patients with transient biochemical response of the non-SVR group of aged patients, HCC development was not reduced (risk ratio 0.67, 95% CI 0.32–1.43, $P = 0.303$, Table 3) in contrast to the patients showing transient biochemical response in the non-aged group.

As the cumulative incidence of HCC calculated by the Kaplan–Meier Method of the patients with SVR in the aged group was much higher than that in the non-aged group, we also carried out Cox proportional-hazards regression analysis to estimate risk factors responsible for HCC development in the 175 patients achieving SVR. As a result, older age (risk ratio 1.09, 95% CI 1.01–1.18, $P = 0.025$) and higher histological activity before IFN therapy started (10 or more of the total score of components 1–3 in Knodell's histological activity index) (risk ratio 4.16, 95% CI 1.07–16.25, $P = 0.040$) were identified as risk factors associated with HCC among the patients with SVR.

DISCUSSION

In this long-term retrospective cohort study, an inhibitory effect of 6 months-IFN monotherapy in early 1990s on the cumulative incidence of HCC were compared between the patients with histologically proven chronic hepatitis C under 60 years old (non-aged group) and those 60 years old and over (aged group). Because of retrospective analysis, there were some differences in baseline characteristics between the two groups. In the aged group, the histological stage and activity as well as the proportion of male patients were significantly higher than in the non-aged group. Also, SVR rate in the aged group was lower than that in the non-aged group. To avoid the influence of these biases, we performed Cox proportional-hazards regression analysis to see whether IFN monotherapy reduced the risk of HCC in the aged and non-aged groups. Then, we found that IFN therapy for 6 months significantly reduced the risk of HCC (risk ratio 0.52) in the non-aged group, whereas this inhibitory effect of IFN monotherapy on HCC development was recognized only in the patients achieving SVR among the aged-patients.

It is difficult to explain why IFN had no inhibitory effect on HCC development in the aged patients, whereas IFN had significant inhibitory effect in the non-aged patients of this study. Many clinical studies have demonstrated that aging was an independent risk factor associated with HCV-related HCC other than advanced histological staging and male gender [7,11–17,29]. However, molecular mechanism of the impact of aging on haepetocarcinogenesis has not been elucidated. Moriya *et al.* reported that lipid hydroperoxide products accumulated in the liver without inflammation and may play a role in the development of HCC in HCV core gene transgenic mice [30,31]. A long-term infection of HCV may lead to HCC through some molecular alterations.

Recently, there have been two controversial reports from the United States and Japan as to the long-term effect of low-dose IFN therapy on the incidence of HCC in chronic hepatitis C [22,24]. The report from Japan was a non-randomized retrospective study and observed beneficial effect of long-term natural IFN-α therapy on hepatocarcinogenesis in aged chronic hepatitis C patients [22]. The HALT-C Trial from the United States, a large prospective randomized study, reported that treatment with peginterfeon-α2a at a dose of 90 μg weekly for 3.5 years did not prevent HCC development in the patients with bridging fibrosis or cirrhosis who did not obtain SVR by combination therapy of peginterferon and ribavirin [24]. The result was consistent with our data in the aged patients. However, the annual incidence of HCC of the HALT-C Trial, about 1%, was much lower than that in the aged group in this study, about 4%. Accordingly, a randomized prospective study to determine the effect of long-term IFN or peginterferon therapy on the incidence of HCC in chronic hepatitis C, especially in the aged patients, may be needed in Japan.

This study has a limitation, because we used historical controls as control patients. A lead-time bias may have occurred. Detection of HCC by the screening program could be less effective in controls than IFN-treated patients. In that case, we might underestimate the effect of IFN on the cumulative incidence of HCC. However, such underestimation may be unlikely as the tumour sizes at the time of detection were not different between the control and IFN-treated patients.

The 10-year incidence of HCC for SVR patients of the aged group (12.7%) was much higher than that of non-aged group (4.5%) in our study. Makiyama *et al.* [32] studied the risk factors for developing HCC after obtaining sustained biochemical response to IFN therapy in chronic hepatitis C and reported that older age, male gender and advanced fibrosis were associated with HCC. Consistent with their results, we found that older age was an independent risk factor for HCC in the patients with SVR, suggesting a high potential of developing HCC even after eradication of HCV RNA in the aged patients. Another possibility is that malignant foci, which could not be detected by imaging modalities, had already existed before IFN therapy. Our finding indicates that even in the patients showing SVR, a follow-up examination to investigate HCC should be carried out for at least 10 years, particularly in the aged patients.

In conclusion, IFN monotherapy reduced the risk of HCC in the patients with chronic hepatitis C under 60 years old. In contrast, this inhibitory effect of IFN on hepatocacinogenesis was limited to patients showing SVR in the aged-patients when treated with 6 months-IFN monotherapy. These results suggest that combination therapy of peginterferon and ribavirin is recommended even in the aged patients with chronic hepatitis C to obtain better preventive effect of IFN on HCC development. For reasons of relatively high cumulative incidence of HCC in the aged chronic hepatitis C patients with SVR to IFN therapy, they should be followed carefully even after eradication of HCV by IFN therapy.

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Hydroxyoctadecadienoic acid as a potential biomarker for oxidative stress in patients with chronic hepatitis C

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Abstract

Background and Aim: The possible involvement of oxidative stress in hepatitis C virus (HCV)-induced liver damage and hepatocarcinogenesis has been reported. We have recently developed a novel method to measure total hydroxyoctadecadienoic acid (tHODE) and have proposed its usefulness as a biomarker for lipid peroxidation. The present study was undertaken to evaluate oxidative stress in HCV-infected liver diseases by several potential oxidative stress markers including tHODE and further to validate the biomarkers for evaluating the efficacy of iron reduction therapy.

Methods: Total hydroxyoctadecadienoic acid, total 8-iso-prostagrandin $F_{2\alpha}$ (t8-iso-$PGF_{2\alpha}$), selenoprotein P and other antioxidant compounds were measured in the plasma and erythrocytes obtained from 42 healthy controls and 78 HCV patients. Plasma levels of biomarkers and antioxidants were also assessed during the iron reduction therapy for 16 weeks in 12 HCV patients.

Results: The concentrations of tHODE in the plasma and erythrocytes and t8-iso-$PGF_{2\alpha}$ in the plasma of chronic HCV-infected patients were significantly higher than those of healthy controls. Plasma levels of vitamin E and vitamin C of HCV-infected patients were lower than those of the controls. Furthermore, the plasma tHODE significantly correlated with serum aminotransferases and type IV collagen-7S domain in chronic HCV-infected patients. During the iron reduction therapy, the plasma levels of tHODE but not t8-iso-$PGF_{2\alpha}$ decreased and inversely its stereo-isomer ratio (ZE/EE) increased in parallel with the decreases of serum alanine aminotransferase, ferritin and α-fetoprotein.

Conclusion: The levels of tHODE in chronic HCV-infected patients can be a useful biomarker for the evaluation of oxidative stress in chronic hepatitis C.

Introduction

The pathogenesis of chronic inflammation and fibrogenesis in hepatitis C virus (HCV) infection is not completely understood. However, clinical and experimental evidence that suggests an association between oxidative stress and the progression of chronic liver diseases, including chronic hepatitis C, has been accumulated.[1–9] Lipid peroxidation induced by reactive oxygen species (ROS) and reactive nitrogen species has been implicated in oxidative stress-induced diseases.[10–13] It was reported that ROS activate hepatic stellate cells, which then lead to hepatic fibrosis.[3,4] An increase in serum malondialdehyde (MDA) and the formation of MDA-protein adducts have been demonstrated in patients with chronic hepatitis C[5–7] Thus, oxidative stress may contribute to the formation of hepatic fibrosis, and finally result in hepatocarcinogenesis in chronic hepatitis C.[14,15]

Lipid peroxidation has been assessed by several biomarkers such as thiobarbituric acid reactive substances (TBARS), MDA, and 4-hydroxy-2-nonenal (HNE). Recently, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) has been reported as the gold standard for the assessment of lipid peroxidation.[16,17] The oxidation of arachidonic acid by free radicals produces 8-iso-$PGF_{2\alpha}$ independent of the cyclooxygenase pathway.[18] Significant elevation in 8-iso-$PGF_{2\alpha}$ levels was observed in the plasma of HCV-infected patients.[1] More recently, we have developed a novel method to measure total hydroxyoctadecadienoic acid (tHODE) and 7-hydroxycholesterol (t7-OHCh) as oxidative stress markers[19] In this method hydroperoxides and ketones as well as hydroxides of both free and esterified forms of linoleic acid are measured as tHODE after reduction and saponification of the plasma and erythrocytes. Further, linoleates are the most abundant polyunsaturated fatty acid (PUFA) *in vivo* and their oxidation proceeds by a straightforward

mechanism to give hydroperoxyoctadecadienoates (HPODEs) as primary products. Thus, tHODE measured using this method is expected to account for a major portion of the lipid peroxidation occurring *in vivo*.[19,20] Hence, this method has a significant advantage over those measuring aldehydes, aldehyde-modified proteins, and isoprostanes as biomarkers of oxidative stress. More importantly, antioxidant capacity can be also determined by the stereoisomer ratio of HODE (ZE/EE) in this method.[19]

The antioxidant status is also important to estimate the oxidative stress. An extracellular antioxidant system is composed of numerous antioxidants with different functions, molecular weights, and sites of localization. Low molecular weight antioxidants found in the plasma, such as vitamin E, C, and ubiquinol, scavenge free radicals to synergistically inhibit lipid peroxidation.[21] On the other hand, antioxidant enzymes in the plasma, such as extracellular glutathione peroxidase (eGPx) and selenoprotein P (SeP), reduce various hydroperoxides including lipid hydroperoxides to hydroxide derivatives by using reducing equivalents such as glutathione (GSH) and thioredoxin (TRX).[22] The levels of these antioxidants between the patients and healthy controls were also measured and compared in this study.

In the present study, we measured tHODE, its stereoisomer ratio, total 8-isoprostane levels, and antioxidants in the plasma and erythrocytes of patients with chronic hepatitis C and of the control subjects in order to evaluate the oxidative status of HCV-infected patients comprehensively and further to validate these biomarkers for evaluating the effectiveness of iron reduction therapy.

Materials and methods

Patients

We prospectively studied the patients with chronic HCV infection (HCV group). The inclusion criteria of the patients were as follows: (i) positive HCV RNA (Roche Amplicor HCV 2.0 assay, Roche Diagnostics, Tokyo, Japan) in serum; (ii) no evidence of hepatocellular carcinoma (HCC) as assessed by ultrasonography and/or computed tomography; (iii) absence of serum hepatitis B surface antigen; (iv) absence of co-existing diseases which requires medication; (v) no history of drug abuse; (vi) no previous interferon therapy; and (vii) no medication such as ursodeoxycholic acid and glycyrrhizin at least 2 weeks before the assays. Patients with decompensated cirrhosis were excluded. We also studied healthy controls (control group). None of the subjects in the control group had illnesses that required medical treatment, including liver diseases. None of the subjects in the HCV or control group took antioxidant supplements or had daily consumption of alcohol more than 20 g. Finally, 78 HCV-infected patients and 42 healthy controls were enrolled. The other 12 patients with chronic hepatitis C whose serum ferritin levels were over 150 ng/mL received phlebotomy. The patients who received phlebotomy consisted of eight males and four females (mean age ± standard deviation [SD], 66.5 ± 9.5 years). In addition to the inclusion criteria mentioned above in (i) to (vi), only the patients with hemoglobin levels of more than 13 g/dL were included. Of these, five patients had been treated with ursodeoxycholic acid and seven with ursodeoxycholic acid and glycyrrhizin before enrollment. These treatments were continued during the study. Two hundred milliliters of blood was removed from the patients of body weight less than 50 kg or 400 mL for the other patients biweekly until serum ferritin levels reached 20 ng/mL or less for 16 weeks. When the hemoglobin level fell below 10 g/dL, phlebotomy was discontinued.

This study was approved by the institutional review boards of the National Institute of Advanced Industrial Science and Technology and Ikeda Municipal Hospital. All patients gave their written informed consent after the purpose of this study was completely explained. Diagnosis of the chronic hepatitis and cirrhosis were based on histological findings in 31 patients and on ultrasonographic and laboratory findings in the remaining 47 patients. There was no difference in age between the two groups (mean age ± SD, HCV group 63.4 ± 11.8 years *vs* control group 58.1 ± 13.5 years). The age of the patients with cirrhosis was higher than that of the control group (Table 1). Also, there was no difference in gender between the two groups: 40 males and 38 females in the HCV group, and 17 males and 25 females in the control group (Table 1). The laboratory data of the control and HCV groups are shown in Table 1.

Table 1 Laboratory data in the hepatitis C virus (HCV) group

	Control ($n = 42$)	Chronic hepatitis ($n = 47$)	Cirrhosis ($n = 31$)
Age (years)	58.1 ± 13.5	61.2 ± 13.2	66.9 ± 12.6*
Gender (male/female)	17/25	26/21	14/17
AST (IU/L)	22 ± 5	64 ± 32*	83 ± 42*,**
ALT (IU/L)	18 ± 9.7	78 ± 52*	74 ± 41*
Albumin (g/dL)	4.2 ± 0.2	4.0 ± 0.4*	3.5 ± 0.5*,**
Type IV collagen-7S domain (ng/mL)	3.3 ± 0.7	6.1 ± 2.1*	11.6 ± 3.5*,**
Procollagen III peptide (ng/mL)	0.51 ± 0.10	0.91 ± 0.23*	1.13 ± 0.30*,**
Hyaluronic acid (ng/mL)	46.7 ± 35.0	162 ± 215*	551 ± 449*,**
Serum HCV-RNA level (kIU/mL)		1240 ± 868	949 ± 614
Serum ferritin (ng/mL)	79 ± 51	295 ± 257*	361 ± 314*

*$P < 0.05$ against controls; **$P < 0.05$ against chronic hepatitis.

Results are expressed as mean ± standard deviation (SD). Comparisons between groups were carried out by using ANOVA.

ALT, alanine aminotransferase; AST, asparate aminotransferase; kIU, kilo International Units.

Materials

8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), 8-iso-prostaglandin $F_{2\alpha}$-d_4 (8-iso-$PGF_{2\alpha}$-d_4), 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid (9-(*E,Z*)-HODE), 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13-(*Z,E*)-HODE), and 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic-9,10,12,13-d_4 acid (9-HODE-d_4) were obtained from Cayman Chemical Company (MI, USA). 9-Hydroxy-10(*E*),12(*E*)-octadecadienoic acid (9-(*E,E*)-HODE) and 13-hydroxy-9(*E*),11(*E*)-octadecadienoic acid (13-(*E,E*)-HODE) were obtained from Larodan Fine Chemicals AB (Malmo, Sweden). Other materials were of the highest grade available commercially.

Analyses of tHODE and total 8-iso-$PGF_{2\alpha}$

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes after the subjects fasted overnight. The samples were placed on ice immediately after collection. Plasma was obtained by centrifugation at 1580 *g* for 10 min at 4°C and immediately analyzed. The erythrocytes were washed twice with a threefold volume of saline and adjusted to a hematocrit value (HV) that was approximately 40% by using saline. The accurate HV was determined later by using a hematocrit capillary (Cosmo-bio Ltd. Tokyo, Japan). The erythrocyte sample (HV ca 40%) was extracted using a fourfold volume of methanol that contained 100 μM 2,6-di-tert-butyl-4-methylphenol (BHT) by vortexing and centrifugation (20 400 *g* at 4°C for 10 min), and it was then immediately analyzed.

Total hydroxyoctadecadienoic acid and total 8-iso-$PGF_{2\alpha}$ (t8-iso-$PGF_{2\alpha}$) were measured using a previously reported method with slight modifications.[19,23] Internal standards of both 8-iso-$PGF_{2\alpha}$-d_4 (50 ng) and 9-HODE-d_4 (50 ng) and 1 mL methanol were added to the plasma (2 mL) and erythrocytes extract (1 mL), followed by the reduction of hydroperoxides and ketones by using an excessive amount of sodium borohydride (4 mg) at room temperature for 5 min. Next, the reduced sample was mixed with 1 M KOH in methanol (1 mL) under nitrogen and incubated on a shaker for 30 min in the dark at 40°C. The sample was centrifuged (1580 *g* at 4°C for 10 min), and the supernatant was diluted with a fourfold volume of water (pH 3) and adjusted to pH 3 with 2 N HCl. The acidified sample was centrifuged (1580 *g* at 4°C for 10 min) and the supernatant was subjected to solid phase extraction.[19] The eluent obtained was evaporated under nitrogen, and 30 μL of a silylating agent, N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), was added to the dried residue. The solution was vigorously mixed by vortexing for 1 min and incubated for 60 min at 60°C to obtain trimethylsilyl esters and ethers. An aliquot of this sample was injected into the gas chromatograph (GC 6890 N, Agilent Technologies, Palo Alto, CA, USA) that was equipped with a quadruple mass spectrometer (5973 Network, Agilent Technologies). Analytical conditions are the same as a previous report.[19] By using these methods, artificial oxidation of lipids during sample work-up was kept minimal and it was confirmed that the experimental errors were within ± 10%.[19,23]

Analyses of antioxidants

The concentrations of α-tocopherol, γ-tocopherol (T), ascorbic acid, ubiquinol-10, and ubiquinone-10 in plasma, and those of α-T and γ-T in erythrocytes were also measured according to the previous report[24] by using high performance liquid chromatography (HPLC).

Analyses of SeP

Plasma SeP concentrations were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) using rat anti-human SeP monoclonal antibody BD1 and AH5 as described previously.[25] The optical density of the plates was measured at 450 nm using a Multiskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). All measurements were made in duplicate, and the average value was used.

Laboratory tests during phlebotomy

Laboratory tests, including serum alanine aminotransferase (ALT), asparate aminotransferase (AST), complete blood count, ferritin, α-fetoprotein (AFP), type IV collagen-7S domain, procollagen III peptide (PIIIP), and hyaluronic acid in addition to plasma tHODE, t8-iso-$PGF_{2\alpha}$, SeP, and antioxidants, were measured before and during phlebotomy.

Statistics

Data are presented as the mean ± SD and were analyzed using SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to compare distribution of gender between the HCV and the control groups. Comparisons between groups were carried out by using ANOVA. Correlations were presented as scatter plots and analyzed using the Pearson test. The levels of tHODE, ALT, ferritin, AFP, type IV collagen-7S domain, PIIIP, and hyaluronic acid after phlebotomy were compared with the mean value of 4 weeks and just before the onset of phlebotomy by using paired two-tailed *t*-test. A *P*-value of <0.05 was considered significant.

Results

Plasma levels of tHODE, 8-iso-$PGF_{2\alpha}$, and antioxidants

The plasma levels of lipid peroxidation markers including tHODE (9- and 13-(*Z,E*)-HODE (enzymatic and non-enzymatic products) and 9- and 13-(*E,E*)-HODE (non-enzymatic free radical-mediated products)) and t8-iso-$PGF_{2\alpha}$ in the HCV group were found to be significantly higher than those of the control group (Table 2). In the HCV group, plasma levels of tHODE and t8-iso-$PGF_{2\alpha}$ of both chronic hepatitis and cirrhosis were higher than those of the healthy controls; these levels tended to increase with the progression of the disease (chronic hepatitis to cirrhosis, Table 2, Fig. 1a,b). The stereoisomer ratio of HODE, (*Z,E*)-HODE/(*E,E*)-HODE, in the plasma of the HCV group tended to be low as compared with that of the control group, although the difference between the two groups was not significant (Table 2). There was a significant negative correlation ($r = -0.21$, $P = 0.022$) between the plasma tHODE level and the stereoisomer ratio of HODE, indicating that the (*E,E*)-HODE, which is formed by a free radical-mediated oxidation, was increased with

Table 2 Concentrations of hydroxyoctadecadienoic acid and other bio-markers in plasma and erythrocytes in the hepatitis C virus (HCV) and control groups

		HCV group		
	Control group ($n = 42$)	Total ($n = 78$)	Chronic hepatitis ($n = 47$)	Cirrhosis ($n = 31$)
Plasma				
tHODE (nM)	224 ± 74	305 ± 143*	281 ± 118*	341 ± 170*
(Z,E) pHODE (nM)	84 ± 30	112 ± 55*	104 ± 46	124 ± 66*
(E,E)-HODE (nM)	139 ± 50	193 ± 94*	177 ± 78*	217 ± 111*
HODE ratio (ZE/EE)	0.64 ± 0.19	0.61 ± 0.17	0.62 ± 0.16	0.61 ± 0.19
8-iso-PGF2α (nM)	0.61 ± 0.42	1.08 ± 0.92*	1.02 ± 0.83	1.18 ± 1.05*
Erythrocytes				
tHODE (nmol/L-packed cell)	1076 ± 1049	2509 ± 1252*	2301 ± 1241	2824 ± 1220*
(Z,E) pHODE (nmol/L-packed cell)	1286 ± 696	1490 ± 720	1357 ± 640	1692 ± 796*
(E,E)-HODE (nmol/L-packed cell)	691 ± 375	1015 ± 644*	945 ± 638	1120 ± 650*
HODE ratio (ZE/EE)	1.93 ± 0.42	1.66 ± 0.54*	1.63 ± 0.48*	1.71 ± 0.62
8-iso-PGF2α (nmol/L-packed cell)	13.2 ± 8.2	13.4 ± 8.2	13.5 ± 8.6	13.3 ± 7.7

*$P < 0.05$ against controls.
Results are expressed as mean ± standard deviation (SD). Comparisons between groups were carried out by using ANOVA.
HODE, hydroxyoctadecadienoic acid.

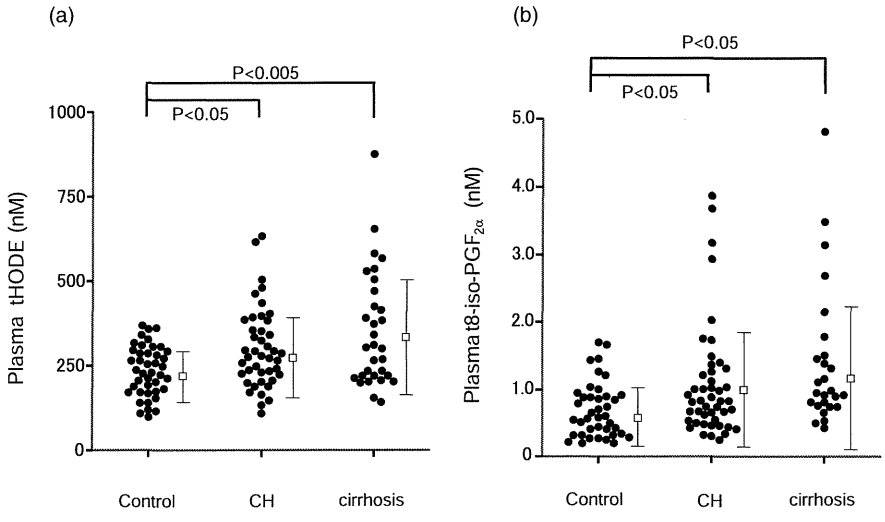

Figure 1 Plasma levels of total hydroxyoctadecadienoic acid (tHODE) (a) and total 8-iso-$PGF_{2\alpha}$ (b) in the control group and patients with chronic hepatitis (CH) and cirrhosis. The plasma levels of total HODE and total 8-iso-$PGF_{2\alpha}$ of patients with both chronic hepatitis and cirrhosis were significantly higher than those of the control group. Statistical analysis was carried out as described in Materials and Methods.

disease progression. There was no significant difference in the plasma SeP levels between the HCV and control groups. However, the plasma SeP levels of cirrhotic patients were significantly lower than those of both chronic hepatitis and the control groups (Table 3). Among plasma antioxidants, the plasma concentrations of α-T, γ-T, and ascorbic acid were significantly decreased in the HCV group as compared with the control group (Table 3).

Erythrocytes levels of tHODE, t8-iso-$PGF_{2\alpha}$, and antioxidants

When compared with the healthy controls, the erythrocyte tHODE level of the HCV group was significantly higher (Table 2). Moreover, there was a significant correlation ($r = 0.26$, $P = 0.047$) between the plasma and erythrocyte tHODE levels. In the HCV group, the erythrocyte tHODE level of patients with cirrhosis was higher than that of the control group (Table 2, Fig. 2). The erythrocyte t8-iso-$PGF_{2\alpha}$ levels of the HCV group tended to be high as compared with those of the control group, although there was no significant difference between the two groups (Table 2). The stereoisomer ratio of HODE, (*Z,E*)-HODE/(*E,E*)-HODE, in the erythrocytes of the HCV group was significantly lower than that of the control group (Table 2), suggesting diminished antioxidant capacity in the erythrocytes of the patients. Furthermore, there was a significant negative correlation ($r = -0.21$, $P = 0.023$) between the tHODE level and the stereoisomer ratio of HODE in the erythrocytes. The erythrocyte levels of α-T and γ-T of the HCV group were significantly decreased as compared with those of the control group (Table 3).

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Table 3 Concentrations of antioxidants in plasma and erythrocytes in the hepatitis C virus (HCV) and control groups

		HCV group		
	Control group ($n = 42$)	Total ($n = 78$)	Chronic hepatitis ($n = 47$)	Cirrhosis ($n = 31$)
Plasma (μM)				
α-tocopherol	24.2 ± 8.96	18.2 ± 6.96*	16.9 ± 5.45*	20.1 ± 8.46
γ-tocopherol	2.56 ± 1.52	1.63 ± 1.02*	1.65 ± 0.95*	1.61 ± 1.14*
Ascorbic acid	40.2 ± 19.2	33.9 ± 17.8*	34.1 ± 16.1	33.7 ± 20.2
Ubiquinol-10 (A)	0.81 ± 0.45	0.69 ± 0.49	0.63 ± 0.44	0.78 ± 0.55
Ubiquinone-10 (B)	0.19 ± 0.12	0.14 ± 0.08*	0.12 ± 0.07*	0.16 ± 0.09
(A)/(A) + (B) (%)	79.9 ± 10.8	81.5 ± 8.2	81.6 ± 8.2	81.3 ± 8.5
Selenoprotein P (μg/mL)	6.24 ± 1.37	6.02 ± 1.38	6.54 ± 1.28	5.34 ± 1.22*,**
Erythrocytes (μmol/L-packed cell)				
α-tocopherol	2.38 ± 0.85	1.53 ± 0.79*	1.52 ± 0.71*	1.53 ± 0.91*
γ-tocopherol	0.44 ± 0.49	0.23 ± 0.16*	0.24 ± 0.15*	0.22 ± 0.16*

*$P < 0.05$ against controls; **$P < 0.05$ against chronic hepatitis.
Results are expressed as mean ± standard deviation (SD). Comparisons between groups were carried out by using ANOVA.

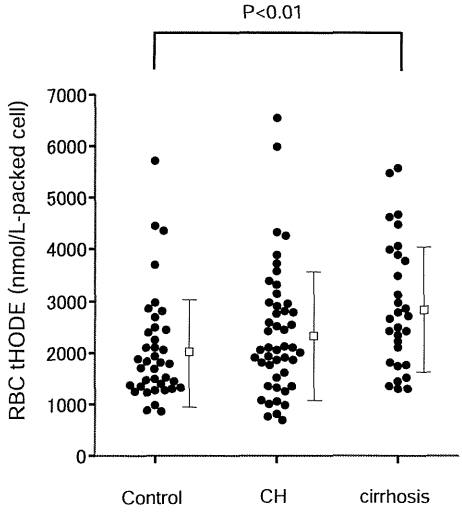

Figure 2 Total hydroxyoctadecadienoic acid (tHODE) of erythrocytes in the control group and patients with chronic hepatitis (CH) and cirrhosis. The erythrocyte levels of tHODE of patients with cirrhosis were significantly higher than those of the control group. Statistical analysis was carried out as described in Materials and Methods.

Plasma tHODE and clinical laboratory data

We investigated correlations between the plasma and erythrocyte tHODE levels and clinical laboratory data, including serum AST, ALT, ferritin, iron, hyaluronic acid, and the type IV collagen-7S domain. The plasma tHODE level correlated significantly with serum AST (Fig. 3a, $r = 0.37$, $P = 0.00006$) and ALT (Fig. 3b, $r = 0.28$, $P = 0.003$) in the chronic HCV-infected patients. Additionally, a significant correlation was observed between the plasma tHODE levels and the serum type IV collagen-7S domain (Fig. 4a, $r = 0.40$, $P = 0.00005$), PIIIP (Fig. 4b, $r = 0.37$, $P = 0.00016$) and hyaluronic acid (data not shown, $r = 0.38$, $P = 0.0002$) in the chronic HCV-infected patients. We also examined the effect of different genotypes of HCV on plasma tHODE levels. Then, we found that there was no difference between plasma levels of tHODE of genotype 1 ($n = 55$, 322 ± 155 nM) and genotype 2 ($n = 13$, 270 ± 114 nM).

Plasma levels of biomarkers during phlebotomy

We then investigated whether the biomarker tHODE is able to evaluate an efficacy of iron reduction therapy (phlebotomy) in HCV-infected patients. Twelve patients were chosen according to the experimental protocol mentioned above. As shown in Figure 5, the plasma levels of tHODE and its stereo-isomer ratio (ZE/EE) significantly decreased and increased, respectively, 8 weeks after the start of phlebotomy and thereafter. Moreover, as expected, clinical laboratory data including ALT, ferritin, and α-fetoprotein (AFP) decreased significantly (Fig. 6). It was also found that the markers for hepatic fibrosis, type IV collagen-7S and PIIIP, but not hyaluronic acid, decreased significantly during the treatment (Fig. 7). These data suggest that tHODE can be a useful biomarker for evaluating oxidative stress during the iron reduction therapy in chronic hepatitis C. On the other hand, none of the plasma level of SeP, t8-iso-$PGF_{2\alpha}$, or antioxidants, including vitamin E, vitamin C, and CoQ_{10}, changed significantly by the therapy.

Discussion

The present study was carried out to elucidate the involvement of oxidative stress in HCV-infected liver diseases by several potential oxidative stress markers and further to validate the biomarkers for evaluating the efficacy of iron reduction therapy. The involvement of free radical-induced lipid peroxidation in liver diseases has been the subject of extensive studies from the initial years of research in the area of free radicals in medicine.[26] Several papers have reported the possible involvement of oxidative stress in HCV-induced liver damage.[27–29] The 8-hydroxydeoxyguanosine content in livers with chronic hepatitis has been reported to be significantly

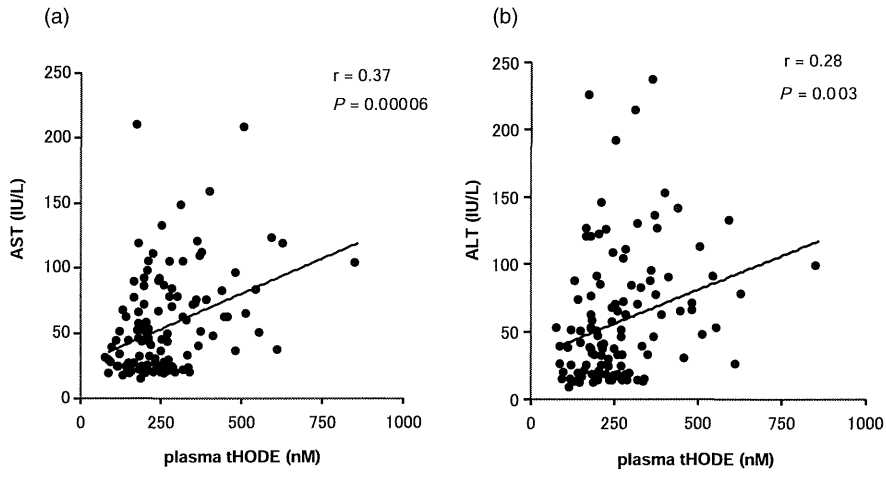

Figure 3 Correlations between plasma levels of total hydroxyoctadecadienoic acid (tHODE) and serum asparate aminotransferase (AST) (6A) and alanine aminotransferase (ALT) (6B) levels in the hepatitis C virus (HCV)-infected patients. There was significant correlation between plasma total HODE and serum AST ($r = 0.37$, $P = 0.009$) and ALT ($r = 0.28$, $P = 0.003$) levels when Pearson's test was carried out.

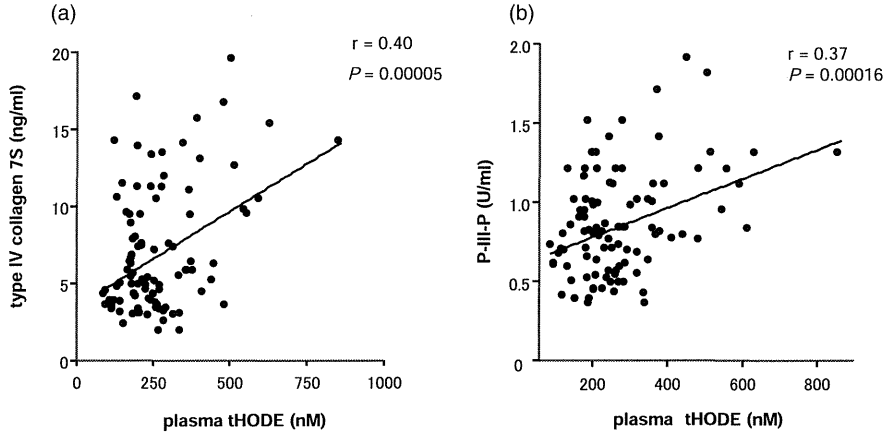

Figure 4 Correlations between plasma levels of total hydroxyoctadecadienoic acid (tHODE) and serum type IV collagen-7S domain (a) and PIIIP (b) levels in the hepatitis C virus (HCV)-infected patients. Significant correlation between plasma total HODE and serum type IV collagen-7S domain ($r = 0.40$, $P = 0.00005$) and PIIIP ($r = 0.37$, $P = 0.00016$) levels was observed when Pearson's test was carried out.

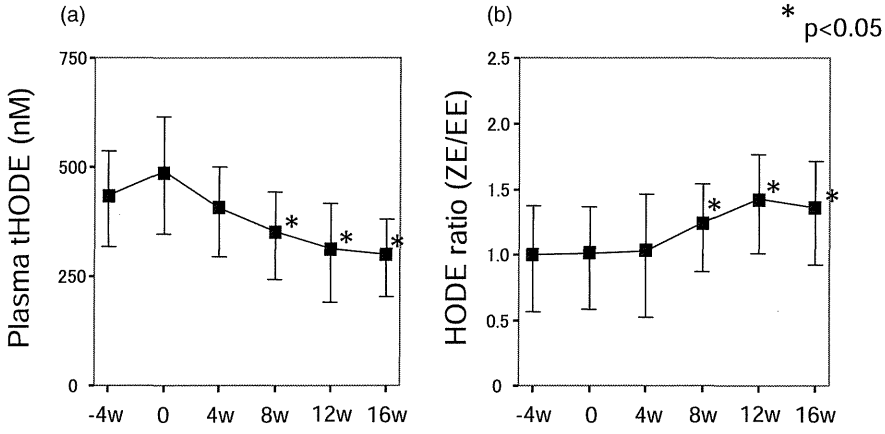

Figure 5 Changes in total hydroxyoctadecadienoic acid (tHODE) (a) and its stereo-isomer ratio (ZE/EE) (b) before and during phlebotomy. The plasma levels of tHODE and its stereo-isomer ratio (ZE/EE) significantly decreased and increased, respectively, 8 weeks after the start of phlebotomy and thereafter as compared with the mean value of 4 weeks and just before the start of phlebotomy by using paired two-tailed *t*-test.

higher than that in normal livers,[30] and chronic HCV infection has been reported to increase hepatic MDA[31] and isoprostane[32] levels. Recently, Moriya *et al.* reported that the HCV core protein increased lipid hydroperoxide products in the liver in the absence of inflammation and that it may play a role in the development of HCC in HCV core gene transgenic mice.[33,34] Based on these reports, it is expected that biomarkers reflecting the extent of oxidative stress *in vivo* as closely as possible from both qualitative and quantitative viewpoints will be developed.

We have recently developed a novel method to measure tHODE from biological fluids and tissues; using this method, a considerable amount of the oxidation products of linoleic acid can be measured.[19,20] Reduction and saponification enabled us to measure hydroperoxides, ketones, and hydroxides of both free and

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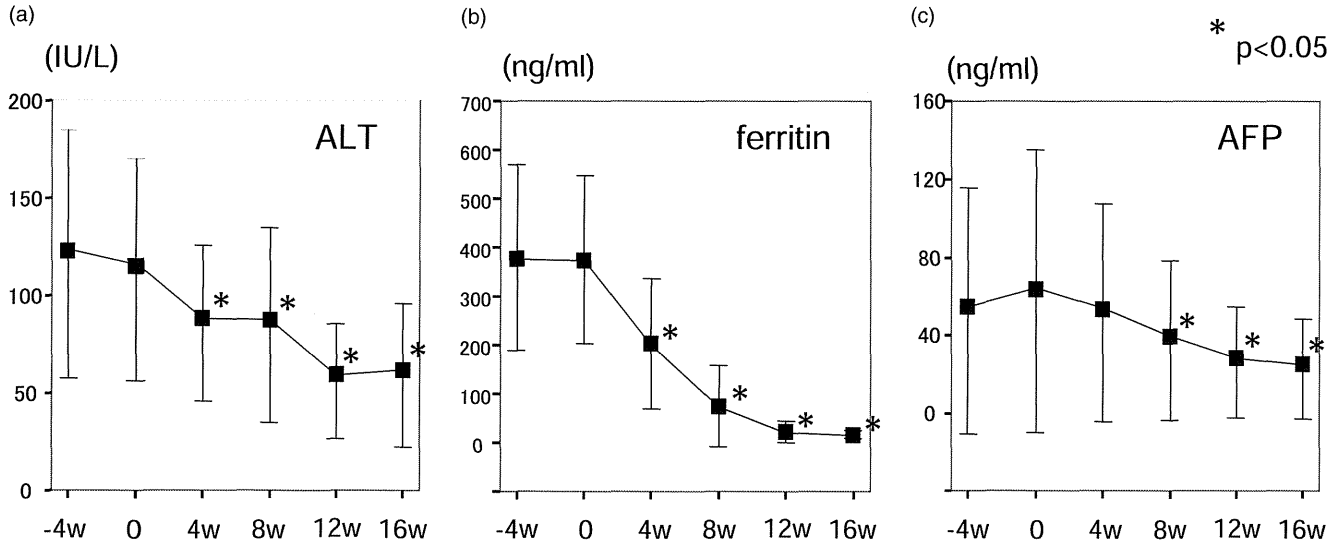

Figure 6 Changes in serum alanine aminotransferase (ALT) (a), ferritin (b) and α-fetoprotein (AFP) (c) levels before and during phlebotomy. The ALT, ferritin, and AFP levels significantly decreased after phlebotomy as compared with the mean value of 4 weeks and just before the start of phlebotomy by using paired two-tailed *t*-test.

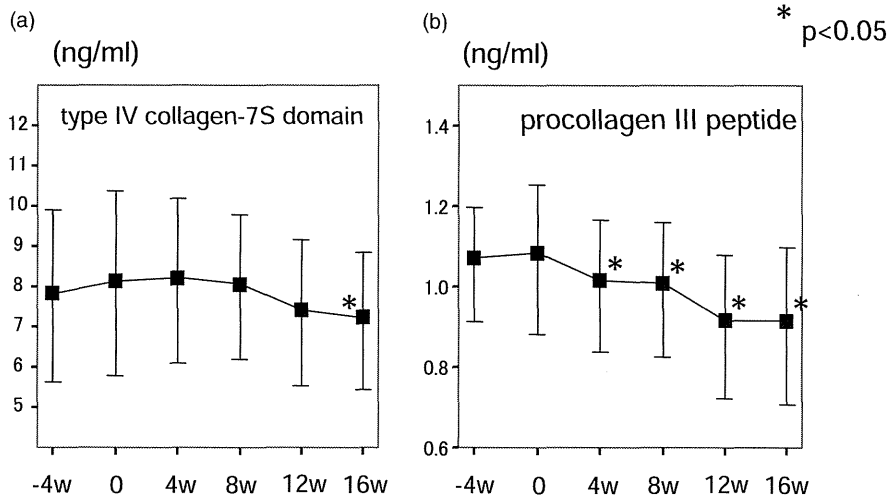

Figure 7 Changes in serum type IV collagen-7S (a) and PIIIP (b) levels before and during phlebotomy. The serum level of PIIIP decreased significantly after the start of the treatment as compared with the mean value of 4 weeks and just before the start of phlebotomy by using paired two-tailed *t*-test.

esterified forms of linoleic acid as tHODE, which includes enzymatic and non-enzymatic products; 9- and 13-(*Z,E*)-HODE and non-enzymatic free radical-mediated products; 9- and 13-(*E,E*)-HODE. As stated above, the tHODE levels in plasma and in erythrocytes were two to three orders of magnitude higher than those of t8-iso-$PGF_{2\alpha}$. Furthermore, we have recently reported that the tHODE level of plasma in healthy volunteers correlated significantly with the corresponding oxidized LDL level.[35] Thus, tHODE is a useful biomarker for the assessment of oxidative status in human. Another advantage of this method is the measurement of the stereoisomer ratio of HODE, which helps in evaluating the antioxidant capacity *in vivo* under the same oxidative stress. The experimental data and theoretical examination show that the ratio (*Z,E*)-HODE/(*E,E*)-HODE is proportional to the antioxidant capacity of the environment.[20,23,24,36] The results of the present study clearly demonstrate that the plasma and erythrocyte levels of tHODE and t8-iso-$PGF_{2\alpha}$ were higher in the HCV patients than in the healthy controls, and the stereoisomer ratio of HODE of the patients was lower than that of the controls, suggesting enhanced oxidative stress in the patients than in the controls. A noteworthy fact is that there was a positive correlation between the plasma tHODE level and the serum type IV collagen-7S domain in the chronic HCV-infected patients. It seems likely that oxidative stress increased with the progression of hepatic fibrosis in chronic hepatitis C. Additionally, a positive correlation was observed between the plasma tHODE level and serum AST and ALT. However, it should be noted that further study is needed for the elucidation of effects of fibrosis, inflammation, and steatosis. Also, it remains to be clarified that the increase of tHODE in plasma and erythrocytes is specific for HCV-related chronic liver diseases.

In agreement with the above observation, the levels of antioxidant compounds such as α-T, γ-T, and vitamin C in the plasma and

erythrocytes from HCV patients were considerably lower than those from the controls and tended to decrease with the progression of the disease. Jain *et al.*[1] also observed lower levels of antioxidants including glutathione; selenium; and vitamins A, C, and E in chronic HCV patients. Further, a randomized, double-blind, placebo-controlled study showed that vitamin E improved the AST status of HCV patients.[37]

SeP is a selenium-rich glycoprotein, and it is mostly synthesized in the liver. It has been reported that SeP levels are decreased in patients with cirrhosis,[38] probably because of the reduced production resulting from liver dysfunction. Consistent with this report, this study revealed that the plasma SeP level was significantly decreased in cirrhotic patients as compared with the chronic hepatitis patients and the healthy controls, indicating that SeP is a suitable marker for the later stage of chronic hepatitis C. SeP has recently been reported to catalyze the reduction of phospholipid hydroperoxide by reducing agents such as glutathione.[22] The significant decrease of SeP might be one of reasons for the increase of lipid peroxidation products with progression of the disease. It has been demonstrated that SeP also functions as a selenium-supply protein and delivers selenium to the cells.[39] Thus, the plasma SeP level is important not only for the plasma capacity to reduce the lipid hydroperoxide but also for the maintenance of tissue selenoproteins such as GSH peroxidase. Therefore, our observation may suggest the necessity to supplement cirrhotic patients with SeP.

Hepatitis C virus infection has been shown to be associated with iron accumulation in the liver and there exists a relationship between iron accumulation and the severity of histological activity and fibrosis and iron reduction therapy has been reported to improve serum aminotransferases.[40,41] Kato *et al.*[42] reported that long-term iron reduction therapy decreased elevated hepatic 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels to almost normal levels with concomitant improvement of liver histology in patients with chronic hepatitis C. More recently, Fujita *et al.*[43] showed the same results, suggesting that iron overload is an important mediator of hepatic oxidative stress and disease progression in chronic HCV infection. Furthermore, it was reported by Kato *et al.* that long-term phlebotomy combined with low-iron diet therapy lowered the risk of hepatocellular carcinoma in patients with chronic hepatitis C.[44] Consistent with the report, we found that serum AFP levels decreased in parallel with serum ALT and ferritin in the present study. It was also found that the serum type IV collagen-7S and PIIIP decreased during the phlebotomy. These results, together with the recoveries of tHODE and its stereoisomer ratio (ZE/EE), suggest that iron reduction therapy is able to reduce oxidative stress induced by hepatic iron overload and may diminish proliferative activity resulting from reduced necroinflammation.

In conclusion, the results of the present study clearly show that the levels of tHODE and 8-sio-$PGF_{2\alpha}$, which are biomarkers for lipid peroxidation *in vivo*, in the HCV-infected patients are significantly higher, and the antioxidant levels of the patients are lower than those of healthy controls. These findings indicate that oxidative stress is involved in HCV-induced liver diseases. More importantly, the level of tHODE tended to increase with disease progression, although overlap exists, and decreased by iron reduction therapy in parallel with markers for hepatic fibrosis and serum AFP. Thus, it can be concluded that tHODE is a suitable biomarker for the evaluation of HCV disease. However, one might argue that these lipid peroxidation products are by no means specific to liver damage. Admittedly, this lack of specificity is an inherent drawback associated with oxidative stress biomarkers of lipids. Nevertheless, the combination of several biomarkers should be effective in improving the accuracy, and they may be used as surrogate biomarkers to evaluate oxidative stress in patients with chronic hepatitis C.

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Secondary Structure of the Amino-Terminal Region of HCV NS3 and Virological Response to Pegylated Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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The aim of the study was to identify a predictive marker for the virological response in hepatitis C virus 1b (HCV-1b)-infected patients treated with pegylated interferon plus ribavirin therapy. A total of 139 patients with chronic hepatitis C who received therapy for 48 weeks were enrolled. The secondary structure of the 120 residues of the amino-terminal HCV-1b non-structural region 3 (NS3) deduced from the amino acid sequence was classified into two major groups: A and B. The association between HCV NS3 protein polymorphism and virological response was analyzed in patients infected with group A ($n = 28$) and B ($n = 40$) isolates who had good adherence to both pegylated interferon and ribavirin administration (>95% of the scheduled dosage) for 48 weeks. A sustained virological response (SVR) representing successful HCV eradication occurred in 33 (49%) in the 68 patients. Of the 28 patients infected with the group A isolate, 18 (64%) were SVR, whereas of the 40 patients infected with the group B isolate only 15 (38%) were SVR. The proportion of virological responses differed significantly between the two groups ($P < 0.05$). These results suggest that polymorphism in the secondary structure of the HCV-1b NS3 amino-terminal region influences the virological response to pegylated interferon plus ribavirin therapy, and that virus grouping based on this polymorphism can contribute to prediction of the outcome of this therapy. ***J. Med. Virol. 82:1364–1370, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis C; interferon; ribavirin; interaction; polymorphism

INTRODUCTION

Hepatitis C virus (HCV) is the major pathogen that causes chronic liver diseases with a risk of progression to cirrhosis and hepatocellular carcinoma. Currently, the standard treatment for chronic hepatitis C is antiviral therapy using pegylated interferon (Peg-IFN) plus ribavirin (RBV), and this approach is most effective for eradication of HCV viremia. However, even with the widely used treatment regimen of 48 weeks, the rate of sustained virological response (SVR), which indicates eradication of viremia, is still approximately 50% for patients infected with the therapy-resistant HCV genotype 1b (HCV-1b) with a high viral load [Manns et al., 2001; Bruno et al., 2004; Hadziyannis et al., 2004]. It would be useful to predict the virological response to this therapy and to identify patients who would obtain beneficial therapeutic effects before treatment, in order to avoid any serious side effect and to eliminate those who would not be helped by the treatment. In the future it will be important to establish a protocol of tailor-made medicine for chronic hepatitis C.

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Both the HCV genotype and pre-treatment viral load are major viral factors that influence the response to IFN-based antiviral therapy, but IFN resistance is also partly due to variation of the amino acid sequence encoded by HCV itself. Enomoto et al. [1996] proposed that variation of 40 amino acids within the NS5A region (aa 2,209–2,248), which is referred to as the IFN sensitivity-determining region (ISDR), is well correlated with IFN responsiveness. ISDR and its adjacent sequence bind and inhibit the enzymatic activity of a double-stranded RNA-activated protein kinase (PKR), which can have an antiviral effect, and therefore the combined region is referred to as the PKR-binding domain (PKR-BD) [Gale et al., 1997, 1998]. A correlation between sequence variation in the PKR-BD and IFN responsiveness has been reported [Nousbaum et al., 2000], and some reports show a correlation between IFN responsiveness and the sequence diversity of variable region 3 (V3) (aa 2,356–2,379) or surrounding regions near the carboxy terminus of NS5A [Murphy et al., 2002; Sarrazin et al., 2002; Puig-Basagoiti et al., 2005]. A high degree of amino acid substitution in the V3 and pre-V3 regions (aa 2,334–2,355) of NS5A, which is referred to as the IFN/RBV resistance-determining region (IRRDR) (aa 2,334–2,379), has been associated with SVR in Peg-IFN/RBV combination therapy for patients infected with HCV-1b [El-Shamy et al., 2007, 2008]. In addition to these findings in non-structural proteins of the virus, amino acid substitution in a structural region of HCV has been reported to be a predictive viral marker for the virological response to PegIFN/RBV therapy. Amino acid polymorphisms in the HCV core region (Arg70 vs. Gln70 and Leu91 vs. Met91) correlate with virological outcome and on-treatment viral kinetics in Peg-IFN/RBV therapy [Akuta et al., 2006, 2007], and a double wild-type HCV core (Arg70 and Leu91) may be a significant predictor of SVR in Peg-IFN/RBV therapy [Akuta et al., 2007].

Interactions between viral and host proteins in infected cells may influence therapeutic effects and the natural history of infection, since the HCV NS3 region has a significant effect on immunity. The amino-terminal part of this region encodes a serine protease, for which the minimum activity has been mapped to a region between aa 1,059 and 1,204 [Yamada et al., 1998]. The serine protease inactivates Cardif, a caspase recruitment domain (CARD)-containing adaptor protein that interacts with the RNA helicase retinoic acid inducible gene 1 (RIG-1)-dependent antiviral pathway in infected cells [Foy et al., 2003; Meylan et al., 2005; Evans and Seeger, 2006]. This action inhibits phosphorylation and subsequent heterodimerization of interferon regulatory factor-3 (IRF-3), which is essential for activation of IFN signaling through translocation of IRF-3 heterodimers into the nucleus, and eventually blocks IFN-beta production. In addition, inactivation of IRF-3 is postulated to influence the therapeutic effect of IFN-based antiviral therapy, because the IRF-3 heterodimer translocates into the nucleus to bind to the IFN-stimulated response element that produces many antiviral proteins, including 2′,5′-oligoadenylate synthetase and PKR [Nakaya et al., 2001; Grandvaux et al., 2002]. Collectively, these findings suggest that polymorphisms in HCV NS3 structure deduced from sequence variation may influence IFN-related signaling and the antiviral effect of IFN-based anti-HCV therapy.

We have focused on polymorphisms in the secondary structure of the viral polyprotein that interacts with host proteins involved in immunity, with the aim of identification of predictive viral markers for the response to Peg-IFN/RBV therapy. In this study, we examined the potential correlation between polymorphisms in the secondary structure of the HCV NS3 amino-terminal region and virological responses to Peg-IFN/RBV therapy in patients infected with HCV-1b with a high viral load.

PATIENTS AND METHODS

Patients and Treatment Regimen With Peg-IFN Plus Ribavirin

A total of 139 consecutive patients diagnosed with chronic hepatitis C were enrolled in the study from December 2004 to March 2007. These patients included 81 men and 58 women, and were aged from 31 to 75 years old (mean ± SD, 56.8 ± 8.7 years old). All patients were infected with HCV-1b with a high viral load of over 100 KIU/ml, and all received Peg-IFN/RBV therapy. Patients with alcoholic liver injury, autoimmune liver disease, and those who had symptoms of decompensated cirrhosis including ascites were excluded. Briefly, all patients were treated with a combination of Peg-IFN-alpha 2b (Pegintron®; Schering-Plough, Kenilworth, NJ) and RBV (Rebetol®; Schering-Plough) for 48 weeks. Peg-IFN was administered subcutaneously once a week and RBV was given orally twice a day for the total dose. The dosages were determined on the basis of body weight according to the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare, and there was a limit for calculating the optimized dose: patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given Peg-IFN at doses of 60, 80, 100, and 120 μg, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1,000 mg, respectively. The dose of Peg-IFN or RBV was reduced according to the Japanese standard criteria based on the white blood cell count, neutrophil count, hemoglobin concentration and platelet count [Hiramatsu et al., 2008].

Virological Tests and Response to Peg-IFN Plus Ribavirin

Virological responses were evaluated at 12 weeks after the start of treatment with an early depletion of viremia referred to as an early virological response (EVR), at the end of treatment with depletion of viremia referred to as an end of treatment virological response (ETR), and at 24 weeks after completion of treatment,

with a clinical outcome of a sustained virological response (SVR) representing successful HCV eradication. All patients were negative for hepatitis B surface antigen. Quantification of serum HCV RNA was performed using an RT-PCR-based commercial kit (Amplicor HCV monitor test, ver. 2.0, Roche Diagnostics, Tokyo, Japan). This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml. SVR was determined by monitering negativity for HCV RNA monthly for 6 months. The real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics) for more precise quantitation of HCV viremia has recently become available and pre-treatment viral titers were re-evaluated using preserved serum samples. This real-time PCR assay has a lower limit of detection of 15 IU/ml. The study protocol was approved by the Ethics Committee of Yamagata University Hospital. Informed consent was obtained from all patients.

PCR Amplification of the Amino-Terminal Region of NS3

RNA was extracted from 50 μl of serum using an RNeasy Mini kit (Qiagen, Tokyo, Japan). To amplify the region of the HCV genome encoding the amino-terminal region of NS3 (1,027–1,206), a one-step PCR was performed in a tube using the Superscript One-Step RT-PCR kit with Platinum Taq (Gibco-BRL, Tokyo, Japan) and an outer set of primers: NS3-F1 (sense primer; 5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3,295–3,316) and NS3-AS2 (antisense primer; 5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4,040–4,019), as reported previously [Ogata et al., 2002a, 2003]. PCR was initially performed at 45°C for 30 min at RT and then at 94°C for 2 min, followed by the first-round PCR for forty 3-min cycles at 94°, 55°, and 72°C for 1 min each. The second-round PCR was performed with *Pfu* DNA polymerase (Promega, Tokyo, Japan) and an inner set of primers: NS3-F3 (sense primer; 5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3,390–3,407) and NS3-AS1 (antisense primer; 5′-GCC ACT TGG AAT GTT TGC GGT A-3′; nucleotides 4,006–3,985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed amplification of the corresponding portion of the HCV genome from HCV-1b RNA-positive samples. The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced (without being subcloned) in both directions using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (Applied Biosystems, Tokyo, Japan).

Classification of the Secondary Structure of the HCV-1b NS3 Amino-Terminal Region

The secondary structure of the amino-terminal region of HCV NS3 was predicted by computer-assisted Robson analysis [Garnier et al., 1978] with Genetyx-Mac software (ver.10.1; Software Development Co., Tokyo, Japan). Previously, the full-length secondary structure of the HCV-1b NS3 region was analyzed, and this showed that the secondary structure deduced from the carboxy-terminal 60 residues was well conserved in terms of linear structure, without any turn structure [Ogata et al., 2002a]. We have shown that the secondary structure of the 120 residues in the amino-terminal region of HCV-1b NS3 can be classified into two major groups: A and B (Fig. 1) [Ogata et al., 2002a, 2003]. Briefly, the criteria for this classification are as follows: in group A isolates, the carboxy-terminal 20 residues (aa 1,125–1,146) are oriented leftward relative to a domain composed of the remaining amino-terminal region; whereas in group B isolates, the same 20 residues are oriented rightward relative to the rest of the amino-terminal domain.

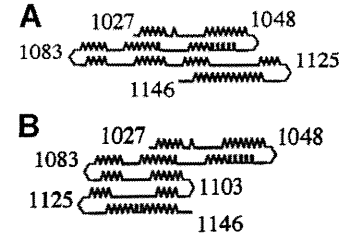

Fig. 1. Secondary structure of the 120 amino-terminal residues of HCV-1b nonstructural 3 (NS3) region classified into two major groups: A and B. The looped, zigzag, straight, and bent lines represent α-helix, β-sheet, coil, and turn structures, respectively. The numbers indicate amino acid positions. **A**: Group A, (**B**) Group B.

Analysis of Amino Acid Substitutions in the Core Region

To amplify a region of the HCV genome encoding the core region including positions 70 and 91, reverse transcription and the first-round PCR were performed in a tube by the Superscript One-Step RT-PCR kit with Platinum *Taq* (Gibco-BRL) and an outer set of primers, followed by second-round PCR with an inner set of primers in accordance with procedures reported previously [Ogata et al., 2002b]. The sequences of the amplified fragments were determined by direct sequencing.

Statistical Analysis

Data were analyzed by a χ^2 test for independence with a two-by-two contingency table and a Student *t*-test. A *P*-value <0.05 was considered significant.

RESULTS

Virological Response *and Adherence* to the Peg-IFN Plus Ribavirin Regimen

Rates of virological responses in patients treated with PegIFN/RBV combination therapy for 48 weeks are shown in Figure 2. Of the 139 patients enrolled in the study, SVR, non-SVR and cessation of therapy occurred in 58 (42%), 62 (45%), and 19 (14%), respectively. Serious

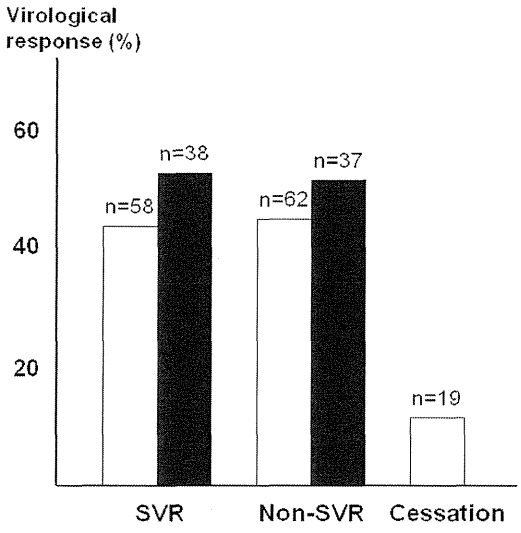

Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

adverse events that necessitated discontinuation of this therapy were depression in one patient, thyroid function disorder in 2, general itching in 2, infection in 2, anorexia in 2, occurrence of hepatocellular carcinoma in 2, and a decreased neutrophil count in 2. Six patients also terminated this therapy at their own request. Of the 139 patients, 75 (54%) received >80% of the scheduled dosage of Peg-IFN and RBV designated before treatment, and of these 75 cases SVR and non-SVR occurred in 38 (51%) and 37 (49%), respectively.

Prevalence of Types of Secondary Structure of the Amino-Terminal Region of HCV NS3

The prevalence of the types of secondary structure of HCV NS3 in the 139 subjects is shown in Table I. Among these subjects, 43 (31%), 70 (50%), and 26 (19%) were classified into groups A, B, and others, including 3 of mixed type (A plus B) and 23 of non-A, non-B type. Of the 75 cases with good adherence to administration of >80% of the scheduled dosage, 28 (37%), 40 (53%) and 7 (9%) were classified into groups A, B, and others. The amino acid data of group A and B in the cases with good adherence to administration are available in the DDBJ/EMBL/GenBank databases with the accession numbers AB548070–AB548137. Our analysis revealed no specific correlations between amino acid sequences and the secondary structure deduced by the Robson method, as we have reported previously [Ogata et al., 2003].

TABLE I. Prevalence of the HCV NS3 Secondary Structure Type

	Group A (%)	Group B (%)	Others (%)
Enrolled cases (n = 139)	43 (31)	70 (50)	26 (19)
Adherent cases (n = 75)	28 (37)	40 (53)	7 (9)

Characteristics of Adherent Patients Based on Different HCV NS3 Structure Types

The virological responses to Peg-IFN/RBV combination therapy for patients infected with group A and B isolates were assessed in the 68 subjects with good adherence to the scheduled dosage of Peg-IFN and RBV. The characteristics of patients infected with group A and B isolates are shown in Table II. Age, gender, pre-treatment level of serum HCV RNA and ALT, and frequency of fibrosis stage did not differ significantly between the two groups. Peg-IFN/RBV combination therapy was completed in all the patients, and the total administered dosages of Peg-IFN and RBV was >95% of the scheduled dosage in both groups.

Relationship Between Virological Responses and Polymorphisms in the HCV NS3 Amino-Terminal Region

In the 68 patients who received >95% of the scheduled doses of Peg-IFN and RBV for 48 weeks, SVR and non-SVR occurred in 33 (49%) and 35 (51%), respectively. The EVR, ETR, and SVR rates in patients infected with group A and B isolates are shown in Table III. There was a significant difference in the rates of EVR between subjects infected with group A and B isolates: EVR was achieved in 19 of 28 (68%) patients with group A infection, compared to 17 of 40 (43%) with group B infection ($P < 0.05$). The final outcome also differed significantly between subjects infected with group A and B isolates: SVR was achieved in 18 of 28 (64%) patients with group A infection, compared to 15 of 40 (38%) with group B infection ($P < 0.05$).

Polymorphisms in Core Amino Acids 70/91 and in the HCV NS3 Secondary Structure

The wild-type core sequence (Arg70, Leu91) has been associated with SVR in Peg-IFN/RBV combination therapy, while the non-double wild-type containing one or two substitutions at positions 70 and/or 91 was associated with non-SVR [Akuta et al., 2007]. Therefore, we examined substitutions at positions 70 and 91 in the HCV core region in pre-treatment serum samples of 44 cases that were available for testing. The double wild-type 70/91 sequence was found in 22 of the 44 cases (50%), of which 12 were SVR and 10 were non-SVR. Combination analysis of polymorphisms of the HCV core 70/91 positions and the NS3 amino-terminal region showed that 10 (83%) of the 12 SVR cases and only 3 (30%) of the 10 non-SVR cases with the double wild-type core had a group A polymorphism in HCV NS3 (Table IV). Thus, combination analysis of the core and NS3 regions may improve prediction of the outcome of Peg-IFN/RBV therapy.

TABLE II. Characteristics of Adherent Patients Infected With HCV Group A and B Isolates

	Group A (n = 28)	Group B (n = 40)	*P*
Age (years)	55.5 ± 9.5	55.5 ± 8.9	NS[a]
Sex (men/women)	18/10	21/19	NS[b]
Pre-treatment HCV RNA (KIU/ml)	1,635 ± 930	2,087 ± 1,422	NS[a]
Alanine aminotransferase level (U/L)	80 ± 62	71 ± 47	NS[a]
Stage of liver fibrosis			
F1 or F2/F3 or F4	19/9	28/12	NS[b]
Drug adherence dosage (%)			
Pegylated interferon	97.7 ± 5.2	95.2 ± 7.3	NS[a]
Ribavirin	96.8 ± 6.4	95.3 ± 7.7	NS[a]

NS, not significant.
[a]*t*-test.
[b]χ^2 test.

Re-Evaluation of Pre-Treatment HCV Viremia Status Using Real-Time PCR

Since the viral titer before treatment is a major predictive marker of the outcome of Peg-IFN/RBV therapy, we re-evaluated the pre-treatment viral titers more precisely using preserved serum samples taken within 1 month before treatment, using a real-time PCR assay. The pre-treatment viral titers did not differ significantly between sera with group A and B isolates (5.98 ± 0.94 vs. 6.25 ± 0.62 logIU/ml) (Table V). The secondary structure polymorphisms of HCV NS3 were independent of the pre-treatment viral titers.

DISCUSSION

Antiviral therapy with Peg-IFN/RBV for 48 weeks fails to eradicate HCV in about half of patients infected with a high titer of HCV genotype 1b, and the severe adverse events and high costs associated with this therapy require outcome prediction to allow targeted treatment for chronic hepatitis C. The pre-treatment viral titer, viral factors that influence the virological response to IFN-based anti-HCV therapy have been widely investigated. Viral kinetics showing prompt seronegativity after the start of treatment is a critical factor for achieving SVR, and thus the possible correlation between an early virological response and genetic sequence variation of the HCV has been studied. In particular, amino acid substitutions in the HCV core region at positions 70 and 91 or multiple mutations detected in the IRRDR of the HCV NS5A region are useful markers for predicting EVR and subsequent SVR.

TABLE III. Virological Responses in Subjects With Different Polymorphisms in the Secondary Structure of HCV NS3

	EVR*	ETR**	SVR*
Group A (n = 28)	19 (68%)	23 (82%)	18 (64%)
Group B (n = 40)	17 (43%)	25 (63%)	15 (38%)

EVR: early virological response at 12 weeks after the start of treatment.
ETR: virological response at the end of treatment.
SVR: sustained virological response 24 weeks after completion of treatment.
*$P < 0.05$.
**$P = 0.08$; χ^2 test.

To date, the influence of several single amino acid substitutions and accumulation of these changes in the viral genome on the effect of IFN-based anti-HCV therapy has been examined. Since interactions between host and viral proteins in infected cells may influence the therapeutic effect of an antiviral agent, we focused on the association of structural polymorphism of a viral protein with the effect of Peg-IFN/RBV combination therapy in this study. Our results suggest that polymorphism analysis of secondary structure deduced from sequence variations in the HCV NS3 amino-terminal region can be used to predict viral responses to this therapy.

Amino acid sequences of the HCV NS3 amino-terminal region, which encodes a serine protease, vary greatly among HCV isolates. Interactions between HCV NS3 and host proteins may influence both oncogenesis and immunity, and thus elucidation of the biological significance of these interactions could result in a new prognostic marker for HCC or a predictive marker for anti-HCV therapy. First, HCV NS3 interacts with the p53 tumor suppressor to suppress p53-dependent apoptosis or p21 transcriptional activity [Ishido and Hotta, 1998; Kwun et al., 2001; Deng et al., 2006]. Transfection of a plasmid expressing the amino-terminal portion of HCV NS3 induces cell transformation in vitro, and transplanted cells proliferate with sarcoma-like features in vivo [Sakamuro et al., 1995]. These findings suggest that NS3 may be involved in the oncogenic pathway in HCV infection. We have shown that the secondary structure of the 120-residue amino-terminal region of NS3 (1,027–1,146) is classifiable into two major groups: A and B. This region encodes a serine protease and also includes p53-binding sites. Our

TABLE IV. Treatment Outcome of Cases With a Double Wild-Type Core Region and Different HCV NS3 Structural Polymorphism

	Group A (%)	Group B (%)	*P*
SVR (n = 12)	10 (83)	2 (17)	0.02[a]
Non-SVR (n = 10)	3 (30)	7 (70)	

SVR, sustained virological response.
[a]χ^2 test.

TABLE V. Pre-Treatment HCV RNA Levels Measured by Real-Time PCR for Subjects With Different HCV NS3 Structural Polymorphism

	Group A	Group B	*P*
SVR (n = 33)	5.78 ± 1.05	6.I3 ± 0.71	NS[a]
Non-SVR (n = 35)	6.33 ± 0.59	6.32 ± 0.55	NS[a]
Total (n = 68)	5.98 ± 0.94	6.25 ± 0.62	NS[a]

SVR, sustained virological response. NS, not significant.
[a]*t* test.

previous cross-sectional studies revealed that the prevalence of group B infection is significantly higher in HCC cases than in non-HCC cases [Ogata et al., 2003], and that the group B infection is an independent risk factor for development of HCC in patients with chronic HCV infection [Nishise et al., 2007]. Second, NS3 interacts with host proteins associated with IFN signaling and thus influences cellular immunity. Since the serine protease encoded by the amino-terminal region of NS3 inhibits the IFN-signaling pathway, polymorphism of this region is likely to influence the effect of Peg-IFN/RBV combination therapy.

Several factors associated with the virological response to this therapy are well known, with adherence to both IFN and RBV strongly influencing outcome [Pearlman, 2004; Arase et al., 2005; Yamada et al., 2008]. In this study, we analyzed 75 cases in which >80% of the scheduled dosage of both drugs was administered. Of these cases, 28 (37%) and 40 (53%) were infected with group A and B isolates, respectively, which were similar rates to those for the 139 cases in the overall study. Age, gender, viral load before treatment, ALT level, proportion of fibrosis stage and adherence to Peg-IFN and RBV did not differ between the group A and B cases. However, the frequencies of SVR and EVR were significantly higher in group A, and those for non-EVR and non-SVR were significantly higher in group B. The results suggest that infection with the group B isolate, which correlates with a higher rate of HCC, is resistant to Peg-IFN/RBV therapy. The pre-treatment viremia status in the 68 cases with group A or B isolates showed no significant differences between the two groups of patients. Therefore, these results suggest that the secondary structure of the HCV NS3 amino-terminal region may be useful for prediction of the outcome of Peg-IFN/RBV combination therapy. In this initial study setting, the relationship of these polymorphisms to the frequency of rapid viral response at 4 weeks after the start of treatment was not evaluated. It will be important to assess this relationship in a future study.

The polymorphism in HCV core region (Arg70/Leu91) is a useful predictive marker for virological responses in Peg-IFN/RBV therapy [Akuta et al., 2007]. Interestingly, a combined analysis of polymorphisms of the core region (which encodes a structural protein) and HCV NS3 (a nonstructural protein) improved the prediction rate. Therefore, analysis of NS3 polymorphism in combination with the core structural polymorphism appears to improve prediction of the outcome of Peg-IFN/RBV therapy. A larger, multi-center prospective study would be necessary to validate the present results. In conclusion, the results of this study suggest that secondary structure polymorphism in the amino-terminal region of HCV NS3 is a useful predictive marker of the effect of Peg-IFN/RBV combination therapy for chronic hepatitis C. Although the present findings are clinically important, and will be helpful for predicting the outcome of Peg-IFN/RBV therapy, further in vitro studies will be needed to elucidate the molecular mechanism underlying the association of HCV NS3polymorphisms with clinical outcome.

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Usefulness of the Multimodality Fusion Imaging for the Diagnosis and Treatment of Hepatocellular Carcinoma

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Key Words
US fusion imaging · CT fusion imaging · Fusion imaging · Volume Navigation System · Hepatocellular carcinoma · Radiofrequency ablation · Gd-EOB-DTPA · Sonazoid

Abstract
A multimodality fusion imaging system has been introduced for the clinical practice of diagnosis and treatment of hepatocellular carcinoma (HCC), especially for loco-regional treatment. An ultrasonography (US) fusion imaging system can provide a side-by-side display of real-time US images and any cross-sectional images of multiplanar reconstruction of CT or MRI that synchronize real-time US. The US fusion imaging system enables us to perform radiofrequency ablation (RFA) for HCCs difficult to detect on conventional US safely. Besides, we can evaluate the treatment effects of RFA easily at the bedside by combining the contrast-enhanced US and the US fusion imaging system. Fusion images of pre- and post-RFA CT have been utilized for the assessment of the treatment effects of RFA. Although the treatment effects of RFA have been conventionally evaluated, comparing pre- and post-RFA CT side-by-side, the evaluation tends to be inaccurate. On CT fusion images, the tumor and the ablation zone are overlaid and we can grasp the positional relation easily, leading to quantitative and more accurate evaluation. The multimodality fusion imaging system has become quite an important tool for loco-regional treatment of HCC because of its usefulness for both the guidance during the RFA procedure and the evaluation of its treatment effects.

Introduction

Recently, imaging technology in CT, MRI and ultrasonography (US) for the diagnosis of hepatocellular carcinoma (HCC) has dramatically progressed. In addition, contrast media such as Sonazoid (Daiichi-Sankyo, Tokyo, Japan), one of the second-generation US contrast agents, and gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) (Primovist; Bayer HealthCare, Osaka, Japan), a liver-specific contrast MR agent have become available [1–9]. As a result, the diagnosis of HCC has come to be made at an earlier stage. However, it is occasionally difficult to perform needle-based loco-re-

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gional treatment such as radiofrequency ablation (RFA) and percutaneous ethanol injection therapy for early HCCs, since some of them are not clearly visualized on grayscale US [10].

Along with the progress in diagnostic imaging, a multimodality fusion imaging system has been developed for the clinical practice of treatment of HCC, particularly for the assistance of loco-regional treatment. It has been reported that HCCs hardly detectable on conventional grayscale US could be detected with subsequent loco-regional treatment by virtue of the US fusion imaging system [1, 10–15]. In addition to the US fusion imaging system, a CT fusion imaging system has been reported to be useful for accurate assessment of treatment effects of RFA [16–19]. This report aims to review the usefulness of the multimodality fusion imaging system for percutaneous loco-regional treatment of HCC.

Outline of the US Fusion Imaging System

The US fusion imaging system, such as the Volume Navigation System (GE Healthcare Japan, Tokyo, Japan) [1, 15] and Real-time Virtual Sonography (Hitachi Medico, Co., Tokyo, Japan) [11–14] enables the synchronized display of real-time US images and multiplanar reconstruction (MPR) images of CT or MRI corresponding to the cross section of real-time US. The MPR images are reconstructed based on the volume data of CT or MR images and displayed as a reference, side-by-side, with real-time US images on a single screen.

The US fusion imaging system is useful for the accurate diagnosis and treatment of HCC with safety, in particular at the time of percutaneous loco-regional procedures. It has been reported that the US fusion imaging system is helpful in the detection of HCCs which are difficult to recognize on grayscale US [1, 14, 15]. Therefore, even if the diagnosis of HCC is made at an early stage and the tumor is not detected on US, percutaneous loco-regional treatments can be conducted using the US fusion imaging system. Besides the guidance of loco-regional treatments, the US fusion imaging system can be applied to the evaluation of treatment effects of RFA [13].

The Volume Navigation System, one of the multimodality fusion imaging systems commercially available since 2009 in Japan, is equipped with an ultrasound unit LOGIQ E9 (GE Healthcare Japan) (fig. 1). In Volume Navigation System, the following steps are needed for the synchronized display of real-time US images and CT or MR images.

First, the volume data of CT or MR images for reference should be imported into the system in the digital imaging and communication in medicine (DICOM) format, through a network or a recording media such as CD-ROM or USB-HDD. After the volume data is imported, US images are displayed on the left side of the screen and CT or MR images on the right side, as a reference. In order to synchronize real-time US images to the reference, the cross section of US images approximately parallel to the axial image of the reference has to be registered. Next, two magnetic positioning sensors attached to the probe of an ultrasound scanner (a in fig. 1) detect the magnetic field radiated from a magnetic field transmitter (b in fig. 1) and transmit the information of spatial location and orientation of the probe to a magnetic position-detecting unit (c in fig. 1) equipped with LOGIQ E9. In this way, a magnetic position-detecting unit integrates the positional information of two magnetic positioning sensors and reconstructs MPR images which match the 3D information of the sensors. Subsequently, real-time US and reference images are synchronously displayed in accordance with the movement of the probe. However, since these two image sets are not exactly matched at this time yet, further positional registration of real-time US and reference images is needed. A common point on US and reference images should be visualized for the registration. Practically, we have to visualize a characteristic landmark on US images and mark the point. After marking the landmark point, the US image is fixed and we should operate the probe to seek the corresponding landmark on reference images, by comparing it with the fixed US image. When the landmark point on the reference image is marked, the registration is completed. Then, US and reference images are matched and simultaneously displayed, side by side, on the same screen.

In addition to the simultaneous display of real-time US images and a reference, the Volume Navigation System is equipped with the global positioning system (GPS) function. After positional registration, when GPS markers are indicated on the target on reference images, corresponding sites are pinpointed on real-time US images. This GPS function provides several advantages: firstly, since the site where the target should be visible is pinpointed on real-time US images, it is helpful to detect the target on US, even if it is difficult to perceive on conventional US, and secondly, once the GPS markers are indicated on a target, the target can easily be detected by anyone and from any scanning section, by referring to the GPS markers. Thirdly, it can be applied to the evaluation of treatment effects of RFA, as described later.