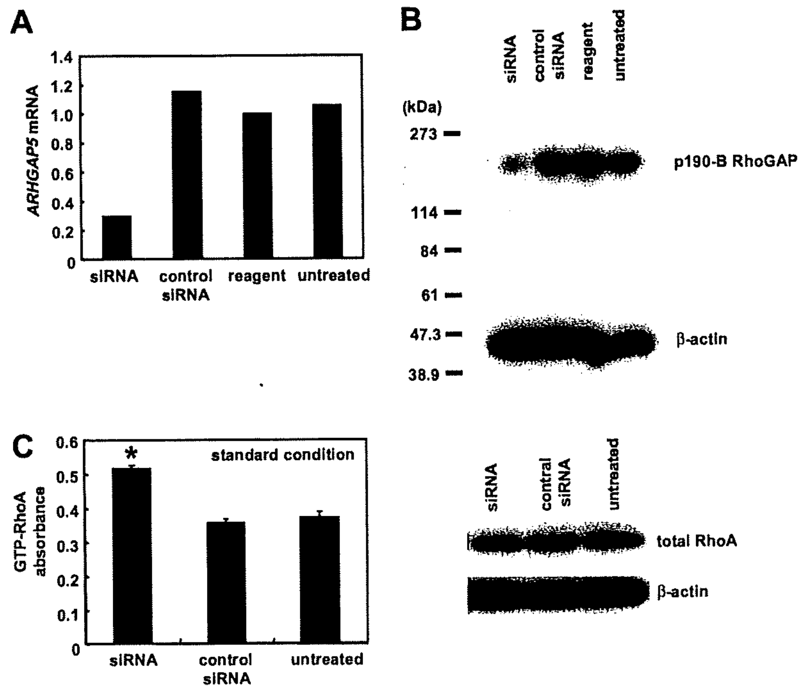

Fig. 3. Knockdown of *ARHGAP5* increases RhoA activity. (A) Relative expression levels of *ARHGAP5* mRNA as determined by real-time quantitative PCR. Huh-7 cells were treated with siRNA targeting *ARHGAP5*, negative control siRNA or transfection agent alone. Untreated cells were maintained under identical experimental conditions. Results are presented as a ratio between the expression level of *ARHGAP5* and that of a reference gene (*GAPDH*) to correct for variation in the amount of RNA. Relative expression levels were normalized such that the ratio in untreated cells was 1. (B) Levels of p190-B RhoGAP and β-actin, an internal control, determined by immunoblotting. (C) (*left*) Levels of RhoA activity under standard culture conditions (DMEM containing 10% FCS). RhoA activity was measured using a G-LISA kit (see Methods section). Values are represented as the mean ± S.D. Differences were analyzed by ANOVA ($^{*}P < 0.05$). (*right*) Total RhoA and β-actin were determined by immunoblotting.

3.3. Regulation of RhoA activity by p190-B RhoGAP in Huh-7 cells

To investigate the biological function of p190-B RhoGAP in HCC cells, knockdown of *ARHGAP5* expression in Huh-7 cells was carried out using RNAi. Following treatment of Huh-7 cells with siRNA targeting *ARHGAP5*, we observed a decrease in both *ARHGAP5* mRNA and p190-B RhoGAP protein levels relative to what was observed for cells receiving control siRNA, transfection agent alone or left untreated (Fig. 3A and B). Because p190-B RhoGAP negatively regulates RhoA activity, we examined the effect of the siRNA-mediated knockdown of *ARHGAP5* on RhoA activity. Huh-7 cells were treated with *ARHGAP5* siRNA or control siRNA or were left untreated. Cells were then cultured in DMEM containing 10% FCS for 48 h under standard conditions. RhoA activity levels were higher in cells treated with *ARHGAP5* siRNA than in cells treated with control siRNA or in untreated cells, whereas total RhoA levels were similar among the three groups (Fig. 3C). These findings suggest that overexpression of *ARHGAP5* contributes to downregulation of RhoA activity in Huh-7 cells.

3.4. Regulation of cell spreading by p190-B RhoGAP in Huh-7 cells

It is known that integrin-mediated adhesion regulates the activity of p190-B RhoGAP and RhoA [3,9]. We therefore examined the function of p190-B RhoGAP when Huh-7 cells were plated on fibronectin, a specific ligand for α5β1 integrin. Huh-7 cells treated with *ARHGAP5* siRNA or control siRNA or left untreated were suspended and plated on fibronectin. Prior to and during plating, cells were maintained in DMEM containing 1% FCS. Adhesion to fibronectin regulated RhoA activity in a triphasic or biphasic manner (Fig. 4A). Prior to plating (0 min), RhoA activity was significantly higher in *ARHGAP5* siRNA-treated cells than in control siRNA-treated cells or untreated cells. In *ARHGAP5* siRNA-treated cells, RhoA activity rapidly and transiently decreased (20 min). This initial decline was followed by an increase that peaked at 60 min. In the final phase, RhoA activity gradually decreased. In control siRNA-treated cells or untreated cells, an initial period of low RhoA activity was followed by a slight increase that peaked between 40–60 min and then returned to basal level. RhoA activity was significantly higher in *ARHGAP5* siRNA-treated cells than control siRNA-treated cells or untreated cells between 40 and 180 min. During the experimental period, expression of p190-B RhoGAP was continuously knocked down by *ARHGAP5* siRNA and total RhoA levels were similar among the three groups (Fig. 4A).

Because RhoA affects cell motility by stimulating reorganization of actin, we examined whether p190-B RhoGAP regulates the spreading of Huh-7 cells on fibronectin. Using immunofluorescence, we observed morphological changes in Huh-7 cells during attachment and spreading on fibronectin (Fig. 4B). Phalloidin staining revealed that *ARHGAP5* siRNA-treated cells exhibited more robust actin stress fibers but less membrane ruffling and protrusion at the cell periphery than control siRNA-treated cells or untreated cells. The actin stress fiber formation and reduced membrane ruffling and protrusion observed in *ARHGAP5* siRNA-treated cells corresponded with higher RhoA activity (Fig. 4)

p190-B RhoGAP was expressed diffusely in the cytoplasm of control siRNA-treated cells and untreated cells, whereas it was hardly detected in *ARHGAP5* siRNA-treated cells. We found that p190-B RhoGAP had partially translocated to the membrane protrusions in control siRNA-treated cells and untreated cells by 40 min after plating (Fig. 4B). Taken together, these findings suggest that RhoA inactivation by p190-B RhoGAP results in inhibition of actin stress fiber formation, enhanced membrane ruffling and protrusion and promotion of cell spreading on fibronectin.

3.5. Regulation of cell migration by p190-B RhoGAP in Huh-7 cells

To investigate the role of p190-B RhoGAP in cell motility, we performed a monolayer wound healing assay. Wound closure was delayed in *ARHGAP5* siRNA-treated cells relative to control siRNA-treated cells or untreated cells, whether cultured in the presence of mitomycin C or in its absence (Figs. 5A–E). Mitomycin C blocks mitosis and thus allows analysis of cell migration in the absence of cell proliferation. These results show that cell migration, rather than cell proliferation, is the major factor

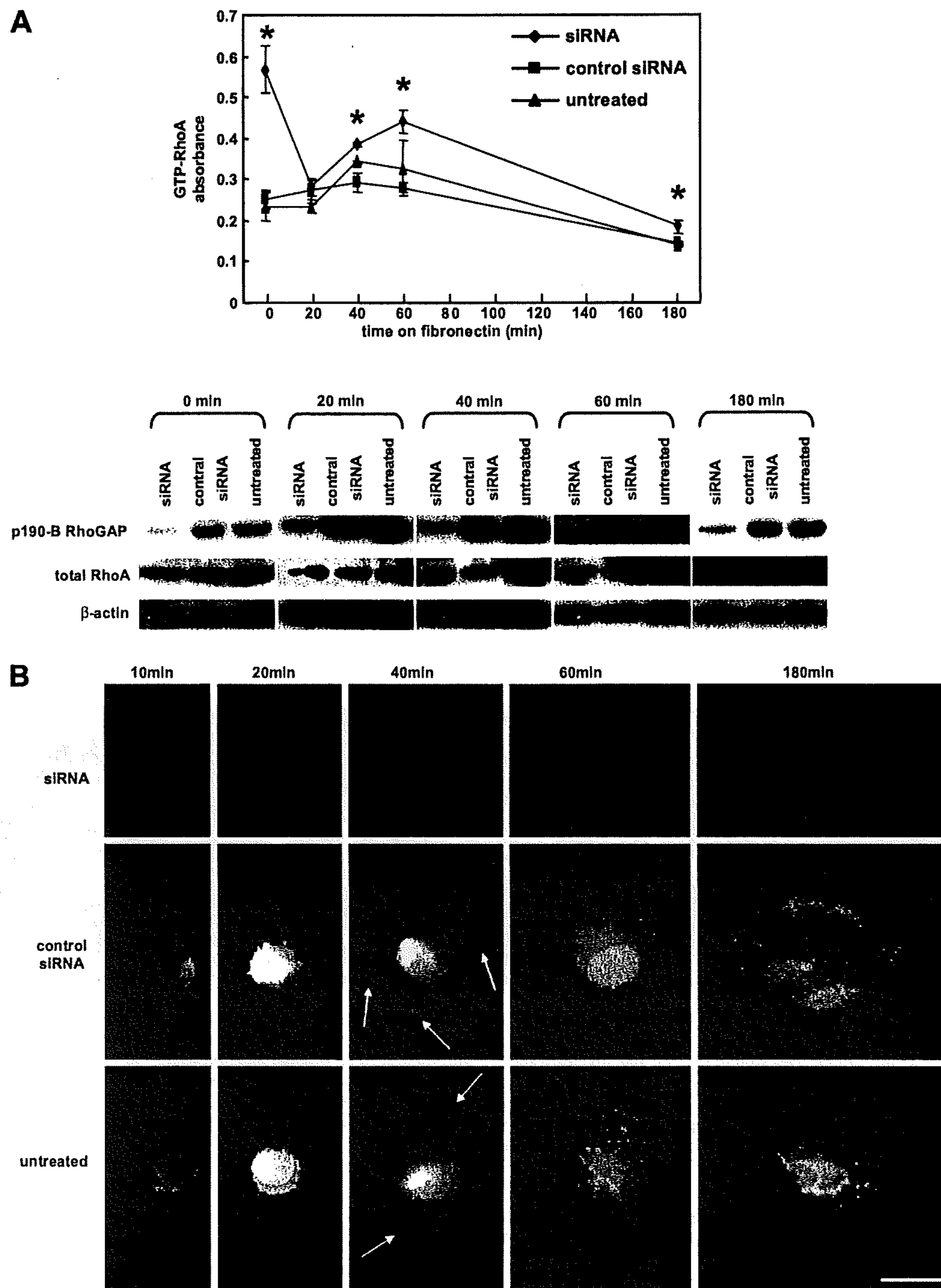

Fig. 4. Knockdown of *ARHGAP5* inhibits Huh-7 cell spreading on fibronectin. (A) Time course of changes in RhoA activity (*upper*) and levels of p190-B RhoGAP and total RhoA (*lower*). Huh-7 cells treated with siRNA targeting *ARHGAP5* or control siRNA or left untreated were plated on fibronectin as described in Materials and Methods and harvested at the indicated time points. Values of RhoA activity are represented as the mean ± SD. Differences were analyzed by ANOVA ($^*P < 0.05$). Levels of p190-B RhoGAP, total RhoA and β-actin were determined by immunoblotting. (B) Time course of cell spreading on fibronectin. Huh-7 cells treated with siRNA targeting *ARHGAP5* or control siRNA or left untreated were plated on fibronectin, fixed at the indicated time points and then triple-labeled with anti-p190-B RhoGAP, rhodamine-conjugated phalloidin and DAPI to reveal p190-B RhoGAP (green), actin filaments (red), and nuclei (blue), respectively. Arrows indicate p190-B RhoGAP on membrane protrusions. Scale bar = 10 μm.

in the retarded wound repair process in *ARHGAP5* siRNA-treated cells. Wound edge cells in *ARHGAP5* siRNA-treated cells had more abundant actin stress fibers but less membrane ruffling and protrusion at the leading edge than control siRNA-treated or untreated cells (Figs. 5F–H). p190-B RhoGAP translocated to the membrane protrusions of control siRNA-treated or untreated cells at the edge of the wound, but not in *ARHGAP5*-siR-

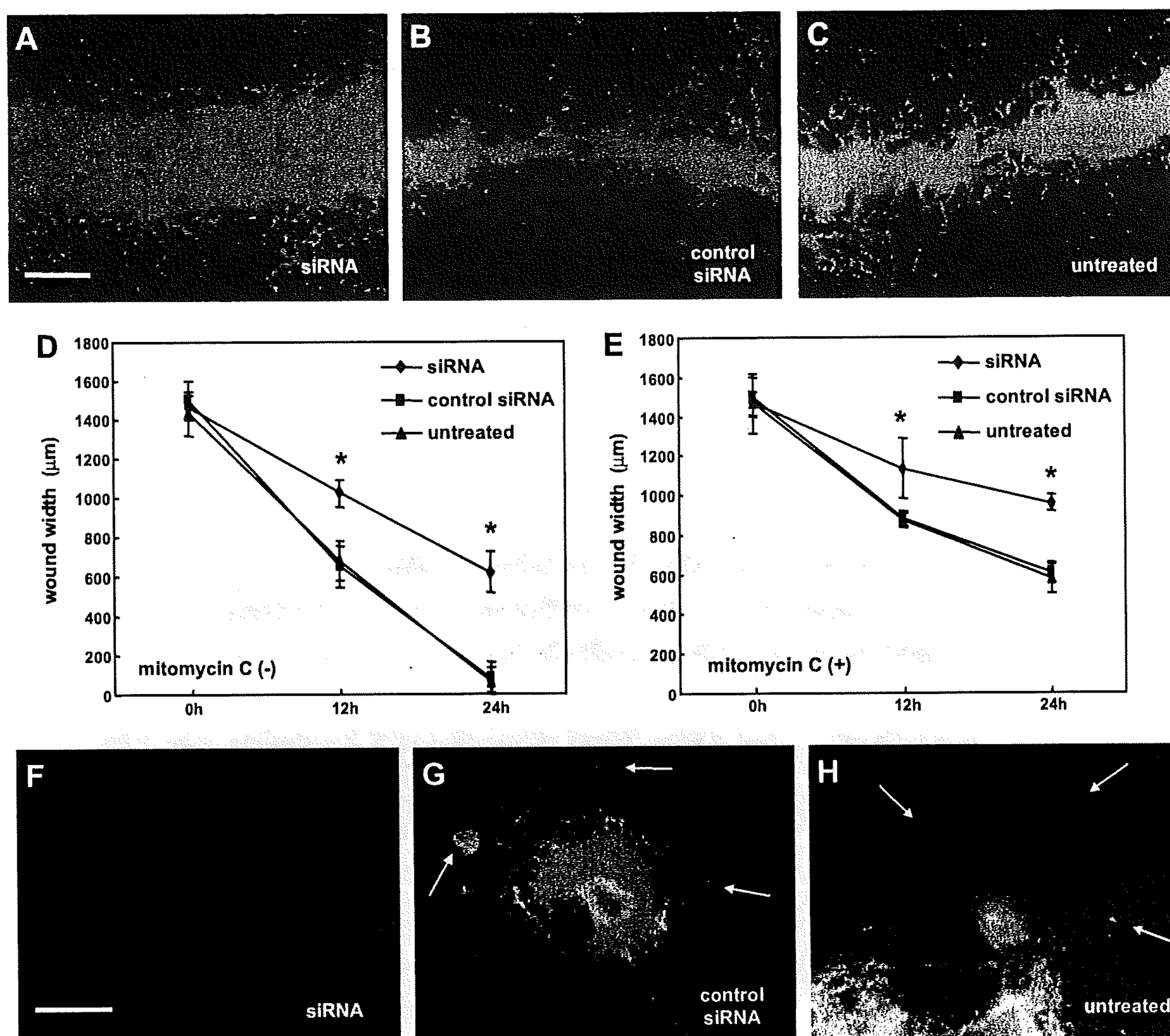

Fig. 5. Knockdown of *ARHGAP5* inhibits migration in Huh-7 cells. Monolayer wound healing assay in Huh-7 cells transfected with siRNA targeting *ARHGAP5* (A, F) or control siRNA (B and G), or left untreated (C and H). Cells were cultured in the absence (A–D, F–H) or presence (E) of mitomycin C. (A–C) Cells were allowed to migrate into a monolayer wound for 24 h and afterward stained with Giemsa stain. Original magnifications: 40×. Scale bar = 500 µm. (D and E) Cells were cultured in the absence (D) or presence (E) of mitomycin C. Wound widths were measured in three randomly chosen regions at the indicated time after wounding. Values are represented as the mean ± SD. Differences were analyzed by ANOVA ($^*P < 0.05$). (F–H) Wound edge cells were triple-labeled with anti-p190-B RhoGAP, rhodamine-conjugated phalloidin and DAPI to reveal p190-B RhoGAP (green), actin filaments (red) and nuclei (blue), respectively. Arrows indicate p190-B RhoGAP on membrane protrusions. Scale bar = 10 µm.

NA cells. Taken together, these observations suggest that the inhibition of RhoA activity by p190-B RhoGAP promotes cell movement and formation of membrane protrusions in migrating cells.

4. Discussion

We report here the amplification of *ARHGAP5* in HCC and ESCC cell lines. We undertook a molecular definition of the amplicon at 14q12 that is present in HCC and ESCC cell lines. The amplification at 14q12 has been reported in various types of cancers, including HCC [10], ESCC [7], nasopharyngeal carcinoma [11] and non-squamous cell lung carcinoma [12], although the frequency of 14q12 gain is low in primary HCC (4–6%) [10,13]. The range of the amplicon varies among these tumors, and their boundaries have not been determined in each case. Moreover, the target oncogene(s) in the amplified regions have not been fully identified. Here we defined the amplified regions in one HCC and two ESCC cell lines and narrowed the site of the amplification to a relatively short section. Among the four genes within the smallest region of the amplification, only *HEATR5A* and *ARHGAP5* were overexpressed in all the tested lines exhibiting copy number gains in the region; hence they are thought to be candidate targets in the amplicon. Of the two genes, we chose to focus further analysis on *ARHGAP5* because its protein product, p190-B RhoGAP, is purported to play an important role in dynamic cellular processes by regulating RhoA activity, while little is known about *HEATR5A*. During the preparation of this manuscript, amplification of *ARHGAP5* was reported in Huh-7 cells [14].

Although several studies have suggested an association of p190-B RhoGAP with tumors [15–17], its biological function in cancer cells is poorly understood. Therefore, using siRNA, we studied its function in Huh-7 cells, the HCC cell line that exhibited the most remarkable copy number gain and overexpression of *ARHGAP5*. We found that p190-B RhoGAP negatively regulates RhoA activity in Huh-7 cells cultured in medium containing 10% FCS and plated on fibronectin. Adhesion to fibronectin regulated RhoA activity in a triphasic or biphasic manner, as previously reported in fibroblasts [18,19]. Although some RhoA activity is required for migration, possibly to maintain sufficient adhesion to the substrate, high activity inhibits movement [19–22]. Our results showed that RhoA inactivation by p190-B RhoGAP results in inhibition of actin stress fiber formation, enhanced membrane ruffling and protrusion, and promotion of spreading and migration of Huh-7 cells. These findings are in agreement with results obtained from previous studies. A dominant negative (loss-of-function) p190-B RhoGAP mutation elevates RhoA activity in fibroblasts cultured on fibronectin and inhibits their migration, whereas overexpression of wild-type p190-B RhoGAP decreases RhoA activity, promotes the formation of membrane protrusions and enhances mobility [19]. Activation of β1 integrin signaling stimulates tyrosine phosphorylation of p190-B RhoGAP and promotes membrane protrusion at invadopodia in a melanoma cell line [17]. p190-B RhoGAP is also involved in invasion by breast cancer cells [15].

In conclusion, we have identified *ARHGAP5* as a probable target for the amplification at 14q12 detected in a subgroup of HCCs and ESCCs. Our results indicate that p190-B RhoGAP, the protein product of *ARHGAP5*, promotes cell spreading and migration in Huh-7 cells. Further studies are needed to determine the importance of *ARHGAP5* and p190-B RhoGAP in the development and progression of not only HCC and ESCC but also other types of tumors.

Conflicts of interest statement

My co-authors and I declare that we have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled, "A novel amplification target, ARHGAP5, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells".

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Review Article

Role of hepatic iron in non-alcoholic steatohepatitis

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Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of clinical entities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with possible evolution to cirrhosis and hepatocellular carcinoma. Iron is considered a putative element that interacts with oxygen radicals in inducing liver damage and fibrosis. The role of hepatic iron in the progression of NASH remains controversial, but in some patients, iron may have a role in the pathogenesis of NASH. Though genetic factors, insulin resistance, dysregulation of iron-regulatory molecules, erythrophagocytosis by Kupffer cells may be responsible for hepatic iron accumulation in NASH, exact mechanisms involved in iron overload remain to be clarified. Iron reduction therapy such as phlebotomy or dietary iron restriction may be promising in patients with NASH/NAFLD to reduce insulin resistance as well as serum transaminase activities.

Key words: insulin resistance, iron, nonalcoholic fatty liver disease, non-alcoholic steatohepatitis, oxidative stress, phlebotomy

INTRODUCTION

IRON IS A potent catalyst of oxidative stress and may act synergistically with other promoters of lipid peroxidation by catalyzing these reactions. Iron overload can also directly cause lipid peroxidation, and one of the subsequent products, malondialdehyde, has been shown to activate hepatic stellate cells in vitro, the major source of fibrogenesis in liver injury.[1]

Non-alcoholic liver disease (NAFLD) is defined as a constellation of clinical conditions characterized by predominantly macrovesicular steatosis of the liver. The histologic spectrum of this disease ranges broadly from simple steatosis and non-alcoholic steatohepatitis (NASH) through to cirrhosis. Although simple steatosis seems to be benign, NASH can have a progressive course. Diagnosis of NASH is defined by liver histology, which typically shows macrovesicular steatosis and lobular inflammation with or without fibrosis. Mallory bodies are occasionally seen in the absence of a history of excessive ethanol ingestion.[2] The exact mechanism of this progression is not known but probably involves two steps.[3] Excessive triglyceride accumulation is the most likely first step. The second step may relate to an increase in oxidative stress, which, in turn, triggers liver cell necrosis and activation of hepatic stellate cells, both leading to fibrosis and ultimately to the development of cirrhosis. One of the potential cofactors suspected to enhance this oxidative stress is excessive hepatic iron accumulation.

This article explores the role of hepatic iron in NASH/NAFLD, and the possible therapeutic implications of iron reduction therapy.

Iron indices and hepatic iron deposition in NASH/NAFLD

There is controversial evidence that hepatic iron may play a role in the pathogenesis of NASH/NAFLD. Bacon *et al.*[4] documented abnormal iron indices (serum ferritin and/or transferrin saturation), and elevated hepatic iron concentration in NASH. George *et al.*[5] first proposed the hypothesis of iron-related liver injury in NASH. In their study of 51 patients, increased hepatic iron was present in 41%, and 23% had hepatic iron concentration (HIC)

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above the upper limit of normal. Their most significant conclusion was that increased hepatic iron had the greatest association with the severity of fibrosis; Perl's stain grade was assigned a relative risk of 5.5 (95% CI, 2.5–13.6). We also previously reported high frequencies of hyperferritinemia and increased hepatic iron stores in Japanese patients with NASH.[6,7] Serum thioredoxin (TRX) levels, an indicator of oxidative stress, were increased in proportion of the grade of hepatic iron accumulation.[6] Our data imply that the presence of excessive hepatic iron may be one of the possible cofactors for the induction of oxidative stress in NASH. Iron could potentially play a supporting role in the lipid peroxidation and fibrogenesis central to the development and progression of NASH.

In contrast, Younossi *et al.*,[8] Angulo *et al.*,[9] and Chitturi *et al.*[10] documented that significant iron accumulation is not seen in most patients with NASH. Younossi *et al.*[8] found no significant iron accumulation in NAFLD patients and no association between hepatic iron and aggressive histological or clinical outcome. Angulo *et al.*[9] studied 132 patients with NASH from Mayo Clinic database to find independent predictors of hepatic fibrosis. HIC and hepatic iron index were normal in all patients with abnormal iron indices (53% had elevated serum ferritin; 11% had elevated transferrin saturation). They found no association between increased iron indices and degree of fibrosis in multivariate analysis. Chitturi *et al.*[10] demonstrated that hyperferritinemia was present in 38 (40%) of 93 patients with NASH, but that only nine (10%) patients showed increased iron: 7 with grade 2 and 2 with grade 3. In India, Duseja *et al.*[11] showed that 71% of thirty-one NASH patients had negative iron staining. There was no association the degree of iron staining and fibrosis stage. These authors conclude that hyperferritinemia in NASH is a nonspecific effect of hepatic necroinflammation, reflecting its function as an acute phase protein. Serum ferritin is known to increase because of release from damaged hepatocytes. We also previously suggested that serum ferritin levels reflect oxidative stress as well as hepatic iron concentration and hepatocyte damage in chronic liver disease,[12] because the synthesis of ferritin seems to be influenced by oxidative stress or reduction/oxidation (redox) state.[13,14] It is possible that elevated serum ferritin in NASH may be derived from iron-unrelated oxidative stress,[12] such as free fatty acid, lipid peroxide, cytokines, and induction of cytochrome P450 enzymes (CYP2E1 and CYP4A).[15]

In this way, the role of hepatic iron in the pathogenesis of NASH or abnormal iron indices in NASH remains controversial and unsettled as of this time.[16]

The role of HFE mutation in hepatic iron deposition of NASH/NAFLD

The significance of hemochromatosis gene (HFE) mutations in the pathogenesis and progression of NASH/NAFLD also remains controversial.[5,10,17] George *et al.*[5] demonstrated a higher prevalence of the Cys282Tyr mutation of the HFE gene in patients with NASH, although there was no difference in the frequency of the His63Asp mutation. The presence of the Cys282Tyr mutation was associated with increased hepatic iron staining and hepatic iron concentration. Bonkovsky *et al.*[18] also reported a significantly higher prevalence of certain HFE mutation in patients with NASH. These authors found that Cys282Tyr heterozygotes had significantly more hepatic fibrosis than those without HFE mutations. However, the HIC was normal in all subjects and did not differ between those with and without HFE mutations. According to data from 126 NASH patients which were collected from 6 North American conters,[19] Cys282Tyr heterozygotes is associated with advanced hepatic fibrosis and stainable hepatic iron in Caucasians with NASH. They speculate that the mechanism is related to increased oxidative stress in the liver due to increased iron deposition. Chitturi *et al.*[10] found a trend toward higher serum ferritin levels among Cys282Tyr heterozygotes with NASH. Neither hepatic iron nor the presence of HFE mutations were identified as risk factors for fibrotic severity. Similarly, no evidence of an association of hepatic iron overload and HFE mutations with NASH was found in Brazilian or Asian Indian patients.[11,20,21] In Japan, there were no NASH/NAFLD patients with HFE mutations,[22] in agreement with a previous report indicating that the frequencies of HFE mutations in the Japanese population are extremely low (Cys282Tyr, 0%; and His63Asp, 0.99%).[23]

In this way, HEF gene mutation which is related to ethnicity[10] does not seem to have important roles in hepatic iron deposition in NASH/NAFLD.

Possible mechanisms involved in hepatic iron overload in NASH/NAFLD

As mentioned above, the precise mechanisms underlying hepatic iron deposition in NASH remain unknown. However, several possible mechanisms have been suggested in clinical or experimental studies (Fig. 1).

An association between insulin resistance (IR) and hepatic iron overload has been recently described.[24] First, it is quite plausible that the unhealthy diets contribute to IR not only through excess fat intake but also through excess iron supply (for example, in meat or in

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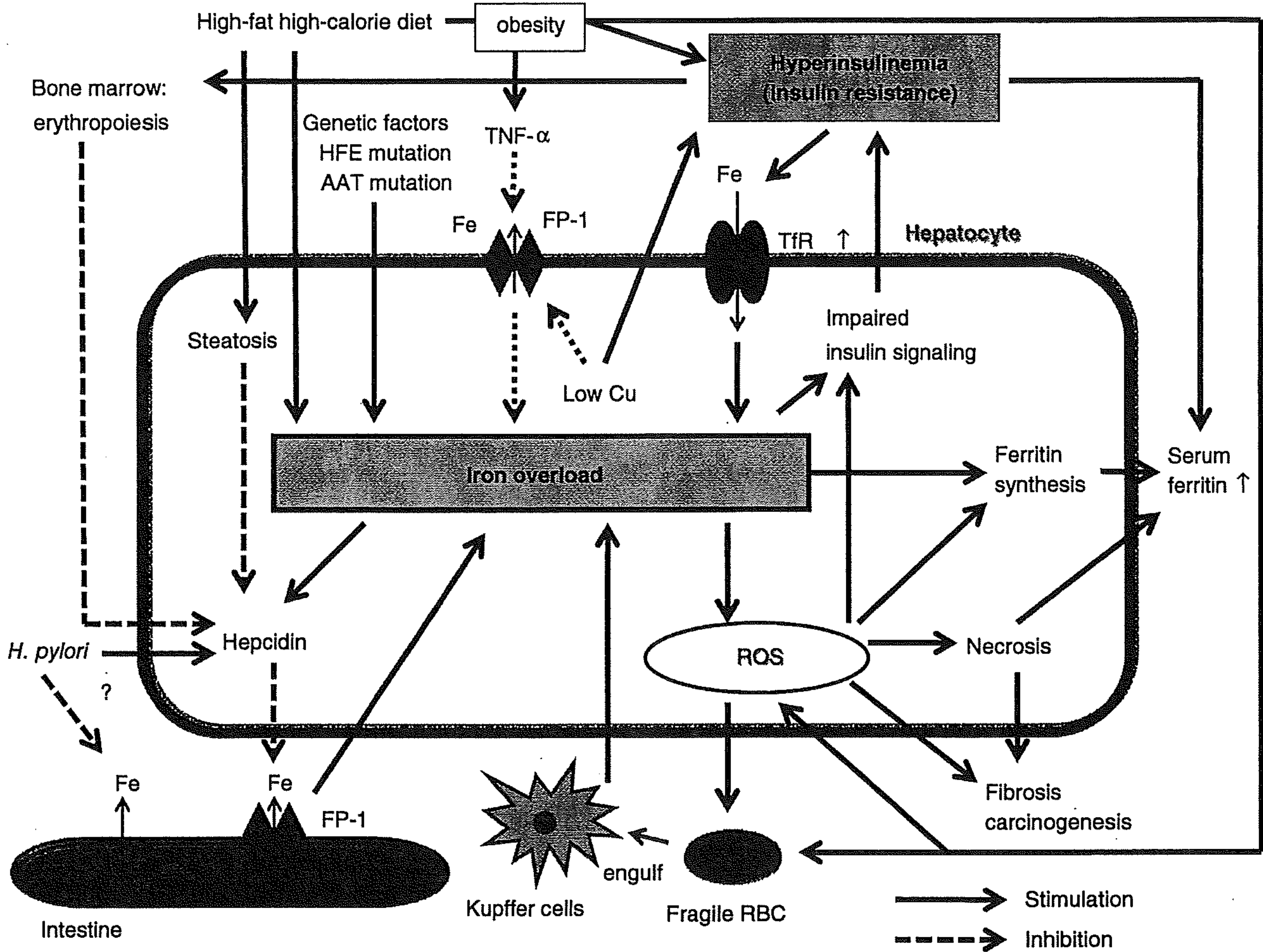

Figure 1 Possible mechanisms of hepatic iron deposition and pathogenetic roles of iron in nonalcoholic steatohepatitis/nonalcoholic fatty liver disease. AAT, alpha 1-antitrypsin; FP-1, ferroporitn-1; *H. pylori, Helicobacter pylori*; RBC, red blood cell; ROS, reactive oxygen species; TfR, transferrin receptor; TNF-α, tumor necrosis factor-α.

iron-supplemented food). Second, iron overload can interfere with insulin signaling through the induction of reactive oxygen species (ROS) (Fig. 1), the latter impairing insulin uptake through a direct effect on insulin receptor function,[25] by inhibiting the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and iron induces IR of glucose transport in adipocytes through a mechanism independent of fatty acids.[26] Moreover, iron has been found to reduce hepatic extraction/metabolism of insulin and to interfere with insulin action on the liver, leading to peripheral hyperinsulinemia.[27,28] Another mechanism is the enhancement of GLUT 1 and 4 activities in skeletal muscle after iron depletion.[29] By contrast, hyperinsulinemia may cause rapid stimulation of iron uptake into the liver, because insulin is known to redistribute transferrin receptors from an intracellular membrane compartment to the cell surface (Fig. 1).[30,31] The association of unexplained hepatic iron overload with metabolic disorders has recently been coined as the insulin resistance-associated hepatic iron overload syndrome (IR-HIO) proposed by Mendler *et al.*[32] Although patients with IR-HIO have a high prevalence of IR-related metabolic disorders, the relationship of IR-HIO to NASH is unclear. It is plausible that IR, which is often associated with NASH,[33] may be directly responsible for the accumulation of iron in the liver.[34] In general population, the association of elevated serum ferritin concentrations and IR was already demonstrated.[35–37] Several studies have demonstrated that serum ferritin is a good indica-

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Table 1 Similarities between chronic hepatitis C and non-alcoholic steatohepatitis

1. Histological findings
Steatosis
Iron deposition (hyperferritinemia)
2. Pathogenesis
Oxidative stress
Insulin resistance
3. Progression
Cirrhosis or hepatic failure
Hepatocellular carcinoma
4. Treatments
Diet therapy
Medication (ursodeoxycholic acid, vitamin E)
Iron reduction therapy (phlebotomy, dietary iron restriction)

tor of IR also in hepatitis C patients.[38,39] In Japan, we also previously reported that serum ferritin levels were positively correlated with homeostasis assessment model for IR (HOMA-IR) in non-diabetic patients with chronic hepatitis C.[40] One problem of these previous studies is that the iron concentration of liver tissue was not measured, because serum ferritin cannot always reflect total body iron stores as mentioned above. We recently studied 56 non-obese non-diabetic patients with chronic hepatitis C to investigate whether hepatic iron deposition is really correlated with IR. HOMA-IR was significantly correlated not only with serum ferritin but also with the grade of hepatic iron deposition.[41] Our results suggest that chronic hepatitis C may be one of the IR-HIO. Marcheisini *et al.*[42] have reported that serum indices of iron overload are present in 10 or 43% patients with NAFLD, but that those do not correlate with measures of insulin sensitivities. We demonstrated that serum ferritin levels and HOMA-IR in patients with NASH were significantly higher than in those with simple steatosis.[43] Our study did not show the correlation of HOMA-IR with serum ferritin levels or hepatic iron concentrations in patients with NASH/NAFLD,[43] in contrast with previous studies showing the positive correlation of IR with hepatic iron deposition in hepatitis C.[40,41] In this way, further studies are required to clarify the association of IR with iron deposition in NASH/NAFLD. On the other hand, high frequencies of hepatic steatosis in hepatitis C as previously shown[44] have suggested that chronic hepatitis C may be named a virus-associated steatohepatitis (VASH).[45] Between chronic hepatitis C and NASH, there are several similarities such as steatosis, insulin resistance, hepatic iron accumulation, and oxidative stress (Table 1).

Hepcidin is a disulfide-bonded peptide that was first identified as an antimicrobial peptide and was subsequently shown to be central player in systemic iron homeostasis.[46,47] Hepcidin is believed to be a negative regulator of dietary iron absorption and of iron release by macrophages via inducing internalization and degradation of the iron exporter ferroportin in absorptive enterocytes and reticuloendothelial cells.[48] The synthesis of hepcidin, which is specifically produced by the liver,[49] is greatly stimulated by inflammation or by iron overload.[50] The expression is down-regulated by hypoxia, anemia, iron deficiency, erythropoietin, and erythropoietic stimulation.[51] Though the role of hepcidin in the iron loading of patients with hepatitis C is unknown, one hypothesis is that low expression of hepcidin in the liver may be responsible for hepatic iron overload in hepatitis C.[52–54] Nagashima *et al.* reported that serum prohepcidin levels were decreased and negatively correlated with serum ferritin levels or hepatic iron concentration in hepatitis C patients.[52] They have suggested that failure of homeostatic regulation of serum prohepcidin concentrations may be induced by HCV infection. Fujita *et al.* demonstrated that hepatic hepcidin mRNA was relatively low in chronic hepatitis C patients, as compared to chronic hepatitis B.[53] This relative impairment of hepcidin production was fully reversible after successful eradication of HCV.[54] In contrast, Aoki *et al.* concluded that liver hepcidin, whose mRNA was correlated with iron concentration, cannot play a role in the hepatic iron accumulation in hepatitis C.[55] According to a recent experimental study using transgenic mice expressing HCV polyprotein by Nishina *et al.*, HCV-induced ROS may down-regulate hepcidin transcription, which in turn leads to increased duodenal iron transport and macrophage iron release, causing hepatic iron accumulation.[56] In alcoholic liver disease, hepatic iron loading seems to be contributed to the down-regulated hepcidin leading to the increase of iron absorption from the intestine.[57,58] The expression of hepcidin in NASH/NAFLD patients remains unknown. In animal model of IR, IR can lead to a downregulation of hepcidin expression via stimulation of erythropoiesis, which in turn increases the needs for iron (Fig. 1).[59] It has been recently shown that hepcidin is expressed, at both the mRNA and protein levels, in adipose tissue and that this expression is enhanced in severely obese patients.[60] Human studies focused on iron absorption rates and hepcidin expression in NASH/NAFLD or metabolic syndrome should be performed to unravel the mechanisms behind the iron metabolism disturbance.[61] According to a recent study from Australia,[62] analysis of

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iron-regulatory molecules in liver tissue revealed a striking down-regulation of the liver iron exporter ferroporitn-1 (FP-1) and the iron sensing molecule hemojuvelin (HJV). They suggest that TNF-α, highly expressed in NAFLD patients, play a role in exerting these regulatory changes, because they found inverse correlations of TNF-α concentrations and expression of FP-1 or HVJ in vivo and decreased formation of FP-1 and HVJ in HepG2 cells on stimulation with TNF-α in vitro. Thus, they concluded that iron accumulation in NAFLD may result from decreased iron mobilization from hepatocytes due to low expression of FP-1 and HVJ (Fig. 1). The same group found that NAFLD patients with iron overload had low levels of serum and hepatic copper along with low serum ceruloplasmin levels.[63] They suggested that copper deficiency may be responsible for hepatic iron overload, because the copper-dependent ferroxidase ceruloplasmin is required for the mobilization of iron from storage sites such as the liver. Moreover, FP-1 mRNA expression and protein were found to be lowest in NAFLD patients with low hepatic copper concentrations. They detected significantly lower FP-1 protein levels in rats kept on a copper-deficient diet as compared with rats with a normal copper diet. Lower copper bioavailability causes increased hepatic iron stores via decreased FP-1 expression and ceruloplasmin ferroxidase activity thus blocking liver iron export in copper-deficient NAFLD patients (Fig. 1). These studies observed hepatic expression of hepcidin, which reflects the physiologic response to liver iron accumulation.

On the other hand, several lines of evidences have suggested an association between *Helicobacter pylori* (Hp) infection and iron deficiency.[64] If Hp infection is associated with iron deficiency anemia, then eradicating the organism should increase iron stores and resolve the anemia. Reversal of iron deficiency anemia after successful eradication of Hp has been observed not only in children[65,66] but also in adults.[67–69] These findings have supported an association between Hp infection and iron deficiency. Several possible mechanisms may correlate Hp infection with decreased iron accumulation.[70] Recent findings support the hypothesis that in patients with Hp positive gastritis, concomitant changes in intragastric pH and ascorbic acid are present that might play a role in impairing alimentary iron absorption with consequent iron deficiency.[64,71] It has also been speculated that Hp infected antrum could act as a sequestering focus for iron. Hp infection enhances gastric lactoferrin,[72,73] which captures iron from transferrin. The iron bound to lactoferrin is in turn picked up by the bacterium, by means of its outer membrane receptors,[74] for its own growth.[64] Though the exact mechanisms of iron deficiency in Hp-infected individuals are unknown, one hypothesis[75] is that up-regulated expression of hepcidin by Hp infection via releasing cytokines such as IL-6 could impair intestinal iron absorption with consequent lower iron deposit in the liver (Fig. 1). Dr Beutler hypothesizes that Hp may produce hepcidin mimicks preventing the absorption of iron in a manner analogous with the suppression of iron absorption by hepcidin associated with inflammation.[76] We previously reported that Hp infection may decrease hepatic iron deposition in hepatitis C patients.[77] Our preliminary study also demonstrated that the grades of hepatic iron deposition in NAFLD patients with Hp infection were lower than those without.[43]

Otogawa K *et al.*[78] newly proposed that hepatic iron in NASH may be derived from erythrophagocytosis by liver macrophages (Kupffer cells), using rabbits fed a cholesterol-rich high-fat diet (HFD) with IR who exhibit pathological changes very similar to those of human NASH (Fig. 1). In addition, immunohistochemistry in liver tissue from NASH patients revealed that the aggregation of erythrocytes in inflammatory hepatic sinusoids was increased, leading to hepatic iron deposition and oxidative stress. How erythrocytes in patients with NASH become easy to be engulfed by Kupffer cells is not fully understood, though ROS may be involved in the mechanism (Fig. 1).

Recently, another element has been examined that could affect iron metabolisim. Alpha 1-antitrypsin (AAT) protein, an acute-phase protein, has been demonstrated to interact with transferrin receptor inducing ferritin synthesis. Valenti *et al.*[79] demonstrated that NAFLD patients with AAT mutations had higher ferritin levels than those without. In liver histology, AAT mutations were associated with higher prevalence of sinusoidal siderosis, but not with more severe liver damage in NAFLD. AAT mutations did not affect parenchymal or portal siderosis.

Iron reduction therapy in NAFLD/NASH

Although the causes of NASH are not well defined and several therapies including diet,[80] antioxidants,[81] and approaches that improve IR[82] have been tried, the optimal therapy for NASH has not been established.

In Japan, we previously confirmed a significant improvement in serum transaminase activities after 3-month iron reduction therapy by phlebotomy for chronic hepatitis C in a multicenter, prospective, randomized, controlled trial.[83] We have also demonstrated that the efficacy of phlebotomy is superior to that of

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Table 2 Phlebotomy in nonalcoholic steatohepatitis/non-alcoholic fatty liver disease

Study	Location	Study design	Patients, n (F/M)	Disease	ALT (IU/mL)	Ferritin (ng/mL)	HFE mutation	Insulin resistance (IRI, HOMA-IR)	Histological evaluation
Facchini *et al.* (2002)[85]	San Francisco, US	Open	17 (5/12)	NAFLD IGT (+)	From 61 ± 5 to 32 ± 2 (P < 0.001)	From 299 ± 41 to 15 ± 1 (P < 0.001)	Excluded	Improved	NA
Valenti *et al.* (2003)[86]	Milano, Italy	Open	12 (0/12)	NAFLD IGT (–)	From 62 ± 45 to 33 ± 22 (P = 0.0074)	From 583 ± 274 to 36 ± 22	C282Y (+/–): 3/12	Improved (independently of serum ferritin levels)	Only at entry
Riquelme *et al.* (2004)[88]	Santiago, Chile	Case report	1 (1/0)	NASH (PCT, β thalassemia minor)	From 68 to normal (<20)	From 762 to normal (<417)	H63D (+/–)	NA	Complete resolution of steatosis and inflammation
Fargion *et al.* (2005)[28]	Milano, Italy	Open	42 (9/33)	NAFLD	From 44 ± 30 to 32 ± 19 (P = 0.03)	From 361 ± 222 to 123 ± 100 (P = 0.0001)	NA	Improved (independently of serum ferritin levels)	NA
Sumida *et al.* (2006)[87]	Nara, Japan	Open	11 (5/6)	NASH	From 126 ± 47 to 56 ± 17 (P = 0.002)	From 563 ± 322 to 18 ± 9 (P = 0.001)	NA	NA	Only at entry
Valenti *et al.* (2007)[89]	Milano, Italy	Case-control	64 (11/53)	NAFLD	From 57.9 ± 47.4 to 34.3 ± 27 (NS *versus* controls)	From 438 {21–628} to 52 {27–96}	C282Y (+/–): 11/54 H63D (+/–): 19/54	Improved (especially in patients with hyperferrtinemia and carrying HFE mutation)	Only at entry

{}: interquartile range. IGT, impaired glucose tolerance; IRI, immuno-reactive insulin; HOMA-IR, homeostasis model assessment for insulin resistance; NA, not assessed; NAFLD, nonalcoholic fatty liver disease; NS, not significant; PCT, porphyria cutanea tarda.

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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

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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.

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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.

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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-

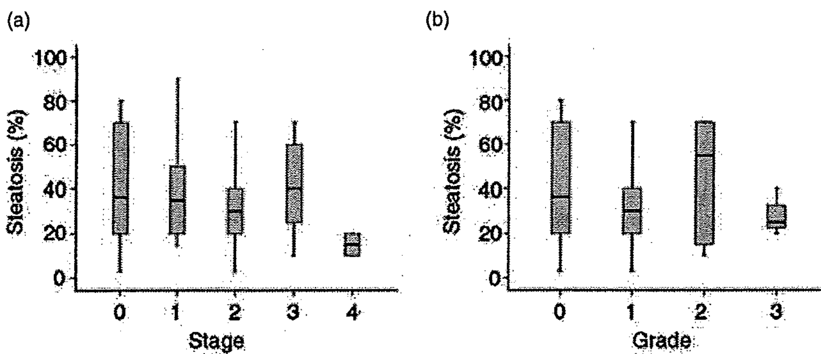

Figure 1 Distributions of the level of hepatic steatosis in association with the stage (a) and grade (b). The level of steatosis decreased as the stage and grade progressed.

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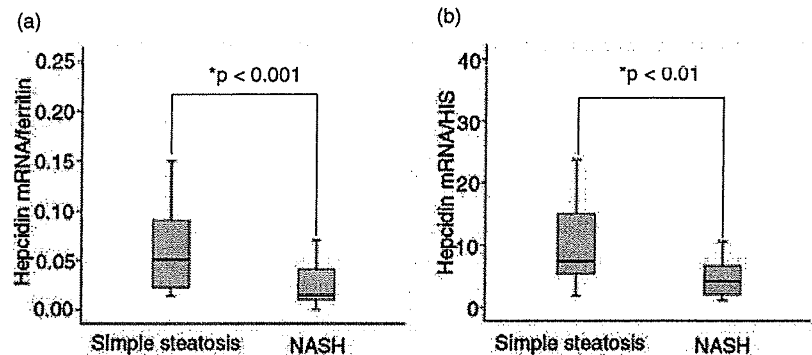

Figure 2 The ratio of hepcidin mRNA levels to serum ferritin levels (a) and that of hepcidin mRNA levels to hepatic iron score (HIS) (b). Hepcidin mRNA levels corrected for iron overload were significantly lower in NASH patients than in simple steatosis patients. *Mann-Whitney U-test.

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 "burn-out" 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]

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