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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.

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.

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).

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-

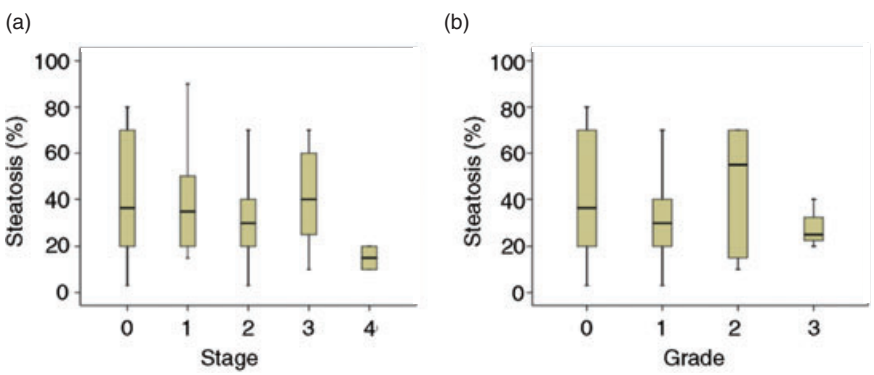

Figure 1 Distributions of the level of hepatic steatosis in association with the stage (a) and grade (b). The level of steatosis decreased as the stage and grade progressed.

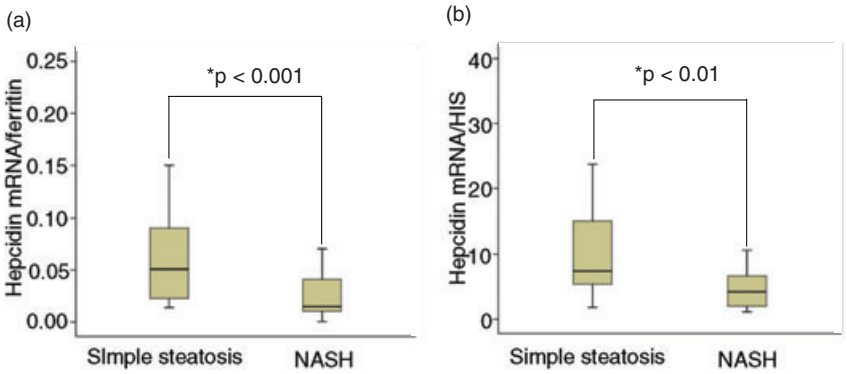

Figure 2 The ratio of hepcidin mRNA levels to serum ferritin levels (a) and that of hepcidin mRNA levels to hepatic iron score (HIS) (b). Hepcidin mRNA levels corrected for iron overload were significantly lower in NASH patients than in simple steatosis patients. *Mann-Whitney U-test.

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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