times 10^4\,mm^{-3}$)	11.7 ± 4.5	16.4 ± 5.8	**<0.0001**[a]
Red blood cell count ($\times 10^4\,mm^{-3}$)	429 ± 48	418 ± 50	0.1993[a]
Haemoglobin ($g\,dl^{-1}$)	13.9 ± 1.3	13.2 ± 1.6	**0.0302**[a]
Serum iron ($\mu g\,dl^{-1}$)	151 ± 68	121 ± 62	**0.0320**[a]
Transferrin saturation (%)	45.7 ± 22.6	36.2 ± 20.0	**0.0289**[a]
Serum ferritin ($ng\,ml^{-1}$)	264 ± 158	151 ± 149	**0.0002**[a]
Serum HCV-RNA ($kIU\,ml^{-1}$) (*N* = 89)	844 ± 900	1720 ± 1260	**0.0068**[a]
HCV genotype (1a/1b/2a/2b) (*N* = 60)	0/5/2/0	0/48/3/2	0.1100[b]
Liver histology			
Inflammatory activity (0/1/2/3)[c]	0/4/18/14	1/37/31/13	**0.0015**[b]
Fibrosis staging (0/1/2/3/4)[d]	0/1/3/10/22	1/28/23/17/13	**<0.0001**[b]
Total iron score[e]	11.09 ± 4.75	6.23 ± 5.62	**<0.0001**[a]
8-OHdG-positive hepatocytes (per $10^5\,\mu m^2$)	65.2 ± 20.2	40.0 ± 23.5	**<0.0001**[a]

Data are expressed as mean ± s.d. HCC = hepatocellular carcinoma; ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; 8-OHdG = 8-hydroxydeoxyguanosine. [a]Mann–Whitney *U*-test. [b]Fisher's exact test, otherwise χ^2 test. [c]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [d]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [e]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

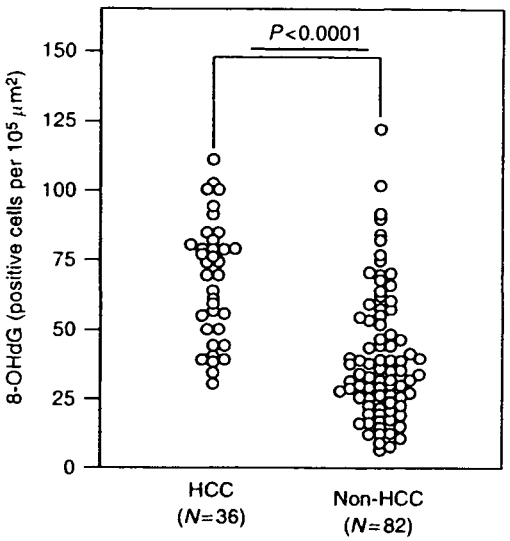

Figure 2 Comparison between 8-OHdG counts in patients who developed HCC ($N=36$) and those who remained free of HCC (non-HCC, $N=82$) during the follow-up period. Baseline 8-OHdG counts were significantly higher in the HCC group than in the non-HCC group in patients with chronic hepatitis C.

Table 3 Factors associated with the occurrence of HCC in patients with chronic hepatitis C by Cox proportional hazard regression analysis

Factor	Odds ratio	95% CI	*P*-value
Count of 8-OHdG-positive hepatocytes (each 10 cells per $10^5\,\mu m^2$ increase)	1.487	1.12–1.97	0.0058
Fibrosis staging (each stage 1 increase)	4.090	1.27–13.15	0.0181

HCC = hepatocellular carcinoma; 8-OHdG = 8-hydroxydeoxyguanosine; CI = confidence interval.

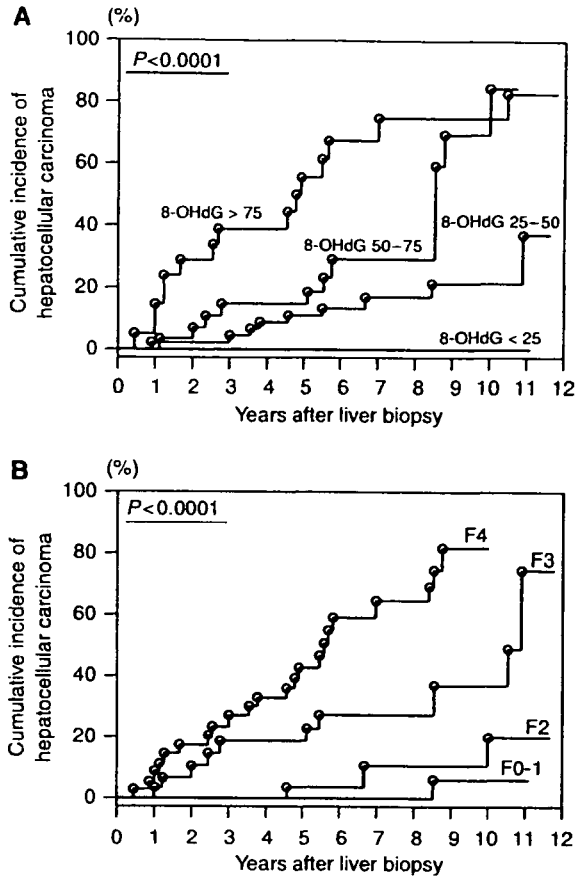

Figure 3 Cumulative incidence of HCC in 118 patients with chronic hepatitis C. Incidence curves were determined using the Kaplan–Meier method and statistical analysis was performed using the long-rank test. (**A**) Cumulative incidence of HCC divided by degrees of hepatic 8-OHdG expression levels. (**B**) Cumulative incidence of HCC divided by degrees of histological hepatic fibrosis staging score.

patients without HCC during the follow-up. The histological grading and staging scores were significantly higher in the HCC group than in the non-HCC group. The prevalence of hepatic iron deposits in patients with HCC was also significantly greater than that in non-HCC patients. Hepatic 8-OHdG expression levels in patients who developed HCC were significantly higher than in those who did not develop HCC (65.2 ± 20.2 *vs* 40.0 ± 23.5 positive cells per $10^5\,\mu m^2$, $P<0.0001$; Mann–Whitney *U*-test) (Figure 2). When hepatic steatosis was evaluated by scoring system as 0, no steatosis; 1, <33% of hepatocytes with steatosis; 2, 33–66% of hepatocytes affected; 3, >66% of hepatocytes affected, the degree of steatosis was not significantly different between HCC and non-HCC groups.

To examine the independent factors that affect the development of HCC, Cox proportional hazard regression analysis was performed using the 16 variables that were significantly different between HCC and non-HCC groups by univariate analyses. The multivariate analysis identified two factors as independent factors for HCC development: degree of hepatocytic 8-OHdG immunoreactivity (odds ratio, 1.487 (each 10 positive cells per $10^5\,\mu m^2$ increase); $P=0.0058$) and histological staging (odds ratio, 4.090 (each stage 1 increase); $P=0.0181$) (Table 3). When the patients were stratified according to the degree of hepatic 8-OHdG counts and histological fibrosis staging, the cumulative incidence of HCC was significantly increased in proportion to these variables (long-rank test) (Figure 3A and B). The cumulative incidences of HCC of 3, 5, and 10 years were 0, 0, and 0% in 8-OHdG counts of <25 (cells per $10^5\,\mu m^2$) subgroup ($n=23$), 4.4, 11.1, and 21.8% in 25–50 (cells per $10^5\,\mu m^2$) subgroup ($n=45$), 14.2, 14.2, and 84.9% in 50–75 (cells per $10^5\,\mu m^2$) subgroup ($n=29$), and 38.8, 55.5, and 74.6% in >75 (cells per $10^5\,\mu m^2$) subgroup ($n=21$), respectively.

Correlation between hepatocytic 8-OHdG counts and clinical characteristics in patients with chronic hepatitis C

To estimate the cause of hepatic oxidative DNA damage, correlation of clinical findings with hepatic 8-OHdG levels was evaluated (Table 4). The age of patients, body mass index, duration of infection, alcohol consumption, and the serum HCV-RNA titre were not related to the degree of oxidative DNA damage. 8-Hydroxydeoxyguanosine immunoreactivity was significantly higher in male than in female patients. Serum transaminases, platelet count, histological inflammation grade, and fibrosis stage were significantly correlated with the hepatic 8-OHdG levels. It is noteworthy that the hepatic 8-OHdG levels were strongly and positively correlated with body and hepatic iron deposition markers; serum ferritin levels and the hepatic iron deposit grade, that is, TIS, were strongly correlated with hepatic 8-OHdG count (8-OHdG *vs* ferritin, $r=0.640$, $P<0.0001$; *vs* TIS, $r=0.768$,

Table 4 Correlations between clinical findings and 8-OHdG levels in the liver of patients with chronic hepatitis C ($N = 118$)

Characteristics	Hepatic 8-OHdG levels (positive cells per $10^5\ \mu m^2$)	Statistics: *r*	Statistics: *P*-values
Age (years)		0.149[a]	0.1059[a]
Gender			
Male ($N = 66$)	57.7 ± 23.3		**< 0.0001**[b]
Female ($N = 52$)	36.7 ± 22.4		
Body mass index ($kg\,m^{-2}$)		0.073[a]	0.4271[a]
Duration of HCV infection (years) ($N = 58$)		0.237[a]	0.0677[a]
Alcohol intake ($g\,day^{-1}$)		0.121[a]	0.2709[a]
Laboratory data			
ALT ($IU\,l^{-1}$)		0.541[a]	**< 0.0001**[a]
AST ($IU\,l^{-1}$)		0.605[a]	**< 0.0001**[a]
Platelet count ($\times 10^4\,mm^{-3}$)		−0.430[a]	**< 0.0001**[a]
Serum ferritin ($ng\,ml^{-1}$)		**0.640**[a]	**< 0.0001**[a]
Serum HCV-RNA ($kIU\,ml^{-1}$) ($N = 89$)		−0.197[a]	0.0721[a]
Inflammatory activity[c]			
A0 or A1 ($N = 42$)	32.2 ± 21.2		
A2 ($N = 49$)	52.3 ± 22.6		**< 0.0001**[d]
A3 ($N = 27$)	62.4 ± 26.0		
Fibrosis staging[e]			
F0 or F1 ($N = 30$)	26.6 ± 14.7		
F2 ($N = 26$)	46.0 ± 22.1		**< 0.0001**[d]
F3 ($N = 27$)	52.4 ± 21.2		
F4 ($N = 35$)	66.3 ± 26.3		
Total iron score[f]		**0.768**[a]	**< 0.0001**[a]

Data are expressed as mean ± s.d. 8-OHdG = 8-hydroxydeoxyguanosine; HCV = hepatitis C virus; ALT = alanine aminotransferase; AST = aspartate aminotransferase. [a]Spearman rank correlation test. [b]Mann–Whitney *U*-test. [c]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [d]Kruskal–Wallis test. [e]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [f]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

$P < 0.0001$) (Table 4 and Figure 4). These results suggest the association between hepatic 8-OHdG production and iron deposition in the liver of patients with chronic hepatitis C.

DISCUSSION

Although free radicals are normally produced by many reactions essential for cell metabolism and energy production, they are also implicated in the pathogenesis of several different diseases (Valko *et al*, 2007). Reactive oxygen species production within the cells is controlled by numerous antioxidant intracellular defence mechanisms, but under certain conditions, ROS overproduction exceeds the cellular defences and damages cell components including nucleic acids (Valko *et al*, 2007). Reactive oxygen species attack on DNA causes the production of stable covalent bonds and the subsequent formation of DNA adducts, such as 8-OHdG (Shigenaga *et al*, 1989). Experiments in which DNA templates containing 8-OHdG were used indicated that this oxidatively modified DNA residue can induce G-C to T-A transversion at DNA replication (Kuchino *et al*, 1987; Shibutani *et al*, 1991), suggesting that the lesion is mutagenic and therefore potentially carcinogenic, but the role of this oxidative DNA adduct in human carcinogenesis is not entirely understood.

Chronic HCV infection is recognised as the most major risk factor for HCC (Nishioka *et al*, 1991; El-Serag, 2002; Seeff, 2002), but little is known about the precise role of HCC development in HCV-related liver disease. It was reported recently that oxidative damage is a peculiar feature of HCV-mediated liver injury. Patients with chronic hepatitis C showed increased oxidative stress markers in serum or in the liver (Shimoda *et al*, 1994; Sumida *et al*, 2000; Mahmood *et al*, 2004; Fujita *et al*, 2007). Therefore, we measured the amount of 8-OHdG in liver biopsy specimen of patients with chronic hepatitis C and examined its relation with future HCC development. Baseline clinical variables were compared between patients with and without HCC development. Based on univariate analysis, the following numerous variables were picked up for potential factors for HCC development: (1) gender and duration of infection, (2) hepatic inflammation (serum ALT and AST levels and histological grade), (3) hepatic fibrosis (hyaluronic acid, platelet count, and histological stage), (4) iron-related markers (haemoglobin, serum iron, transferrin saturation, ferritin, and TIS), (5) serum HCV-RNA titres, and (6) hepatocytic 8-OHdG

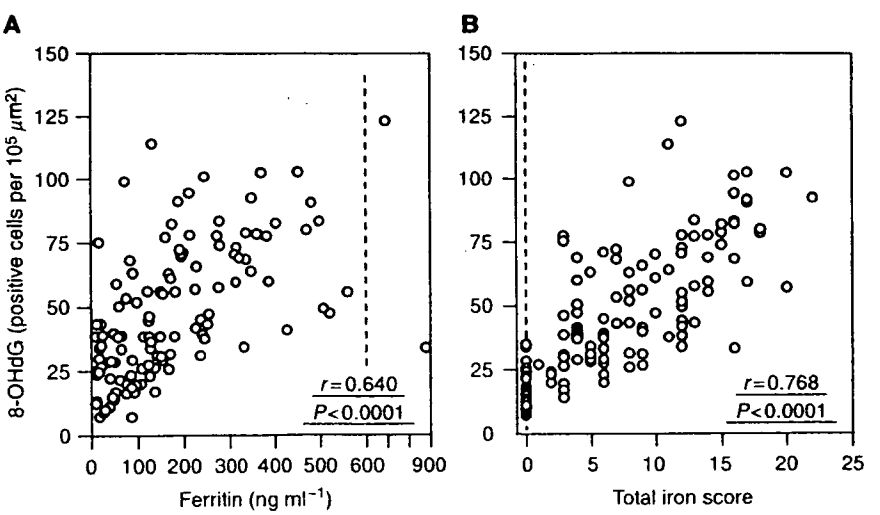

Figure 4 Correlations between hepatic 8-OHdG staining and serum ferritin levels (**A**), and TIS in hepatic tissues (**B**), in 118 patients with chronic hepatitis C.

counts. Cox proportional hazard analysis identified increased hepatic oxidative DNA damage, together with histological fibrosis, which is a well-recognised risk factor for HCC (Greten *et al*, 2005), as an independent risk factor for HCC development. This result suggests that the hepatic oxidative stress plays an important role in hepatocarcinogenesis and it may be a useful marker to predict future HCC development in chronic HCV-infected patients. Especially in the group of patients with hepatic 8-OHdG counts exceeding 75 positive cells per $10^5\,\mu m^2$, the HCC incidence during the first 3 years was extremely high (38.8%) (Figure 3A), indicating that these patients constitute a very high-risk subgroup for developing HCC and should necessarily be carefully monitored by several modalities. Recently, Maki *et al* (2007) evaluated the expression levels of 8-OHdG of non-cancerous hepatic tissues in HCV-infected patients who developed HCC and received curative tumour resection. The postoperative cumulative HCC-free survival was significantly shorter in patients with the highest percentage of 8-OHdG-positive hepatocytes, indicating that the hepatic 8-OHdG levels are also useful for prediction of HCC recurrence in patients with chronic HCV infection who developed HCC.

Several additional risk factors for HCC were identified in patients with chronic hepatitis C in previous reports – increased age (Seeff, 2002), heavy alcohol intake (Donato *et al*, 1997), and chronic coinfection with HBV (Donato *et al*, 1997) – but our results did not identify these factors for HCC. This may be attributable to the fact that our study population excluded heavy alcohol abusers (defined as a chronic consumer of ethanol in excess of 40 g day^{-1} for male and 20 g day^{-1} for female for at least 5 years) and included relatively old patients (median age was 57.5 years). Patients coinfected with HBV were completely excluded from our study because all patients were seronegative for both hepatitis B surface antigen and hepatitis B core antibody. Recently, several reports have suggested that persons with diabetes mellitus are at an increased risk for developing HCC (El-Serag *et al*, 2001), and obesity, which frequently accompanies diabetes, has also been reported to increase the risk for hepatic steatosis and HCC in HCV-infected patients (Ohata *et al*, 2003). But, body mass index and hepatic steatosis were not significantly different between the HCC- and non-HCC-developed groups among our patients.

To determine the factors involved in the occurrence of hepatic oxidative stress during chronic HCV infection, epidemiologic, laboratory, and histological variables were examined for association with hepatocytic 8-OHdG staining counts. Quantitative analysis revealed that hepatocytic 8-OHdG levels were significantly correlated with serum aminotransferase levels and with the histological grading of necroinflammation, suggesting a possible link between hepatic oxidative stress and hepatic inflammation in chronic hepatitis C. It is unclear whether oxidative stress is the cause or the consequence of liver injury, but it has been demonstrated that oxidative stress can directly activate Kupffer cells, causing the release of inflammatory and profibrogenic cytokines such as tumour necrosis factor-α and transforming growth factor-β (Poli and Parola, 1997). Accordingly, the hepatocytic 8-OHdG counts were also significantly correlated with hepatic fibrosis, as assessed by the serum hyaluronic acid, platelet count, and histological staging score. Sumida *et al* (2000) have also shown a significant association between oxidative stress (serum thioredoxin levels) and hepatic fibrosis (hyaluronic acid, type IV collagen-7S domain, procollagen-III peptide) in HCV-positive persons, suggesting that ROS is an important cofactor in accelerating the development of hepatic fibrosis during chronic HCV infection, which may lead to further acceleration of HCC development. In addition, the hepatic 8-OHdG levels were significantly correlated with the serum ferritin and hepatic iron amounts assessed by TIS, suggesting a strong relationship between the damage to hepatocytic DNA and body store of iron in chronic hepatitis C patients. It is known that free iron promotes generation of oxygen radicals by catalysing the Fenton reaction in which Fe^{2+} reacts with H_2O_2 to generate highly reactive $OH^{\bullet}$ radicals, which can cause nucleic acid damage and 8-OHdG adducts. Therefore, iron may cause liver tissue injury by increasing the formation of toxic hydroxyl radicals leading to progression of liver inflammation, fibrosis, and increased risk for developing liver cancer during chronic HCV infection. Increased iron stores were associated with increased oxidative DNA damage, suggesting that removing iron stores in the body, for example by phlebotomy (Yano *et al*, 2004), or dietary iron restriction (Iwasa *et al*, 2004), which has been accepted as a useful treatment option, may delay or reduce the incidence of HCC in patients with chronic hepatitis C. Additional studies are warranted to determine whether these iron-reduction therapies are effective for reducing HCC and for improving the clinical outcomes of patients with chronic HCV infection.

In conclusion, this study clearly showed that in patients with chronic hepatitis C, the oxidative DNA damage in the liver frequently occurred and that it was strongly associated with increased risk for HCC. Strong positive correlations between hepatic oxidative stress and iron overload suggest that iron content may be a mediator of hepatic oxidative stress and that iron reduction may be beneficial to reduce the HCC incidence in chronic HCV-infected patients.

ACKNOWLEDGEMENTS

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