dietary iron reduction in Japanese patients with chronic hepatitis C.[84] The efficacy of phlebotomy for NASH/NAFLD patients has never been established (Table 2). Facchini *et al.* have shown improvement in liver enzymes levels in 17 NAFLD patients with impaired glucose tolerance undergoing serial phlebotomy for iron reduction.[85] The estimated body iron stores (based on phlebotomy need) in these patients were with the normal range. At the end of phlebotomy schedule, however, there was a 40% to 55% improvement of both fasting and glucose-stimulated plasma insulin concentrations. This efficacy of phlebotomy was confirmed even in NAFLD patients with normal glucose tolerance.[86] According to Fargion *et al.*,[28] in 42 NAFLD patients, HOMA-IR was significantly decreased after 4-month hypocaloric diet, and a further reduction was observed after phlebotomies. We also reported that phlebotomy declined serum transaminase activities in Japanese patients with biopsy-proven NASH.[87] One of them obtained improvement of serum TRX after phlebotomies. These studies have not proved histological improvement. Therefore, the effect of phlebotomy on liver histology in NASH/NAFLD must be evaluated further. Riquelme *et al.*[88] reported a 52-year non-obese woman with biopsy-proven NASH obtaining not only improvement of transaminase activities but also complete resolution of fatty infiltration and inflammatory changes after iron depletion therapy. According to a case-control study by Valenti *et al.*,[89] iron depletion produced a significantly larger decrease in IR compared with nutritional counseling alone, independent of changes in BMI, baseline HOMA-IR, and the presence of the metabolic syndrome. Likewise, phlebotomy has been suggested in patients with high-ferritin diabetes, in whom bloodletting led to significant decreases in IR.[90] Similarly, declines in post-glucose load plasma glucose and insulin levels were also observed in healthy volunteers with normal glucose and ferritin levels.[91] Also in patients with IR-HIO, phlebotomy improved the presenting symptoms (chronic fatigue and /or polyarthralgias), serum transaminase activities, and metabolic indices.[92,93] The result of the study by Facchini *et al.*[85,91] raise the question of whether patients with NAFLD and even normal body iron stores should undergo phlebotomies. Fargion *et al.*[28] showed that the effect of phlebotomy was observed also in patients with normal iron parameters at enrolment. In contrast, Valenti L *et al.*[89] indicated that iron depletion was more effective in reducing HOMA-IR and ALT in patients with hyperferritinemia, and in carriers of the HFE mutations. Thus, investigators are needed to examine whether NAFLD patients without hepatic iron overload should be phlebotomized to ameliorate insulin resistance or hepatic inflammation.

Now phase II clinical trials provided by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) are currently recruiting patients. The goal of this pilot study is to determine the effect of iron depletion on insulin sensitivity in patients with type 2 diabetes mellitus and NAFLD. Secondary outcome measures will include the effect of iron depletion on hepatic necroinflammation, markers of oxidative stress and intrahepatic fat content. Because an increase in hepatic iron has been found to correlate with severity of fibrosis,[5] phlebotomy to remove excess iron may potentially have a beneficial effect in preventing the progression of fibrosis. In the HFD-fed rabbits with IR,[78] phlebotomy significantly reduced hepatic fibrosis as well as lipid peroxide. In the future, prospective human studies using a large number of patients are essential to clarify whether phlebotomy can really prevent the progression of fibrosis or carcinogenesis in NASH patients.

According to Yamamoto *et al.*,[94] twelve NAFLD patients (NASH, $n = 9$; simple steatosis, $n = 3$) were given a dietary prescription including restriction of energy, fat and iron (< 6 mg/day). The average energy intake, fat energy fraction and iron intake decreased significantly 6 months after the beginning of the diet in all patients. In addition, the levels of serum transaminase and ferritin were significantly decreased. They suggest that dietary iron reduction should be recommended not only in hepatitis C patients[95] but also in NAFLD patients.

CONCLUSIONS

IRON-ASSOCIATED OXIDATIVE stress may at least partly play a role in the pathogenesis in NASH/NAFLD, but the mechanisms of hepatic iron deposition remains unknown. Iron reduction by phlebotomy, which is well tolerated, may be of clinical use to reduce transaminase activities and insulin resistance. Larger controlled trials of longer duration are warranted to assess the long-term clinical benefit of phlebotomy.

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Original Article

Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease

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Aims: Hepatic steatosis and iron cause oxidative stress, thereby progressing steatosis to steatohepatitis. We quantified the expression of genes involved in the metabolism of fatty acids and iron in patients with nonalcoholic fatty liver disease (NAFLD).

Methods: The levels of transcripts for the following genes were quantified from biopsy specimens of 74 patients with NAFLD: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Twelve samples of human liver RNA were used as controls. Histological evaluation followed the methods of Brunt.

Results: The levels of all genes were significantly higher in the NAFLD patients than in controls. The Trx level increased as the stage progressed. The levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed ($P < 0.05$). Hepatic iron score (HIS) increased as the stage progressed. The TfR1 level significantly increased as the stage progressed ($P < 0.05$), whereas TfR2 level significantly decreased ($P < 0.05$). The ratio of hepcidin mRNA/ferritin ($P < 0.001$) or hepcidin mRNA/HIS ($P < 0.01$) was significantly lower in NASH patients than simple steatosis patients.

Conclusions: Steatosis-related metabolism is attenuated as NAFLD progresses, whereas iron-related metabolism is exacerbated. Appropriate therapies should be considered on the basis of metabolic changes.

Key words: fatty acids, iron, NAFLD, oxidative stress

Abbreviations
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

INTRODUCTION

NON ALCOHOLIC FATTY liver disease (NAFLD) is a wide-spectrum liver disease, ranging from simple steatosis to steatohepatitis.[1] Owing to the obesity epidemic, NAFLD is now recognized as a leading health problem worldwide.[1] Since NAFLD has been documented to progress to liver failure[2] and/or hepatocellular carcinoma,[3] various therapeutic studies for NAFLD or nonalcoholic steatohepatitis (NASH) have been conducted to date.[4–8] These studies included weight reduction,[4] use of insulin sensitizers,[5] antioxidants,[6] phlebotomy[7] and hepato-protective drugs,[8] albeit with limited success. Although these treatments are aimed at addressing the pathogenesis of NAFLD, they would not always be efficient at every stage of this "wide spectrum" disease.

NASH is thought to develop through a "two-hit theory".[9] The first hit includes insulin resistance, mostly due to obesity.[9] The second hits include oxidative stress, inflammatory cytokines, and bacterial endotoxin.[9] In particular, the accumulation of fatty acids in the liver results in oxidative stress through oxidation of fatty

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acids.[10] In addition, hepatic iron load, which also induces oxidative stress, has been reported in some groups of patients with NAFLD.[11] Therefore, hepatic metabolism of fatty acids and iron should be the therapeutic target for NAFLD. However, their roles in the development of NAFLD have not yet been studied

In this study, we quantified the expression of genes involved in hepatic metabolism of fatty acids and iron using liver biopsy specimens from patients with NAFLD, and compared them with liver histology. Based on the results, we explored the role of the metabolism of fatty acids and iron in NAFLD. Our study should improve out understanding of the pathogenesis of NAFLD and contribute to the identification of putative therapeutic pathways.

PATIENTS AND METHODS

Patients

NAFLD PATIENTS WHO underwent liver biopsies in our institute between April 2000 and March 2007 were retrospectively selected according to the following criteria: no excessive alcohol intake (more than 20 g/day), as assessed by interview (on at least three occasions); no history of treatment with steatosis-inducing drugs within the 12 months prior to the study; negative serum hepatitis C virus (HCV) antibody; negative for hepatitis B surface antigen or antibodies to human immunodeficiency virus; and an absence of other forms of chronic liver disease, such as autoimmune liver diseases. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. All patients had given written informed consent for the analysis of metabolic genes and liver biopsies before the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

Laboratory determinations

After a 12-h overnight fast, venous blood samples were drawn to determine asparatate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total cholesterol, triglyceride, fasting plasma glucose (FPG), glycosylated haemoglobin (HbA_{1c}), insulin and ferritin levels. These parameters were measured using standard techniques from clinical chemistry laboratories. The index of insulin resistance was calculated only in patients without overt diabetes (fasting plasma glucose >126 mg/dL), according to the homeostasis model assessment (HOMA).

Histological evaluation

Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin–eosin, Masson's trichrome, and Perl's Prussian blue. The stage of hepatic fibrosis was scored according to Brunt[12]: 1, zone 3 fibrosis; 2, zone 3 fibrosis with periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis. The grade of inflammation was scored as follows[12]: 1, mild; 2, moderate; and 3, severe. We considered the scores of stage and grade of simple steatosis as "0". Steatosis was assessed according to the percentage of hepatocytes containing fat droplets. The degree of iron loading was graded using a Perl's score of 0–4, as described previously.[13]

Quantification of the expression of hepatic genes

Liver specimens were immediately frozen after the biopsy and were stored at −80°C until use. Total RNA was isolated from biopsy specimens using the RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was obtained from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). PCR was performed using the Light Cycler 2.0 System (Roche, Mannheim, Germany), and the mRNA levels were normalized to those of β-actin. Comprehensive target genes were as follows: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase, C4 to C12 straight chain (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Table 1 summarizes the specific primers for these target genes. Twelve samples of human total liver RNA were obtained from commercial sources (Stratagene, CA, USA; Clontech Laboratories, CA, USA; Ambion, TX, USA; Becton, Dickinson, NJ, USA; Cell Applications, CA, USA), and used as controls.

Statistical analysis

Associations between variables were analyzed using the Spearman's correlation coefficient by rank. Differences between variables were analyzed using the Mann–Whitney U-test or Kruskal–Wallis test. All analyses were performed using SPSS software for Windows, version

Table 1 The specific primers used for the target genes

	Sense primers	Antisense primers
Trx	5′-CTGCTTTTCAGGAAGCCTTG-3′	5′-ACCCACCTTTTGTCCCTTCT-3′
FATP5	5′-ACACACTCGGTGTCCCTTTC-3′	5′-CTACAGGGCCCACTGTCATT-3′
SREBP1c	5′-TGCATTTTCTGACACGCTTC-3′	5′-CCAAGCTGTACAGGCTCTCC-3′
FASN	5′-TTCCGAGATTCCATCCTACG-3′	5′-TGTCATCAAAGGTGCTCTCG-3′
ACAC	5′-GAGAACTGCCCTTTCTGCAC-3′	5′-CCAAGCTCCAGGCTTCATAG-3′
PPARα	5′-GGAAAGCCCACTCTGCCCCCT-3′	5′-AGTCACCGAGGAGGGGCTCGA-3′
CYP2E1	5′-CCCAAAGGATATCGACCTCA-3′	5′-AGGGTGTCCTCCACACACTC-3′
ACADM	5′-TTGAGTTCACCGAACAGCAG-3′	5′-AGGGGGACTGGATATTCACC-3′
ACOX	5′-TGATGCGAATGAGTTTCTGC-3′	5′-AGTGCCACAGCTGAGAGGTT-3′
MTP	5′-CATCTGGCGACCCTATCAGT-3′	5′-GGCCAGCTTTCACAAAAGAG-3′
TfR1	5′-ATGCATTTTGCAGCAGTGAG-3′	5′-TCCAAAAGGCCCTACTCCTT-3′
TfR2	5′-GACCCTGCAGTGGGTGTACT-3′	5′-CAGTCGCTCGTCTCTCTCCT-3′
hepcidin	5′-ACCAGAGCAAGCTCAAGACC-3′	5′-AAACAGAGCCACTGGTCAGG-3′

Note: The role of genes analyzed in lipid and iron metabolisms is as follows: oxidative stress-induced, Trx; uptake of fatty acid, FATP5; synthesis of fatty acid, SREBP1c, FASN, ACAC; oxidation of fatty acid, PPARα, CYP2E1, ACADM, ACOX; secretion of triglyceride, MTP; uptake of transferrin-bound iron, TfR1, TfR2; regulation of iron metabolism, hepcidin.
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

14.0 (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered significant.

RESULTS

The characteristics of patients

TABLES 2 AND 3 summarize the characteristics of patients and the results of liver histology, respectively. Of the 16 diabetic patients, 3 had been treated with metformin, 2 with pioglitazone, 2 with sulfonylurea, and the others had been followed with diet restriction. Serum triglyceride levels were greater in the simple steatosis patients than in the NASH patients. Although the values of HbA_{1c} were comparable in the two groups, those of HOMA-IR [index of insulin resistance (IR)] were significantly higher in the NASH

Table 2 Patients characteristics

	Simple steatosis ($n = 33$)	NASH ($n = 41$)	P value
Age	55.4 ± 15.0	61.2 ± 12.7	0.051
BMI (kg /m^2)	27.5 ± 2.4	26.5 ± 4.4	0.748
Sex (male/female)	24/9	25/16	0.208
Diabetes (yes/no)	7/26	9/32	0.584
Plt	21.6 ± 3.9	19.1 ± 6.3	0.006
AST	43.0 ± 21.4	72.9 ± 30.5	0.0002
ALT	62.3 ± 30.8	89.8 ± 50.3	0.006
Alb	4.7 ± 0.3	4.6 ± 0.3	0.023
T-Cho	231.1 ± 50.5	199.9 ± 44.0	0.006
TG	205.0 ± 105.8	140.9 ± 103.2	0.015
FPG	145.1 ± 68.4	116.7 ± 21.5	0.356
HbA_{1c}	6.6 ± 1.8	6.0 ± 0.6	0.533
HOMA-IR	2.9 ± 1.2	4.6 ± 1.8	0.012
ferritin	223.1 ± 106.0	197.7 ± 160.7	0.227

Note: The value is expressed as either mean ± S.D. or the number of patients.
ALT, alanine aminotransferase; AST, asparatate aminotransferase; Alb, albumin; BMI, body mass index; FPG, fasting plasma glucose; HbA_{1c}, glycosylated haemoglobin; HOMA-IR, homeostasis model assessment-index of insulin resistance; T-Cho, total cholesterol; TG, triglyceride.

Table 3 Results of liver biopsy

	Simple steatosis	NASH
Stage: 1/2/3/4		13/13/13/2
Grade: 1/2/3		27/10/4
Iron: 0/1/2/3	11/12/3/1	14/8/6/6
Steatosis:		
<30%	14	18
30%–60%	7	13
60% <	2	10

NASH, nonalcoholic steatohepatitis.

patients than in the simple steatosis patients. Neither significant fibrosis nor inflammation was observed in the biopsy specimens from patients with simple steatosis. Six specimens from simple steatosis patients and seven specimens from NASH patients were not available for iron staining.

Hepatic oxidative stress

We evaluated hepatic oxidative stress by the level of hepatic Trx, since Trx is known to be a redox-sensitive molecule.[14] We have previously reported that serum Trx levels are a marker of NASH.[15] We measured hepatic thioredoxin mRNA, because it would reflect the redox status of the liver more precisely than serum thioredoxin levels. Hepatic thioredoxin consists of both reduced and oxidized forms, whereas serum thioredoxin is an oxidized form. Therefore, hepatic thioredoxin levels do not correlate with serum thioredoxin levels. The Trx level increased in the order of controls, then simple steatosis patients with the highest levels in NASH patients (Table 4). The differences among the groups were significant (Table 4). The Trx level tended to increase as the stage progressed; however, it did not show any association with the grade (Table 5).

Fatty acid metabolism

The levels of transcripts for the genes involved in fatty acid metabolism were increased in the order of controls, then NASH patients with the highest levels in simple steatosis patients (Table 4). The differences among the groups were significant (Table 4). When values were compared between simple steatosis and NASH patients by the Mann–Whitney's test, the difference was significant in FATP5 ($P < 0.01$), ACAC ($P < 0.05$), PPARα ($P < 0.05$), CYP2E1 ($P < 0.05$), ACADM ($P < 0.05$), ACOX ($P < 0.05$), MTP ($P < 0.05$). Levels of all these genes were significantly higher in the simple steatosis patients than the NASH patients. When compared with the liver histology, the levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed (Table 5). The level of ACOX tended to decrease as the stage and grade progressed (Table 5). The level of FASN was similarly decreased, although the difference between groups

Table 4 The levels of hepatic gene involved in lipid and iron metabolism

	Control	Simple steatosis	NASH	*P* value
Trx	1.0 ± 1.1	2.3 ± 0.9	2.5 ± 1.0	$P < 0.00001$
FATP5	1.0 ± 0.4	6.1 ± 3.6	4.3 ± 2.5	$P < 0.00001$
SREBP1c	1.0 ± 0.6	73.9 ± 74.3	56.0 ± 85.4	$P < 0.00001$
FASN	1.0 ± 1.0	28.2 ± 26.8	17.8 ± 15.1	$P < 0.00001$
ACAC	1.0 ± 0.8	12.2 ± 5.9	8.7 ± 3.4	$P < 0.00001$
PPARα	1.0 ± 0.8	21.1 ± 11.3	15.5 ± 8.1	$P < 0.00001$
CYP2E1	1.0 ± 0.4	8.0 ± 4.2	6.2 ± 3.2	$P < 0.00001$
ACADM	1.0 ± 0.9	17.8 ± 9.7	13.1 ± 6.1	$P < 0.00001$
ACOX	1.0 ± 0.9	16.6 ± 9.2	12.0 ± 5.7	$P < 0.00001$
MTP	1.0 ± 1.0	10.8 ± 3.8	8.8 ± 3.3	$P < 0.00001$
TfR1	1.0 ± 1.1	10.8 ± 11.3	11.8 ± 10.3	$P < 0.00001$
TfR2	1.0 ± 0.4	7.6 ± 3.6	5.6 ± 2.8	$P < 0.00001$
hepcidin	1.0 ± 0.9	11.2 ± 9.6	5.7 ± 3.9	$P < 0.00001$

Note: The value is expressed as folds to mean control values (mean ± S.D.). The deference between the groups was determined using the Kruskal–Wallis test.

Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

Table 5 Correlation of the gene levels with liver histology*

	Stage		Grade	
	r	*P* value	*r*	*P* value
Trx	0.209	0.074	0.132	0.266
FATP5	−0.334	0.004	−0.339	0.003
SREBP1c	−0.264	0.024	−0.283	0.015
FASN	−0.158	0.178	−0.182	0.124
ACAC	−0.264	0.024	−0.313	0.007
PPARα	−0.253	0.031	−0.244	0.038
CYP2E1	−0.264	0.024	−0.293	0.012
ACADM	−0.241	0.040	−0.246	0.036
ACOX	−0.213	0.070	−0.213	0.071
MTP	−0.262	0.025	−0.271	0.020
TfR1	0.227	0.037	0.182	0.089
TfR2	−0.307	0.008	−0.318	0.006
hepcidin	−0.251	0.032	−0.221	0.060

*Using Spearman's test. Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

did not reach statistical significance (Table 5). In parallel with these findings, the level of hepatic steatosis decreased as the stage and grade progressed (Fig. 1). None of these genes was independently correlated with hepatic steatosis (not shown).

TfR1 and TfR2

The hepatic iron score (HIS) tended to increase as the stage progressed (Table 6). We examined the levels of TfR1 and TfR2, since the uptake of serum iron by hepatocytes is largely through a transferrin-bound form.[16] The levels of both of these genes were significantly higher in the NAFLD patients than in the controls (Table 4). When values were compared between simple steatosis and NASH using the Mann–Whitney's test, the TfR2 level was significantly ($P < 0.01$) higher in the simple steatosis patients than the NASH patients. The TfR1 level significantly increased as the stage progressed, whereas that of TfR2 significantly decreased as the stage and grade progressed (Table 5). Neither TfR1 nor TfR2 were independently correlated with HIS (not shown).

Table 6 Hepatic iron score and the stage

	Hepatic iron score				
	0	1	2	3	4
Stage 0	11	11	3	0	1
Stage 1	7	1	1	1	0
Stage 2	3	4	3	2	0
Stage 3	4	4	2	2	0
Stage 4	0	0	0	0	1

Note: The value represents the number of patients. Simple steatosis was considered as stage "0". $r = 0.213$, $P = 0.099$, iron score *vs* stage: Spearman's test.

Hepcidin

Hepcidin is known to be secreted from hepatocytes and regulates systemic iron transport.[16] The hepcidin level was significantly different among the controls, the simple steatosis patients and the NASH patients. The value was higher in the simple steatosis patients than in the NASH patients (Table 4). Hepcidin level decreased significantly as the stage progressed (Table 5). Since the ratio of hepcidin to iron load has been reported to evaluate the appropriateness of the hepcidin response to iron overload,[17] we divided hepcidin mRNA levels by serum ferritin levels or HIS. The ratios of hepcidin mRNA/ferritin and hepcidin mNA/HIS were signifi-

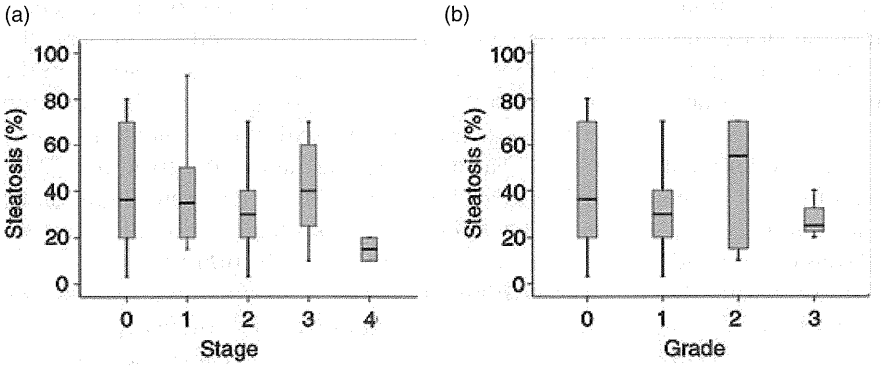

Figure 1 Distributions of the level of hepatic steatosis in association with the stage (a) and grade (b). The level of steatosis decreased as the stage and grade progressed.

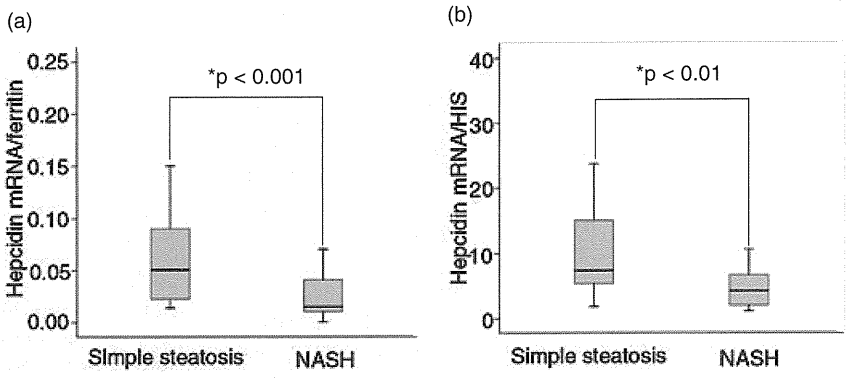

Figure 2 The ratio of hepcidin mRNA levels to serum ferritin levels (a) and that of hepcidin mRNA levels to hepatic iron score (HIS) (b). Hepcidin mRNA levels corrected for iron overload were significantly lower in NASH patients than in simple steatosis patients. *Mann-Whitney U-test.

cantly lower in NASH patients than simple steatosis patients (Fig. 2). The ratio of hepcidin mRNA/ferritin was significantly correlated with stage ($r = -0.523$, $P < 0.00005$) and grade ($r = -0.436$, $P < 0.0005$). The same results were obtained from the ratio of hepcidin mRNA/HIS ($r = -0.424$, $P < 0.01$ vs stage; $r = -0.373$, $P < 0.05$ vs grade). We compared hepcidin mRNA levels with metabolic variables and found that the level of hepcidin was significantly correlated with both total cholesterol ($r = 0.323$, $P < 0.01$) and triglyceride ($r = 0.323$, $P < 0.01$). The ratio of hepcidin mRNA/ferritin was also significantly correlated with total cholesterol ($r = 0.365$, $P < 0.005$).

DISCUSSION

IN THIS STUDY, we investigated the expression levels of hepatic genes that play significant roles in the metabolism of fatty acids and iron. Their roles in hepatocytes include the uptake, synthesis, oxidation, storage and excretion of fatty acids,[10,18,19] the uptake of iron and the regulation of systemic iron transport.[16] We found that the levels of these genes were significantly higher in NAFLD patients than controls. In addition, we found some novel findings. However, none of the individual genes was independently correlated with hepatic steatosis. These results indicated that neither the lack of nor increase in the expression levels of any of these genes plays an independent role in the development of fatty liver.

Insulin resistance is the "first hit" in the development of NASH,[9] which is characterized by an increase in the uptake and synthesis of fatty acids in hepatocytes.[19] Nevertheless, our results showed that the levels of fatty acid-related genes decreased in the later stages despite the presence of insulin resistance. In parallel with these findings, the level of hepatic steatosis also decreased. Considering that fat is the fuel involved in progressive liver injuries,[20] these findings might be associated with "burnout" NASH.[21] Although the underlying reason for this is unclear, some possibilities should be considered. Because hepatic adenosine 5′-triphosphate (ATP) levels tend to be decreased in fatty liver,[22] hepatic adenosine monophosphate-activated protein kinase (AMPK) should be activated.[23] AMPK is known to activate catabolic pathways and switch off protein, carbohydrate and lipid synthesis, such that cellular energy levels remain unchanged.[23] Thus, activated AMPK in hepatocytes might contribute to the decrease in the expression levels of fatty acid-related genes. Anti-diabetic drugs, which ameliorate liver injuries in patients with NASH, have been reported to activate AMPK.[24] Interestingly, the levels of all the genes involved in fatty acid metabolism were lower in the patients treated with insulin sensitizers than in those treated with other agents or followed with diet restriction. Statistical significance was achieved only in FATP5 ($P < 0.05$, Mann–Whitney's test). However, these results may be difficult to evaluate or apply generally, because the numbers of patients were small.

Hepatic iron load has been documented to be another key player in the progression from steatosis to steatohepatitis.[11] Hepatic iron load has been attributed to the Cys282Tyr mutation in the hemochromatosis gene.[11] This mutation decreases hepatic synthesis of hepcidin, resulting in the facilitation of iron absorption from the duodenum.[16] Our results showed that hepatic iron scores tended to correlate with the histological stage of NAFLD. Furthermore, the ratios of hepcidin mRNA/ferritin and hepcidin mRNA/HIS were significantly lower in NASH patients than in simple steatosis patients. This insufficient production of hepcidin may not be attributed to the genetic mutation, since known mutations of hemochromatosis-associated genes have been reported to be rare among Japanese patients.[25]

Interestingly, the hepcidin level was significantly correlated with the levels of total cholesterol and triglycerides. These findings coincide with those recently reported by Barisani *et al.*,[17] who reported that the hepcidin mRNA/ferritin ratio and the hepcidin mRNA/tissue iron score ratio were significantly lower in the NAFLD group with hepatic iron overload than in the NAFLD group without iron overload,[17] and that the level of hepatic hepcidin mRNA was significantly correlated with lipid parameters.[17] Our findings, in concert with those of Barisani *et al.*, suggest that more severe forms of NAFLD are associated with insufficient hepcidin production, and that lipid metabolism might be involved in hepcidin synthesis. Alternatively, the hepatic levels of TfR1 and TfR2 were significantly higher in NAFLD patients than controls. Therefore, TfR1 and TfR2 would be expected to promote hepatic iron load irrespective of iron absorption from the duodenum.

TfR1 is ubiquitously expressed in the human body,[16] while TfR2 is dominantly expressed in specific organs including the liver.[26] TfR1 has a high affinity with transferrin[27] and its expression is regulated by the iron-responsive element (IRE) in the 3′-untranslated regions of mRNAs.[16] In the NAFLD patients, the TfR1 level increased significantly as the stage progressed. Since ROS stabilize TfR1 mRNA via activation of iron regulatory proteins that interact with IRE,[16] hepatic oxidative stress should upregulate TfR1 in NAFLD.

TfR2 was recently identified as a novel transferrin receptor,[26] although the expression mechanisms have not been fully determined.[28] Similarly, neither the physiological nor pathological role of TfR2 in the liver has been documented. The expression level of TfR2 was higher in NAFLD patients than controls. At present, the association between the level of TfR2 and the pathogenesis of NAFLD remains unknown. Regardless of the role of TfR2, we have reported that the TfR2 level is significantly correlated with that of PPARα.[29] It is of much interest to speculate that PPARα might contribute to the regulation of TfR2, since PPARα may be upregulated in NAFLD by intrinsic PPARα ligands. This hypothesis is under investigation in our institute.

In summary, we investigated the metabolism of fatty acids and iron in the livers of NAFLD patients. Steatosis-related metabolism is attenuated as the disease progresses, whereas iron load-related metabolism is exacerbated. Based on these findings, we hypothesize that anti-lipid synthesis should be considered in the early stages and that iron reduction should be considered in the later stages. The former therapies may thus include body weight reduction and insulin-sensitizing drugs, and the latter therapies may include phlebotomy, iron-restriction diets and/or antioxidants.

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Influence of *ITPA* Polymorphisms on Decreases of Hemoglobin During Treatment with Pegylated Interferon, Ribavirin, and Telaprevir

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Polymorphisms of the inosine triphosphatase (*ITPA*) gene influence anemia during pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy, but their effects during triple therapy with PEG-IFN, RBV, and telaprevir are not known. Triple therapy for 12 weeks, followed by PEG-IFN and RBV for 12 weeks, was given to 49 patients with RBV-sensitive (CC at rs1127354) and 12 with RBV-resistant (CA/AA) *ITPA* genotypes who had been infected with hepatitis C virus (HCV) of genotype 1. Decreases in hemoglobin levels were greater in patients with CC than CA/AA genotypes at week 2 (−1.63 ± 0.92 vs. −0.48 ± 0.75 g/dL, $P = 0.001$) and week 4 (−3.5 ± 1.1 vs. −2.2 ± 0.96, $P = 0.001$), as well as at the end of treatment (−2.9 ± 1.1 vs. −2.0 ± 0.86, $P = 0.013$). Risk factors for hemoglobin <11.0 g/dL at week 4 were female gender, age >50 years, body mass index (BMI) <23, and CC at rs1127354 by multivariate analysis. RBV dose during the first 12 weeks was smaller in patients with CC than CA/AA genotypes (52 ± 14% vs. 65 ± 21% of the target dose, $P = 0.039$), but the total RBV dose was no different between them (49 ± 17% and 54 ± 18% of the target, $P = 0.531$). Sustained virological response (SVR) was achieved in 70% and 64% of them, respectively ($P = 0.724$). *Conclusion: ITPA* polymorphism influences hemoglobin levels during triple therapy, particularly during the first 12 weeks while telaprevir is given. With careful monitoring of anemia and prompt adjustment of RBV dose, SVR can be achieved comparably frequently between patients with CC and CA/AA genotypes. (HEPATOLOGY 2011;53:415-421)

Worldwide, 123 million people are estimated to have been infected with hepatitis C virus (HCV),[1] and ≈30% of them develop fatal liver disease such as cirrhosis and hepatocellular carcinoma.[2,3] Currently, the standard of care therapy for patients infected with HCV is pegylated interferon (PEG-IFN) and ribavirin (RBV) for 48 weeks.[4-6] However, the combined treatment can induce a sustained virological response (SVR), judged by the loss of detectable HCV RNA from serum 24 weeks after treatment completion, in at most 50% of patients infected with HCV-1, the genotype most prevalent and least responsive to IFN-based therapies.

Recently, Fellay et al.[7] reported that polymorphisms of the inosine triphosphatase (*ITPA*) gene in chromosome 20 (20p13) influence RBV-induced anemia in a genome-wide association study (GWAS). Single nucleotide polymorphism (SNP) at rs1127354 for proline-to-threonine substitution (P32T) in the second of eight

Abbreviations: BMI, body mass index; GWAS, genome-wide association study; HCV, hepatitis C virus; IFN, interferon; IL28B, *interleukin 28B;* ITPA, *inosine triphosphatase; PEG-IFN, pegylated interferon; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virological response.*

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exons in the *ITPA* gene, as well as that at rs7270101 in the second intron, affects the expression of ITPA.[8-11] Patients infected with HCV-1 carrying the CC genotype at rs1127354 are more prone to develop anemia than those with CA/AA genotypes during the combination therapy, and the decrease in hemoglobin is greater in patients with the AA than AC/CC genotypes at rs7270101.[7] Their observations have been extended to many patients in a large-scale trial with pegIFN-α-2a on Caucasian and African Americans,[12] as well as in the Japanese receiving PEG-IFN-α-2b and RBV who were infected with HCV-1.[13]

For improving SVR in HCV-1 patients, protease inhibitors have been added to the standard treatment with PEG-IFN and RBV, and increased SVR by ≈20%.[14-16] However, such a gain in efficacy is not without trade-offs, represented by aggravation of anemia. Early decreases in hemoglobin levels during the triple therapy reach 4 g/dL, and they exceed ≈3.0 g/dL in the standard treatment.[14,15] Because there have been no reports focusing on the influence of *ITPA* genotypes on anemia developing in patients during triple therapy, hemoglobin levels were followed in 61 Japanese patients with HCV-1 who had received it. The results were correlated with polymorphisms at rs1127354 in the *ITPA* gene because the Japanese are monoallelic at rs7270101 and have the AA genotype exclusively.[11]

Patients and Methods

Study Cohort. This retrospective cohort study was performed in 61 patients with chronic hepatitis C who met the following inclusion and exclusion criteria. Inclusion criteria were: (1) diagnosed with chronic hepatitis C; (2) HCV-1 confirmed by sequence analysis in the NS5B region; (3) HCV RNA levels ≥5.0 log IU/mL determined by the COBAS TaqMan HCV test (Roche Diagnostics K.K. Tokyo, Japan); (4) Japanese aged from 20 to 65 years at the entry; and (5) body weight between ≥40 kg and ≤120 kg at the time of registration. Exclusion criteria were: (1) decompensated liver cirrhosis; (2) hepatitis B surface antigen in serum; (3) hepatocellular carcinoma or its history; (4) autoimmune hepatitis, alcoholic liver disease, hemochromatosis, or chronic liver disease other than chronic hepatitis C; (5) chronic renal disease or creatinine clearance ≤50 mL/min at the baseline; (6) hemoglobin ≤12 g/dL, neutrophil ≤1,500/mm^3 or platelet ≤100,000/mm^3 at baseline.

Of the 61 patients, 44 (72%) had received IFN-based treatment before. Relapse occurred in 29 (47%) and the remaining 15 (25%) did not respond (null-responders). All patients gave consent for analysis of SNPs in *ITPA* and interleukin 28 (*IL28B*) genes. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of Toranomon Hospital. Written informed consent was obtained from each patient.

Triple Treatment with PEG-IFN-α-2b, RBV, and Telaprevir. Telaprevir (MP-424; Mitsubishi Tanabe Pharma, Osaka, Japan), 750 mg, was administered 3 times a day at an 8-hour (q8) interval after each meal. Pegylated-IFN-α-2b (PEG-Intron, Schering Plough, Kenilworth, NJ) was injected subcutaneously at a median dose of 1.5 μg/kg (range: 1.32-1.71 μg/kg) once a week. RBV (Rebetol, Schering Plough) 200-600 mg was administered after breakfast and dinner. The RBV dose was adjusted by body weight: 600 mg for ≤60 kg; 800 mg for >60 kg ≈≤80 kg; and 1,000 mg for ≥80 kg. The triple therapy with PEG-IFN-α-2b, RBV, and telaprevir was continued for 12 weeks, and then switched to PEG-IFN-α-2b and RBV for an additional 12 weeks. It was withdrawn when hemoglobin levels decreased <8.5 g/dL. After the therapy was completed or discontinued, patients were followed for 24 weeks for SVR.

The RBV dose was cut by 200 mg in patients receiving 600 or 800 mg (by 400 mg in those receiving 1,000 mg) when hemoglobin decreased <12 g/dL, and by another 200 mg when it was below <10 g/dL. In addition, RBV was reduced by 200 mg in patients with hemoglobin <13 g/dL at baseline and those in whom it decreased by 1 g/dL to <13 g/dL within a week. PEG-IFN dose was reduced by one-half when the leukocyte count decreased <1,500/mm^3, neutrophil count <750/mm^3, or platelet count <80 × 10^3/mm^3; PEG-IFN was withdrawn when they decreased <1,000/mm^3, 500/mm^3, or 50 × 10^3/mm^3, respectively.

The triple therapy was withdrawn or stopped temporarily when hemoglobin decreased <8.5 g/dL. In patients in whom hemoglobin increased ≥8.5 g/dL within 2 weeks after the withdrawal, treatment was resumed with PEG-IFN and RBV 200 mg. A reduction of telaprevir (MP-424) dose was not permitted. It was discontinued when severe side effects appeared, whereas PEG-IFN and RBV were continued. Growth factors were not used for elevating hemoglobin levels.

Determination of ITPA Genotypes. *ITPA* (rs1127354) and *IL28B* (rs8099917 and rs12979860) were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described.[17,18]

Statistical Analyses. Continuous variables between groups were compared by the Mann-Whitney test (*U* test), and discontinuous variables by the chi-square test

Table 1. Baseline Characteristics of the 61 Patients Infected with HCV-1 Who Received Triple Therapy with Pegylated-Interferon, Ribavirin, and Telaprevir

		ITPA Genotypes at rs1127354	
	Total	CC	CA + AA
Demographic data			
Number	61	49	12
Sex (male/female)	34/27	28/21	6/6
Age (years)	56 (23-65)	55 (23-65)	58 (28-62)
Body weight (kg)	61.5 (41.0-92.9)	61.5 (41.0-92.9)	62.1 (44.4-81.1)
Body mass index (kg/m^2)	22.6 (17.6-32.4)	22.2 (17.6-32.4)	22.9 (17.8-26.5)
Genotypes of the *IL28B* gene			
rs8099917 (for 59 patients)			
(TT/TG + GG)	33/26	27/21	6/7
rs12979860 (for 57 patients)			
(CC/CT + TT)	30/27	36/22	4/5
Laboratory data			
Hemoglobin (g/dL)	14.4 (12.5-16.6)	14.4 (12.5-16.6)	14.2 (12.8-16.3)
Platelets (x 10^4/mm^3)	17.8 (9.1-33.8)	17.7 (9.1-33.8)	19.5 (13.1-31.6)
Albumin (g/dL)	3.9 (3.2-4.6)	3.9 (3.2-4.6)	3.9 (3.5-4.1)
Alanine aminotransferase (U/L)	39 (12-175)	41 (12-175)	28 (17-57)
Aspartate aminotransferase (U/L)	32 (15-137)	35 (15-137)	28 (20-35)
HCV RNA (log IU/mL)	6.7 (5.1-7.6)	6.8 (5.7-7.6)	6.6 (5.1-7.5)
HCV genotype 1a/1b	1/60	1/48	0/12
Previous IFN-based treatment			
Treatment naïve	17	12 (24%)	5 (42%)
Relapsed	29	23 (47%)	6 (50%)
Null response	15	14 (29%)	1 (8%)

Data are median values (range) or n.

and Fisher's exact test. Kaplan-Meier analysis and the log-rank test were applied to estimate and compare decreases of RBV dose between groups. Factors evaluated for influence on hemoglobin decrease by univariate analysis were: sex; age; body mass index (BMI); body weight; hemoglobin levels; initial PEG-IFN and RBV doses; amino acid substitutions in the HCV core protein; number of amino acid substitutions in the interferon sensitivity determining region; and *IL28B* polymorphisms (at rs8099917 and rs12979860). Factors associated with a decrease in hemoglobin levels ($P < 0.10$) were assessed by multiple logistic regression analysis, and the odds ratio (OR) with 95% confidence interval (CI) was determined. All analyses were performed using SPSS software (SPSS II v. 11.0, Chicago, IL), and a P-value < 0.05 was considered significant.

Results

Triple Therapy in Patients with HCV-1 Infection. Baseline characteristics of the 49 patients with CC and the 12 with CA/AA genotypes at rs1127354 in the *ITPA* gene are compared in Table 1. They all were infected with HCV-1. There were no significant differences between them, except that alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were higher in patients with CC than CA/AA genotypes ($P = 0.041$ and $P = 0.008$, respectively). Overall, *IL28B* genotypes resistant to PEG-IFN and RBV, TT/TG at rs8099917, and CC/CT at rs12979860 were rather frequent, and possessed by 44% and 47%, respectively, of the patients. This was due to inclusion of 15 nonresponders to previous IFN-based therapies, corresponding to 25% of the 61 patients studied, most of whom (14/15 [93%]) possessed IFN-resistant genotypes (TT/TG and CC/CT). Six of them had low hemoglobin levels (<13 g/dL) at baseline and were started with an RBV dose decreased by 200 mg; they included five with CC and one with CA genotypes of the *ITPA* gene.

Modification of RBV Dose During Triple Therapy. RBV dose was reduced by ≥200 mg in all 61 patients studied during triple therapy because hemoglobin had decreased <12.0 g/dL in them. During the first 12 weeks of therapy while telaprevir was given, the proportion of patients receiving the full RBV dose differed between those with CC and CA/AA genotypes (Fig. 1). RBV dose reduction was started earlier in the 49 patients with CC than the 12 with CA/AA genotypes (2.6 ± 1.3 vs. 4.8 ± 3.1 weeks after the start, respectively, $P = 0.010$). Thus, during the first 12 weeks with telaprevir the RBV dose was smaller in patients with CC than CA/AA genotypes (52 ± 14% vs. 65 ± 21% of the target dose, $P = 0.039$). During the next 12

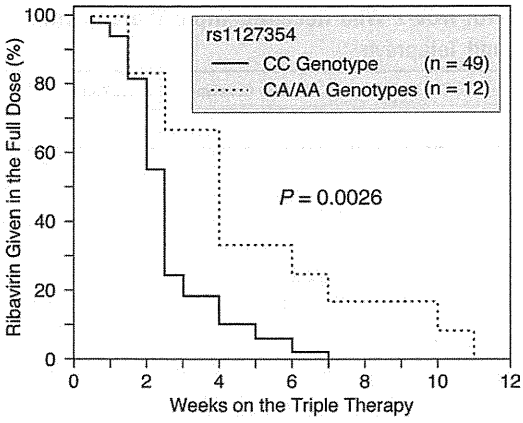

Fig. 1. Patients who received the full ribavirin dose during 12 weeks on triple therapy. The 49 patients with CC and the 12 with CA/AA genotypes at rs1127354 are compared.

weeks without telaprevir, in contrast, the RBV dose was somewhat larger in patients with CC than CA/AA genotypes (47 ± 24% vs. 43 ± 20%, $P = 0.649$). The total RBV dose during 24 weeks on therapy was comparable between the 49 patients with CC and the 12 with CA/AA genotypes (49 ± 17% vs. 54 ± 18%, $P = 0.531$). In patients with the CC genotype, the RBV dose was no different between those who achieved SVR and those who did not (50 ± 18% vs. 47 ± 13%, $P = 0.728$). The RBV dose did not differ either in patients with CA/AA genotypes with and without SVR (57 ± 17% vs. 48 ± 20%, $P = 0.368$).

The total dose of PEG-IFN was comparable among 49 patients with CC and 12 with CA/AA genotypes (87 ± 23% vs. 86 ± 20% of the target, $P = 0.488$). The total telaprevir dose was no different either between them (87 ± 27% vs. 71 ± 36% of the target, $P = 0.098$). Telaprevir was discontinued in 10 of the 49 (20%) patients with CC and 5 of the 12 (42%) with CA/AA genotypes ($P = 0.147$).

Decreases in Hemoglobin Levels During Triple Therapy. Figure 2 compares decreases in hemoglobin levels between 49 patients with CC and 12 with CA/AA genotypes of the *ITPA* gene. Data of six patients were omitted because the triple therapy was withdrawn 4-10 weeks after the start, including five with CC and one with CA genotype. Hemoglobin decreased more in patients with CC than CA/AA genotypes at week 2 (−1.63 ± 0.92 vs. −0.48 ± 0.75 g/dL, $P = 0.001$) and week 4 (−3.5 ± 1.1 vs. −2.2 ± 0.96, $P = 0.001$). During week 8 through 12, hemoglobin reached the nadir of approximately −4 g/dL both in patients with CC and CA/AA genotypes. Thereafter, differences in hemoglobin decrease started to widen between patients with CC and CA/AA genotypes and were significant at week 20 (−3.0 ± 1.2 vs. −2.4 ± 0.88 g/dL, $P = 0.048$) and week 24 (−2.9 ± 1.1 vs. −2.0 ± 0.85 g/dL, $P = 0.013$).

SVR was achieved by 35 (71%) of the 49 patients with CC and 8 (67%) of the 12 with CA/AA genotypes ($P = 0.736$). Hemoglobin levels did not differ between them 24 weeks after the completion of triple therapy (−0.57 ± 1.1 vs. −0.17 ± 0.87 g/dL, $P = 0.271$). Of the 32 patients with TT genotype of the *IL28B* gene at rs8099917, 30 (94%) gained SVR, more frequently than 10 of the 26 (38%) with TG/GG genotypes ($P < 0.001$). Likewise, 29 of the 30 (97%) patients with CC genotype at rs12979860 achieved SVR, more frequently than 11 of the 27 (41%) with CT/TT genotypes ($P < 0.001$).

Factors Influencing Decreases in Hemoglobin Levels. Hemoglobin decreased <11 g/dL at week 4 during the triple therapy in 27 of the 61 (44%) patients. Factors for hemoglobin <11.0 g/dL were female gender, age >50 years, body weight <60 kg, BMI <23, and baseline hemoglobin <15 g/dL, as well as the CC genotype of the *ITPA* gene, in the univariate analysis (Table 2). Of them, female gender, age >50 years, BMI <23, and the CC genotype remained significant in the multivariate analysis. Hemoglobin levels lowered <8.5 g/dL during the triple therapy in 13 of the 61 (21%) patients. Factors for hemoglobin <8.5 g/dL were female gender, age >60 years, body weight <60 kg, BMI <23, and baseline hemoglobin <14 g/dL in the univariate analysis (Table 3). Of

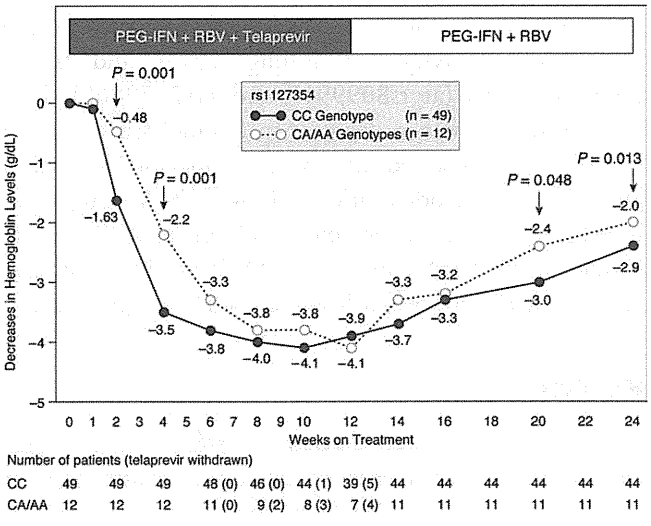

Fig. 2. Decreases in hemoglobin levels during triple therapy with telaprevir, PEG-IFN, and RBV. The 49 patients with CC and the 12 with CA/AA genotypes at rs1127354 are compared. Patients evaluated at each timepoint are indicated below, with the number of patients in whom telaprevir was withdrawn (PEG-IFN and RBV continued) in parentheses.

Table 2. Univariate and Multivariate Analyses of Host and Viral Factors Associated with Low Hemoglobin Levels (< 11.0 g/dL) at Week 4 of Triple Therapy

	Univariate Analysis		Multivariate Analysis	
Parameter	OR (95% CI)	P	OR (95% CI)	P
Sex (female)	14.3 (4.1-50.0)	< 0.001	29.41 (3.8-250.0)	0.001
Age (> 50 years)	4.3 (1.0-17.5)	0.030	7.3 (1.1-47.6)	0.039
Body weight (< 60 kg)	11.5 (3.4-38.2)	< 0.001		
Body mass index (< 23)	8.4 (2.6-27.1)	< 0.001	17.2 (2.6-112.0)	0.003
Hemoglobin (< 15g/dL)	14.2 (3.5-57.4)	< 0.001		
ITPA gene (CC genotype)		0.062	36.8 (2.5-550.2)	0.009

Abbreviations: OR, odds ratio; CI, confidence level.

them, only age and body weight remained significant in the multivariate analysis.

Discussion

Anemia is a substantial risk in the standard of care therapy with PEG-IFN and RBV.[4-6] Triphosphorylated RBV accumulates in erythrocytes of patients who receive RBV, increasingly with RBV dose and duration, and causes oxidative damage to erythrocyte membranes toward extravascular hemolysis by the reticuloendothelial system.[19,20] Inosine triphosphate accumulates also in erythrocytes of individuals who have mutations in the *ITPA* gene, and results in benign red-cell enzymopathy.[8] The expression of *ITPA* is genetically controlled and reduced in individuals who have point mutations in the *ITPA* gene.[8-11] As another achievement of GWAS in hepatology,[21] in the wake of polymorphisms of the *IL28B* gene that influence the response to PEG-IFN and RBV,[22-24] polymorphisms in the *ITPA* gene has been reported to influence anemia caused by RBV.[7] How inosine triphosphate protects erythrocytes from hemolysis caused by RBV needs to be sorted out by *in vivo* and *in vitro* experiments. Inosine triphosphate may prohibit the accumulation of RBV in erythrocytes, or rather, it might act directly toward prohibition of hemolysis.

In the present study, 61 patients infected with HCV-1 received triple therapy with PEG-IFN, RBV, and telaprevir in the first 12 weeks followed by PEG-IFN and RBV in the second 12 weeks. Then the RBV dose and hemoglobin were compared between patients with CC and CA/AA genotypes in the *ITPA* gene. Two polymorphisms in the *ITPA* gene, in close linkage disequilibrium with an r^2 value of 0.65,[7] have been recognized in Caucasians (rs1127354 and rs7270107); the respective CA/AA and AC/CC genotypes decrease the activity of inosine triphosphatase and protect against anemia induced by RBV.[7,12] Because the Japanese are monoallelic at rs7270107 and possess the AA genotype exclusively,[11,25] only polymorphisms at rs1127354 were examined.

Of the 61 patients, 49 possessed the RBV-sensitive CC genotype and the remaining 12 had RBV-resistant CA/AA genotypes. Hemoglobin levels decreased both in patients with CC and CA/AA genotypes. They lowered ≈4 g/dL during weeks 8-12 on the triple therapy with telaprevir, and increased thereafter (Fig. 2). Between the two groups of patients, differences in hemoglobin decrease were greatest at week 4 (1.3 g/dL), as in the standard treatment with PEG-IFN and RBV.[7,12,13]

When anemia and other side effects occurred, doses of RBV, PEG-IFN, and telaprevir were modified. Of the 61 patients studied, 27 (44%) were women and most of them were in old age. Beyond 50 years of age, women are less responsive than men to the standard treatment with PEG-IFN and RBV, probably because estrogens with an antifibrotic potential decrease after menopause.[26] Stringent precautions had to be taken, therefore, by reducing the RBV dose in the patients in whom hemoglobin levels decreased <12 g/dL, rather than the conventional threshold of <10 g/dL.

Reductions of RBV dose due to anemia in patients who receive PEG-IFN and RBV are influenced by *ITPA* polymorphisms.[12] Also, in patients who had received the triple therapy the RBV dose had to be reduced more in

Table 3. Univariate and Multivariate Analyses of Host and Viral Factors Associated with Very Low Hemoglobin Levels (<8.5 g/dL) During Triple Therapy

	Univariate Analysis		Multivariate Analysis	
Parameter	OR (95% CI)	P	OR (95% CI)	P
Sex (female)	6.1 (1.5-25.1)	0.007		
Age (>60 years)	6.8 (1.8-26.0)	0.004	10.1 (1.9-53.9)	0.007
Body weight (<60 kg)	23.8 (2.9-200.0)	<0.001	33.3 (3.4-333.3)	0.003
Body mass index (<23)	14.1 (1.7-125.0)	0.001		
Hemoglobin (<14 g/dL)	4.3 (1.2-15.6)	0.023		

Abbreviations: OR, odds ratio; CI, confidence level.

patients with CC than CA/AA genotypes during the first 12 weeks while they received telaprevir (52 ± 14% vs. 65 ± 21% of the target dose, $P = 0.039$). During the second 12 weeks off telaprevir, the RBV dose was somewhat greater in patients with CC than CA/AA genotypes (47 ± 24% vs. 43 ± 20%, $P = 0.649$). Thus, the total RBV dose during 24 weeks of therapy was comparable between patients with CC and CA/AA genotypes (51 ± 15% and 57 ± 18%, $P = 0.724$). Likewise, the total dose of PEG-IFN (87 ± 23% vs. 86 ± 20% of the target, $P = 0.806$), as well as that of telaprevir (87 ± 27% vs. 71 ± 36% of the target, $P = 0.098$), was no different between patients with CC and CA/AA genotypes. SVR was achieved comparably frequently in them (71% vs. 67%, $P = 0.736$).

Decreases in hemoglobin levels during the first 12 week were similar between the current triple therapy cohort and previous patients receiving PEG-IFN and RBV.[12,13] The conservative hemoglobin levels chosen for RBV dose reduction may be a possible confounding factor on the impact of *ITPA* variants in anemia, which would have been greater should the RBV dose not be reduced in patients with RBV-sensitive CC genotypes.

ITPA polymorphisms at rs1127354 were associated with RBV-induced anemia in Japanese patients, without involvement of those at rs7270107 reported in Caucasian and African-American patients.[13] Thus, *ITPA* polymorphisms at rs1127354 would play a major role in protecting patients from RBV-induced anemia. CC/CA genotypes at rs1127354 occurs in 6% of the Caucasian population, much less often in the Oriental population, at 16%.[25,27] Although AC/CC genotypes at rs7270107 occurs in 13% of Caucasians, they do not exist in Orientals.[11,25] Obviously, different polymorphisms need to be examined in patients of distinct ethnicities when the influence on RBV-induced anemia is to be evaluated.

In confirmation of our previous report,[28] the triple therapy achieved SVR more frequently in patients with CC than CT/TT genotypes of *IL28* at rs12979860 (96% vs. 41%, $P < 0.001$). About two-thirds of studied patients accomplished SVR with the triple treatment, although one-fourth of them were nonresponders to previous IFN-based treatments; they are known to respond poorly to repeated treatments. This would lend further support to the efficacy of triple therapy being higher than treatment with pegylated IFN and RBV.

There are strong points in this study. First, *ITPA* polymorphisms influence RBV-induced anemia in the triple therapy. Second, polymorphisms at rs1127350, without involvement of those at rs7270107, protect against RBV-induced anemia. Third, the triple therapy can be applied with high efficacy by careful monitoring of hemoglobin and prompt modification of RBV dose. There are weak points in this study as well. First, it was a retrospective cohort study conducted in a small size of patients, especially those with CA/AA genotypes at rs1127350, and included null-responders to previous IFN-based therapies; the real impact of *ITPA* polymorphisms on RBV-induced anemia may have been obscured. Second, the study was conducted in Japanese patients, and the results may or may not be extended to patients of different ethnicities with distinct genetic backgrounds. Hopefully, the results presented herein will promote future studies in which the influence of the *ITPA* polymorphism on RBV-induced anemia will be pursued in larger scale and on patients of various ethnicities around the world.

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MAJOR ARTICLE

IL28B But Not ITPA Polymorphism Is Predictive of Response to Pegylated Interferon, Ribavirin, and Telaprevir Triple Therapy in Patients With Genotype 1 Hepatitis C

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Background. Pegylated interferon, ribavirin, and telaprevir triple therapy is a new strategy expected to eradicate the hepatitis C virus (HCV) even in patients infected with difficult-to-treat genotype 1 strains, although adverse effects, such as anemia and rash, are frequent.

Methods. We assessed efficacy and predictive factors for sustained virological response (SVR) for triple therapy in 94 Japanese patients with HCV genotype 1. We included recently identified predictive factors, such as IL28B and ITPA polymorphism, and substitutions in the HCV core and NS5A proteins.

Results. Patients treated with triple therapy achieved comparatively high SVR rates (73%), especially among treatment-naive patients (80%). Of note, however, patients who experienced relapse during prior pegylated interferon plus ribavirin combination therapy were highly likely to achieve SVR while receiving triple therapy (93%); conversely, prior nonresponders were much less likely to respond to triple therapy (32%). In addition to prior treatment response, IL28B SNP genotype and rapid viral response were significant independent predictors for SVR. Patients with the anemia-susceptible ITPA SNP rs1127354 genotype typically required ribavirin dose reduction earlier than did patients with other genotypes.

Conclusions. Analysis of predictive factors identified IL28B SNP, rapid viral response, and transient response to previous therapy as significant independent predictors of SVR after triple therapy.

Hepatitis C virus (HCV) establishes a chronic infection in 80% of infected individuals, and currently, >100 million persons are chronically infected and at increased risk of cirrhosis, hepatocellular carcinoma, and end-stage liver disease [1–3]. The current standard of care is combination treatment with pegylated interferon (PEG-IFN) and ribavirin, but this costly and poorly tolerated treatment achieves sustained virological response in only 50% of patients [4]. Options are limited in the event of treatment failure, and alternative therapies are needed.

Of the many drugs under investigation, the most promising are the direct-acting antiviral agents, which directly target essential aspects of viral replication, including internal ribosome entry site inhibitors, protease and polymerase inhibitors, and assembly inhibitors [5]. Several protease inhibitors, including telaprevir and boceprevir, are in phase III clinical trials and will likely become the first direct-acting antiviral agents approved for clinical use [6].

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