Table 4 Logistic regression models of the association of NAFLD (advanced vs mild) with DHEA-S levels and other clinical variables

Variables	OR	95% CI	*P*-value
Model 1			
DHEA-S ≤66 μg/dL	8.9113	2.7009–29.4014	0.0003
Model 2			
DHEA-S ≤66 μg/dL	7.1201	2.0811–24.3606	0.0018
Age ≥65 years	2.1324	0.6899–6.5910	0.1884
Model 3			
DHEA-S ≤66 μg/dL	5.4624	1.5555–19.1822	0.0081
Age ≥65 years	2.2978	0.7440–7.0964	0.1482
Sex (female)	2.7458	0.6797–11.0932	0.1562
Model 4			
DHEA-S ≤66 μg/dL	8.5274	2.2958–31.6740	0.0014
HOMA-IR ≥5	2.4319	0.6799–8.6982	0.1717
BMI ≥2 kg/m^2	0.9328	0.2546–3.4177	0.9164
Diabetes	1.5532	0.4324–5.5796	0.4998
Dyslipidemia	0.2547	0.0727–0.8926	0.0326
Hypertension	0.5488	0.1473–2.0446	0.3713
Model 5			
DHEA-S ≤66 μg/dL	4.9549	1.1691–20.9996	0.0229
Age ≥65 years	2.8962	0.7843–10.6948	0.1106
Sex (female)	1.9494	0.3765–10.0935	0.4264
HOMA-IR ≥5	2.3671	0.6276–8.9273	0.2033
BMI ≥28 kg/m^2	1.0446	0.2619–4.1658	0.9508
Diabetes	1.6007	0.3904–6.5023	0.5107
Dyslipidemia	0.2500	0.0682–0.9162	0.0364
Hypertension	0.4184	0.1022–1.7126	0.2256

BMI, body mass index; CI, confidence interval; DHEA-S, dehydroepiandrosterone sulfate; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

aged more than 65 years (mean 71.4 ± 3.4 years), with patients with mild NAFLD, aged more than 65 years (mean 72.0 ± 5.5 years, $P = 0.887$). DHEA-S levels were significantly lower in patients with advanced NAFLD than in patients with mild NAFLD (37.6 ± 22.8 vs 68.4 ± 37.5 μg/dL, $P = 0.026$). As levels of DHEA-S are different between men and women and lower in older individuals as mentioned above, DHEA-S levels were adjusted for age and sex. Several multivariate logistic regression models were run in order to determine the association of DHEA levels with the presence or absence of advanced NAFLD while adjusting for the effect of age and sex. As shown in Table 4, the unadjusted (model 1) association of DHEA levels with severity of NAFLD remained highly significant when adjusted by age (model 2) and age plus sex (model 3). The AUC for DHEA in separating patients with and without advanced fibrosis was 0.788 (Fig. 1b). The sensitivity of a DHEA-S-value of 66 μg/dL or less for the presence of more advanced NAFLD was 76.5% (13/17) and specificity was 73.3% (85/116). The positive predictive value (PPV) of the cut-off value was 29.5 % (13/44) and negative predictive value (NPV) was 95.5% (85/89). Almost all of the predictivity for histological severity of NAFLD could be attributed to DHEA-S levels independent of age and sex. DHEA levels remained highly significantly associated with advanced NAFLD after adjustment by metabolic disease or insulin resistance (Table 4, models 4 and 5). A "dose effect" of lower DHEA-S and advanced fibrosis was observed with a mean DHEA-S of 170.4 ± 129.2, 137.6 ± 110.5, 96.2 ± 79.3, 61.2 ± 46.3 and 30.0 ± 32.0 μg/dL for fibrosis stages 0, 1, 2, 3 and 4,

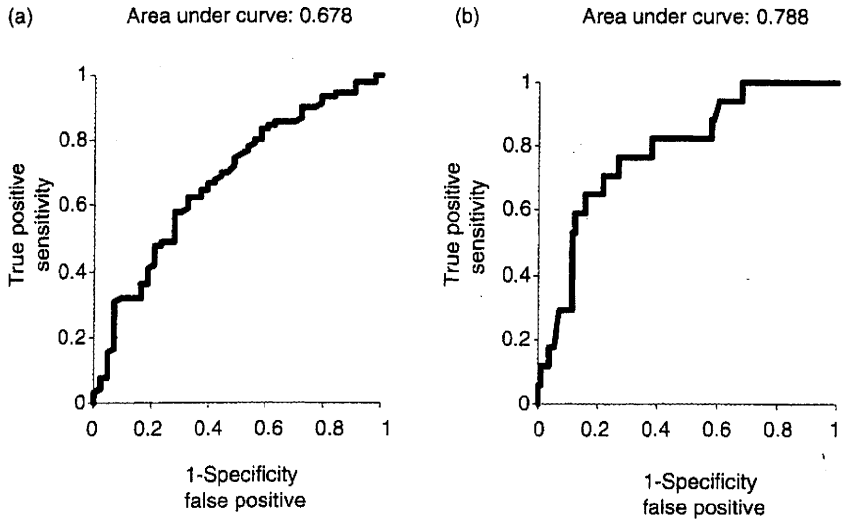

Figure 1 The area under the receiver-operator curve for dehydroepiandrosterone in separating patients with and without non-alcoholic steatohepatitis (a) or separating patients with and without advanced fibrosis (b).

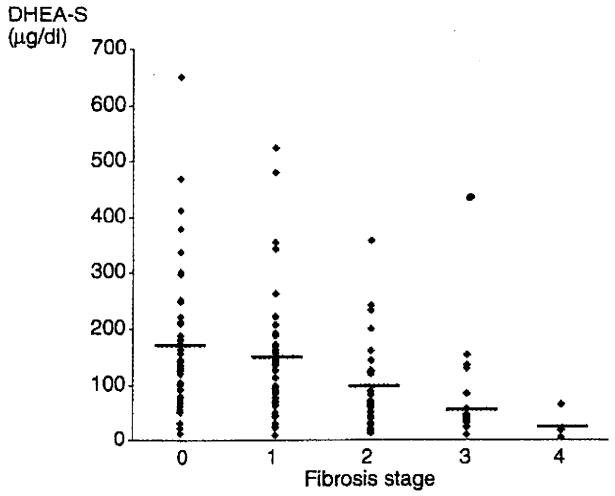

Figure 2 Variation in dehydroepiandrosterone sulfate (DHEA-S) levels with fibrosis stage for participants with non-alcoholic fatty liver disease. Mean DHEA-S levels are indicated by horizontal lines. A "dose effect" of lower DHEA-S and advanced fibrosis was observed, with a mean DHEA-S of 170.4 ± 129.2, 137.6 ± 110.5, 96.2 ± 79.3, 61.2 ± 46.3 and 30.0 ± 32.0 for fibrosis stages 0, 1, 2, 3 and 4, respectively. Significant inverse correlations were detected by Spearman's rank correlation analysis (correlation coefficient value −0.4141, $P < 0.0001$).

respectively (Fig. 2). DHEA-S levels were inversely correlated with the progression of fibrosis by Spearman's rank correlation analysis (correlation coefficient value −0.4141, $P < 0.0001$).

DISCUSSION

THE PRINCIPAL FINDING of this study is that circulating DHEA-S levels are strongly associated with the most important feature of histologically advanced NAFLD, though DHEA-S levels in a total of NAFLD patients were not different from those in sex- and age-matched healthy people. DHEA, and its interchangeable sulfated form, DHEA-S, are the most abundant circulating steroid hormones in healthy individuals. They are derived from the zona reticularis of the adrenal cortex. Both cross-sectional and longitudinal data[26] have clearly indicated that serum concentrations of DHES-S decrease with age. DHEA and DHEA-S levels peak at approximately age 25 years and decrease progressively thereafter, falling to 5% of peak levels by the ninth decade. Though it is important to consider whether the lower DHEA levels observed in patients with advanced NAFLD in our study were simply a surrogate of older age, age was less predictive of severity of NAFLD than DHEA-S by logistic regression analysis (Table 4). In an middle-aged population, there was no significant difference in serum DHEA-S levels between mild and advanced NAFLD, but in aged people, DHEA-S was significantly lower in advanced NAFLD than in mild NAFLD.

The role of DHEA-S deficiency in histological progression of NAFLD is likely to involve effects on insulin sensitivity, hepatic susceptibility to oxidative stress injury and/or stimulation of fibrosis. Hyperinsulinemia and increased insulin resistance may have important roles in the pathogenesis of NASH in both Western and Asian countries.[6,27–29] Hyperinsulinemia in NASH patients is attributable to increased insulin secretion compensatory to reduced insulin sensitivity, and is not the consequence of decreased hepatic extraction of insulin that occurs in all forms of chronic liver diseases at the stage of advanced fibrosis or cirrhosis.[27,28] The HOMA model[23] or the QUICKI model[24] have been validated and widely used for determining the degree of insulin resistance and strongly predicts the development of type 2 DM.[2] In agreement with our result, patients with NASH have higher HOMA index or lower QUICKI index compared with those with SS.[27,30] With regard to results obtained from the logistic regression model (Table 4), we intended to support the concept that the association between low levels of DHEA and worsening histology is independent of age, sex and insulin resistance. Though Charlton *et al.* observed that levels of DHEA are significantly lower in patients with histologically advanced NASH, independent of age or sex, they did not capture a specific index of insulin resistance, such as HOMA or QUICKI. We examined whether serum DHEA-S is correlated with these indices, but did not find any correlations. Therefore, it is highly likely that the association of DHEA levels and severity of NAFLD found in our patients was not confounded by the degree of insulin resistance. Several studies found an association between a decline in serum DHEA concentration and reduced insulin sensitivity.[31] In this way, the published work concerning the role of DHEA in mediating insulin sensitivity in humans is conflicting.

On the other hand, DHEA has been shown to exert a protective effect in hepatocytes against oxidative injury by decreasing malondialdehyde concentration and increasing superoxide dismutase activity and total glutathione concentrations in animal models of oxidative stress.[32,33] FFA, which lead to oxidative stress in NASH, are the major source of DHEA. In the presence of severe insulin resistance, increased circulating FFA are not converted into DHEA. It is suggested that the inability to

produce appropriate amounts of DHEA in response to FFA may translate into a more rapid and worsening progression toward NASH.[34] Although we found no correlations between serum DHEA-S and serum FFA (Table 2), it remains unknown whether serum DHEA-S is correlated with hepatic FFA.

Although several relatively non-invasive parameters have been identified as predictive of more advanced fibrosis stage in patients with NAFLD, none has sufficient sensitivity or specificity to be of clinical utility to negate the need for liver biopsy.[35,36] By ROC analysis, serum DHEA-S levels seem to be useful for differentiating advanced NAFLD rather than for detecting NASH. Our data suggest that patients with DHEA-S levels greater than 66 μg/dL are highly unlikely to have advanced NAFLD (4/89 patients, sensitivity 76% and specificity 73%). This cut-off value was lower than that proposed by Charlton *et al.* (<100 μg/dL).[19] First, racial/ethnic differences in DHEA-S levels could account for this difference.[37] DHEA-S seems to be significantly higher in white versus Chinese men, but not in the women.[38] However, the PPV is too low (29.5%) to pick up advanced NAFLD, because the prevalence of advanced NAFLD is extremely low in our population (12.7%, 17/133). Thus, serum DHEA-S levels can be applicable to exclude advanced NAFLD rather than to detect the stage.

It was also important to consider whether low levels of DHEA-S might occur as a result of chronic liver disease in general versus a specific phenomenon of histologically more advanced NAFLD. Serum DHEA-S levels depend on adrenal DHEA production and its hepatic metabolism mediated by DHEA sulfotransferase (DHEA-ST) which catalyzes sulfonation of DHEA to form DHEA-S. The relationship between adrenal function and liver function remains unclear. It is probable that adrenal DHEA production may decrease in accordance with fibrosis progression, because adrenal insufficiency is increasingly reported with end-stage liver disease.[39] In the present study, however, we excluded patients with decompensated LC. In cirrhotic patients, serum DHEA-S levels are lower than normal control subjects.[40] It is hypothesized that a low level of DHEA-S was due to a defect in sulfurylation in patients with hepatic cirrhosis. However, that study has a few limitations: the number of subjects was very small, and cirrhotic patients were older (mean 49 years, range 21–70) than normal men (mean 25 years, range 21–38). On the other hand, histochemical analysis revealed that the immunopositive area for DHEA-ST was significantly larger in chronic hepatitis than in normal liver, but was not different between LC and normal liver.[41] In another study,[42] DHEA-ST activity and concentration were significantly reduced in PBC, primary sclerosing cholangitis (PSC), chronic active hepatitis and alcoholic cirrhosis, but not in cryptogenic cirrhosis when compared to normal liver. Based on these controversial results, it is unknown whether reduced activity of DHEA-ST is responsible for low levels of DHEA-S in the advanced stage of NAFLD. According to Charlton *et al.*[19] DHEA-S levels were not significantly predictive of severity of disease in patients with cholestatic liver disease, PBC and PSC. In the future, we should examine the serum levels of DHEA-S in other chronic liver diseases and hepatic expression of DHEA-ST in NAFLD patients.

There are thus several potential mechanisms for DHEA deficiency to promote histological progression in NAFLD. DHEA deficiency (patients with advanced NAFLD had levels of DHEA-S associated with hypoadrenalism) presents an appealing new therapeutic target for the treatment and prevention of NASH. A protective effect of DHEA was reported in an orotic acid-induced animal model of fatty liver disease.[33] However, therapeutic benefits of hormone supplementation for the treatment of aging, insulin resistance and cardiovascular disease remain obscure and controversial.[43]

Our study has a few important limitations. Patient selection bias can also exist, because liver biopsy might be considered for NAFLD patients who are likely to have NASH. First, the proportion of subjects with advanced fibrosis was small as reported in other Asian series.[44,45] We acknowledge that pathological diagnosis was mainly determined using liver tissues received by percutaneous liver biopsy, which is prone to sampling error or inter-observer variability.[46,47] Due to these limitations, the present results need to be validated in independent populations by other investigators.

In conclusion, we have found that patients with more advanced NAFLD have low circulating levels of DHEA-S. These data provide novel evidence for relative DHEA deficiency in Japanese patients with histologically advanced NASH.

REFERENCES

1 Ludwig J, Viggiano TR, McGill DB *et al.* Non-alcoholic steatohepatitis. Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; 55: 434–8.
2 Matteoni CA, Younossi ZM, Gramlich T *et al.* Nonalcoholic fatty liver diseases: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; 116: 1413–19.

3 Rafiq N, Bai C, Fang Y *et al.* Long-term follow-up of patients with nonalcoholic fatty liver. *Clin Gastroenterol Hepatol* 2009; 7: 234–8.
4 Saibara T. Nonalcoholic steatohepatitis in Asia-Oceania. *Hepatol Res* 2005; **33**: 64–7.
5 Hamaguchi M, Kojima T, Takeda N *et al.* The Metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann Intern Med* 2005; **143**: 722–8.
6 Marcheisini G, Bugianesi E, Forlani G *et al.* Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003; **37**: 917–23.
7 Sanyal AJ, Campbell-Sargent C, Mirshahi F *et al.* Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183–92.
8 Oliveira CP, Costa Gayotto LC, Tatai C *et al.* Oxidative stress in the pathogenesis of nonalcoholic fatty liver disease, in rats fed with a choline-deficient diet. *J Cell Mol Med* 2002; **6**: 399–406.
9 Sumida Y, Nakashima T, Yoh T *et al.* Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic liver disease. *J Hepatol* 2003; **38**: 32–8.
10 Roth GS, Lane MA, Ingram DK *et al.* Biomarkers of caloric restriction may predict longevity in humans. *Science* 2002; **297**: 811.
11 Barrett-Connor E, Khaw KT, Yen SS. A prospective study of dehydroepiandrosterone sulfate, mortality, and cardiovascular disease. *N Engl J Med* 1986; **315**: 1519–24.
12 Bednarek-Tupikowska G, Gosk I, Szuba A *et al.* Influence of dehydroepiandrosterone on platelet aggregation, superoxide dismutase activity and serum lipid peroxide concentrations in rabbits with induced hypercholesterolemia. *Med Sci Monit* 2000; **6**: 40–5.
13 Lasco A, Frisina N, Morabito N *et al.* Metabolic effects of dehydroepiandrosterone replacement therapy in postmenopausal women. *Eur J Endocrinol* 2001; **145**: 457–61.
14 Jakubowicz D, Beer N, Rengifo R. Effect of dehydroepiandrosterone on cyclic-guanosine monophosphate in men of advancing age. *Ann NY Acad Sci* 1995; **774**: 312–5.
15 Kawano M. Complement regulatory proteins and autoimmunity. *Arch Immunol Ther Exp* 2000; **48**: 367–72.
16 Dhatariya K, Bigelow ML, Nair KS. Effect of dehydroepiandrosterone replacement on insulin sensitivity and lipids in hypoadrenal women. *Diabetes* 2005; **54**: 765–9.
17 Poynter ME, Daynes RA. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappaB signaling, and reduces inflammatory cytokine production in aging. *J Biol Chem* 1998; **273**: 32833–41.
18 Peters JM, Zhou YC, Ram PA *et al.* Peroxisome proliferator-activated receptor alpha required for gene induction by dehydroepiandrosterone-3 beta-sulfate. *Mol Pharmacol* 1996; **50**: 67–74.
19 Charlton M, Angulo P, Chalasani N *et al.* Low circulating levels of dehydroepiandrosterone in histologically advanced nonalcoholic fatty liver disease. *Hepatology* 2008; **47**: 484–92.
20 Kleiner DE, Brunt EM, Van Natta M *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313–21.
21 Japanese Society for the Study of Obesity. New criteria of obesity (in Japanese). *J Jpn Soc Study Obes* 2000; **6**: 18–28.
22 American Diabetes Association. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997; **20**: 1183–97.
23 Matthews DR, Hosker JP, Rudenski AS *et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–19.
24 Katz A, Nambi SS, Mather K *et al.* Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; **85**: 2402–10.
25 Brunt EM, Janney CG, Di Bisceglie AM *et al.* Non-alcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467–74.
26 Orentreich N, Brind JL, Vogelman JH *et al.* Long-term longitudinal measurements of plasma dehydroepiandrosterone sulfate in normal men. *J Clin Endocrinol Metab* 1992; **75**: 1002–4.
27 Chitturi S, Abeygunasekera S, Farrell GC *et al.* NASH and insulin resistance: insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; **35**: 373–9.
28 Pagano G, Pacini G, Musso G *et al.* Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: further evidence for an etiologic association. *Hepatology* 2002; **35**: 367–72.
29 Ono M, Saibara T. Clinical features of nonalcoholic steatohepatitis in Japan: evidence from literature. *J Gastroenterol* 2006; **41**: 725–32.
30 Boza C, Riquelme A, Ibañez L *et al.* Predictors of nonalcoholic steatohepatitis (NASH) in obese patients undergoing gastric bypass. *Obes Surg* 2005; **15**: 1148–53.
31 Nestler JE, Usiskin KS, Barlascini CO *et al.* Suppression of serum dehydroepiandrosterone sulfate levels by insulin: an evaluation of possible mechanisms. *J Clin Endocrinol Metab* 1989; **69**: 1040–6.
32 Celebi F, Yilmaz I, Aksoy H *et al.* Dehydroepiandrosterone prevents oxidative injury in obstructive jaundice in rats. *J Int Med Res* 2004; **32**: 400–5.
33 Goto H, Yamashita S, Makita T. Preventive effects of dehydroepiandrosterone acetate on the fatty liver induced by orotic acid in male rats. *Exp Anim* 1998; **47**: 257–60.
34 Manco M, Bottazzo G. Does the hormone of eternal youth protect against nonalcoholic steatohepatitis? *Hepatology* 2008; **48**: 1351.
35 Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: selected practical

issues in their evaluation and management. *Hepatology* 2009; **49**: 306–17.
36 Wieckowska A, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. *Hepatology* 2007; **46**: 582–9.
37 Litman HJ, Bhasin S, Link CL *et al.* Serum androgen levels in black, Hispanic, and white men. *J Clin Endocrinol Metab* 2006; **91**: 4326–34.
38 Lookingbill DP, Demers LM, Wang C *et al.* Clinical and biochemical parameters of androgen action in normal healthy Caucasian versus Chinese subjects. *J Clin Endocrinol Metab* 1991; **72**: 1242–8.
39 Loria P, Carulli L, Bertolotti M *et al.* Endocrine and liver interaction: the role of endocrine pathways in NASH. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 236–47.
40 Franz C, Watson D, Longcope C. Estrone sulfate and dehydroepiandrosterone sulfate concentrations in normal subjects and men with cirrhosis. *Steroids* 1979; **34**: 563–73.
41 Narasaka T, Moriya T, Endoh M *et al.* 17Beta-hydroxysteroid dehydrogenase type 2 and dehydroepiandrosterone sulfotransferase in the human liver. *Endocr J* 2000; **47**: 697–705.
42 Elekima OT, Mills CO, Ahmad A *et al.* Reduced hepatic content of dehydroepiandrosterone sulphotransferase in chronic liver diseases. *Liver* 2000; **20**: 45–50.
43 Nair KS, Rizza RA, O'Brien P *et al.* DHEA in elderly women and DHEA or testosterone in elderly men. *N Engl J Med* 2006; **355**: 1647–59.
44 Tsang SW, Ng WF, Wu BP *et al.* Predictors of fibrosis in Asian patients with non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2006; **21**: 116–21.
45 Wong VW, Wong GL, Chim AM *et al.* Validation of the NAFLD fibrosis score in a Chinese population with low prevalence of advanced fibrosis. *Am J Gastroenterol* 2008; **103**: 1682–8.
46 Ratziu V, Charlotte F, Heurtier A *et al.* Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898–906.
47 Merriman RB, Ferrell LD, Patti MG *et al.* Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 874–80.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Steatosis and hepatic expression of genes regulating lipid metabolism in Japanese patients infected with hepatitis C virus

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Abstract
Purpose Steatosis is a histological finding associated with the progression of chronic hepatitis C. The aims of this study were to elucidate risk factors associated with steatosis and to evaluate the association between steatosis and hepatic expression of genes regulating lipid metabolism.
Methods We analyzed 297 Japanese patients infected with hepatitis C virus and a subgroup of 100 patients who lack metabolic factors for steatosis. We determined intrahepatic mRNA levels of 18 genes regulating lipid metabolism in these 100 patients using real-time reverse transcription-polymerase chain reaction. Levels of peroxisome proliferator-activated receptor α and sterol regulatory element-binding protein 1 proteins were assessed by immunohistochemistry.
Results Steatosis was present in 171 (57%) of 297 patients. The presence of steatosis was independently associated with a higher body mass index, higher levels of γ-glutamyl transpeptidase and triglyceride, and a higher fibrosis stage. Steatosis was present in 43 (43%) of 100 patients lacking metabolic factors. Levels of mRNA and protein of peroxisome proliferator-activated receptor α, which regulates β-oxidation of fatty acid, were lower in patients with steatosis than in patients without steatosis.
Conclusions These findings indicate that impaired degradation of lipid may contribute to the development of hepatitis C virus-related steatosis.

Keywords Steatosis · Hepatitis C virus · Fibrosis · Gene expression · Peroxisome proliferator-activated receptor α

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Introduction

The prevalence of hepatic steatosis ranges from 40 to 86% (mean ~55%) in patients infected with hepatitis C virus (HCV) [1]. This range is higher than in the general population of adults in the Western world (20–30%) [2]. Steatosis appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [3–5].

Patients with HCV infection may have metabolic cofactors, such as obesity, diabetes, and alcohol abuse that contribute to the development of fatty liver. It is likely that two types of steatosis, viral and metabolic, coexist in patients with chronic hepatitis C [1, 3]. Known risk factors associated with steatosis include HCV genotype 3, a higher body mass index (BMI), diabetes, hyperlipidemia, ongoing alcohol abuse, older age, the presence of fibrosis, and

hepatic inflammation [1, 5]. However, different populations may have different risk factors for steatosis, and the distribution of HCV genotype differs from region to region. For example, HCV genotype 3, which is thought to be directly responsible for steatosis [6–8], is far less frequent in Japan than in Europe [7] or the United States [9].

Although the mechanisms of HCV-related steatosis are not well known, several viral and host factors appear to be involved [3]. In vitro studies [10] and a transgenic mouse models [11] have shown that HCV core protein can induce steatosis. HCV core protein, in turn, inhibits the activity of microsomal triglyceride transfer protein, which is essential for the assembly and secretion of very low density lipoproteins [12]. The intrahepatic levels of microsomal triglyceride transfer protein mRNA show an inverse correlation with the degree of steatosis in patients with chronic hepatitis C [13]. HCV infection and HCV core protein upregulates the expression of sterol regulatory element-binding protein 1 (SREBP1), a key transcriptional factor that activates the expression of genes involved in lipid synthesis [14, 15]. In addition, HCV core protein binds to retinoid X receptor α, a transcriptional regulator that controls many cellular functions including lipid metabolism [16]. HCV core protein also down-regulates the expression of peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyl transferase 1 (CPT1) [17, 18], and the mRNA levels of PPARα and CPT1 are found to be reduced in patients with chronic HCV infection [19].

In the present study, we investigated the risk factors associated with steatosis in Japanese patients with chronic HCV infection. To elucidate the molecular mechanisms underlying HCV-related (i.e., viral) steatosis, we also systematically measured the intrahepatic expression levels of genes that regulate lipid degradation, secretion, synthesis, and uptake in patients who lack metabolic factors for steatosis.

Methods

Patients

The study included a total of 297 Japanese patients with chronic HCV infection who underwent liver biopsy between April 2004 and June 2006 at the Hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan. To eliminate selection biases, the patients were recruited consecutively. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV (third-generation enzyme immunoassay; Chiron, Emeryville, CA), and positive for serum HCV-RNA (Amplicor HCV assay; Roche Diagnostic Systems, Tokyo, Japan). Exclusion criteria were as follows: positive for hepatitis B virus surface antigen (radioimmunoassay; Dainabot, Tokyo, Japan); other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, Wilson's disease, or hemochromatosis; coinfection with human immunodeficiency virus; treated with antiviral or immunosuppressive agents within 6 months of enrollment; treated with drugs known to produce hepatic steatosis, including corticosteroids, high dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; a history of gastrointestinal bypass surgery.

BMI was calculated using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as a BMI $\geq$25, according to the criteria of the Japan Society for the Study of Obesity [20]. Diabetes was defined as a fasting glucose level $\geq$126 mg/dl or by the use of insulin or oral hypoglycemic agents to control blood glucose. The ongoing alcohol intake per week recorded and converted to average grams per day. Significant alcohol intake was defined as consumption of >20 g/day.

The Ethics Committee of the Kyoto Prefectural University of Medicine approved this study. Informed consent was obtained from each patient in accordance with the Helsinki declaration.

Laboratory tests

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), total cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using the standard clinical chemistry techniques. The HCV genotype was determined according to the classification of Simmonds et al. [21]. The serum HCV-RNA level was quantified by Amplicor HCV monitor assay (version 2.0; Roche). These clinical and laboratory data were collected at the time of liver biopsy.

Histopathological examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes and divided into two parts. One part was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and silver impregnation. The sections were analyzed by an experienced hepatologist (T.O.) who was blinded to the laboratory parameters and clinical data. The degrees of inflammation and fibrosis were evaluated according to the criteria proposed by Desmet et al. [22]. Steatosis was graded based on percent of hepatocytes in the biopsy involved: none (0%), mild (<33%), moderate (33–66%), or severe (>66%) [23, 24]. The other part of the liver

biopsy was frozen immediately in liquid nitrogen and stored at –80°C for mRNA analysis.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

We quantified mRNA by real-time fluorescence detection. Total RNA was obtained using an RNeasy Kit (Qiagen, Tokyo, Japan). Residual genomic DNA was removed and single-stranded complementary DNA was generated using a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time quantitative RT-PCR experiments were performed with the LightCycler system using Faststart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The 18 genes chosen for the current study, their protein products, and the primer sequences for amplifying them are listed in Table 1. The primers were designed using Primer3 version 0.4 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *ACTB* (β-actin gene) was used as an endogenous control.

Immunohistochemistry

Immunohistochemical staining for PPARα and SREBP1 was performed on formalin-fixed, paraffin-embedded sections from 100 liver biopsy specimens using rabbit polyclonal antibodies against human PPARα (clone H-98; Santa Cruz Biotechnology, Santa Cruz, CA) and SREBP1 (clone K-10; Santa Cruz Biotechnology), respectively. Deparaffinized sections were microwaved in a citrate buffer (pH 6.0) for 20 min. After blocking the endogenous peroxidase, the sections were incubated for 90 min at room temperature with 1:100 anti-PPARα or anti-SREBP1 antibodies. The sections were then incubated for 30 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-rabbit immunoglobulin (Histofine Simple Stain Max-Po (Multi); Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated by substituting the primary antibody with nonimmunized rabbit serum. Immunoreactivity was scored according to the intensity of staining as follows: 1+, weak or absent; 2+, moderate; 3+, strong.

Table 1 Genes and primer sequences used for reverse transcription-polymerase chain reaction assays

Function/gene symbol	Alternate symbol	Protein product	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Nuclear receptor				
PPARA	PPARα	Peroxisome proliferator-activative receptor α	ggaaagcccactctgcccct	agtcaccgaggaggggctcga
PPARG	PPARγ	Peroxisome proliferator-activative receptor γ	cattctggcccaccaactttgg	tggagatgcaggctccactttg
NR1H3	LXRα	Liver X receptor α	cgggcttccactacaatgtt	tcaggcggatctgttcttct
RXRA	RXRα	Retinoid X receptor α	tccttctcccaccgctccatc	cagctccgtcttgtccatctg
Fatty acid oxidation				
CPT1A	CPT1	Carnitine palmitoyltransferase 1	catcatcactggcgtgtacc	ttggcgtacatcgttgtcat
ACADS	SCAD	Short chain acyl-CoA dehydrogenase	ctcacgttggggaagaaaga	tgcgacagtcctcaaagatg
ACADM	MCAD	Medium chain acyl-CoA dehydrogenase	ttgagttcaccgaacagcag	agggggactggatattcacc
ACADL	LCAD	Long-chain acyl-CoA dehydrogenase	ttggcaaaacagttgctcac	ctcccacatgtatccccaac
ACADVL	VLCAD	Very long-chain acyl-CoA dehydrogenase	agccgtgaaggagaagatca	tgtgtttgaagccttgatgc
EHHADH	LBP	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	cttcagccctggatgttgat	aaaagaagtgggtgccaatg
HADHA	LCHAD	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	cacctctctgcctgttcctc	ggcaaagatgctgacacaga
ACOX1	AOX	Acly-CoA oxidase	tgatgcgaatgagtttctgc	agtgccacagctgagaggtt
CYP2E1	CYP2E	Cytochrome P450 CYP2E	cccaaaggatatcgacctca	agggtgtcctccacacactc
Intake of fatty acid				
SLC27A5	FATP5	Fatty acid transporor protein 5	acacactcggtgtccctttc	ctacagggcccactgtcatt
Transfer of trigyceride				
MTP	MTP	Microsomal triglyceride transfer protein	catctggcgaccctatcagt	ggccagctttcacaaaagag
Biosynthesis of fatty acid				
SREBF1	SREBP1	Sterol regulatory element-binding protein 1	tgcattttctgacacgcttc	ccaagctgtacaggctctcc
ACACA	ACC	Acetyl CoA carboxylase	gagaactgccctttctgcac	ccaagctccaggcttcatag
FASN	FAS	Fatty acid synthase	ttccgagattccatcctacg	tgtcatcaaaggtgctctcg

Table 2 Patient characteristics

Characteristic	
n	297
Age[a]	58 (20–78)
Male gender (%)	131 (44.9%)
BMI[a]	22.7 (15.6–35.1)
Obesity (%)	76 (25.6%)
Alcohol intake (%)	67 (22.6%)
Diabetes (%)	9 (3.0%)
HCV genotype (%)	
1	212 (71.4%)
2	76 (25.6%)
3	2 (0.7%)
Unknown	7 (2.3%)
HCV-RNA level (KIU/ml)[a]	1100 (5–9400)
Platelet count ($\times 10^4/\mu L$)[a]	17.6 (5.3–37.4)
AST (IU/L)[a]	47 (14–413)
ALT (IU/L)[a]	59 (9–537)
γ-GTP (IU/L)[a]	39 (10–490)
Fasting glucose (mg/dL)[a]	96 (68–223)
Total cholesterol (mg/dL)[a]	173 (19–318)
Triglyceride (mg/dL)[a]	91 (26–930)
Histological activity (%)	
0	3 (1.0%)
1	127 (42.8%)
2	120 (40.4%)
3	47 (15.8%)
Fibrosis (%)	
0	4 (1.3%)
1	100 (33.7%)
2	120 (40.4%)
3	62 (20.9%)
4	11 (3.7%)
Steatosis (%)	
None	126 (42.4%)
Mild (<33%)	163 (54.9%)
Moderate (33–66%)	7 (2.4%)
Severe (>66%)	1 (0.3%)

[a] Median (range)

Table 3 Univariate analysis of factors associated with steatosis

Factors	No steatosis ($n = 126$)	Steatosis ($n = 171$)	*P*
Age[a]	56 (20–78)	59 (27–75)	0.019
Male gender (%)	44 (34.9%)	87 (50.9%)	0.007
BMI[a]	21.8 (16.5–30.7)	23.9 (15.6–35.1)	<0.0001
Alcohol intake (%)	29 (23.0%)	38 (22.2%)	0.89
Diabetes (%)	4 (3.2%)	5 (2.9%)	1.00
HCV genotype (%)			
1	91 (72.2%)	121 (70.8%)	
2	31 (24.6%)	45 (26.3%)	
3	1 (0.8%)	1 (0.9%)	
Unknown	3 (2.4%)	4 (2.4%)	0.78
HCV-RNA level (KIU/ml)[a]	1257 (5–7030)	1063 (5–9400)	0.14
Platelet count ($\times 10^4/\mu L$)[a]	18.4 (5.9–32.7)	17.4 (5.3–37.4)	0.19
AST (IU/L)[a]	36 (15–413)	58 (14–339)	<0.0001
ALT (IU/L)[a]	40 (9–537)	73 (12–509)	<0.0001
γ-GTP (IU/L)[a]	25 (10–298)	56 (12–490)	<0.0001
Fasting glucose (mg/dL)[a]	95 (68–207)	97 (77–223)	0.002
Total cholesterol (mg/dL)[a]	179 (109–285)	171 (104–318)	0.13
Triglyceride (mg/dL)[a]	83 (26–214)	96 (32–930)	<0.0001
Histological activity (%)			
0	2 (1.6%)	1 (0.6%)	
1	72 (57.1%)	55 (32.2%)	
2	42 (33.3%)	78 (45.6%)	
3	10 (7.9%)	37 (21.6%)	<0.0001
Fibrosis (%)			
0	3 (2.4%)	1 (0.6%)	
1	62 (49.2%)	38 (22.2%)	
2	47 (37.3%)	73 (42.7%)	
3	11 (8.7%)	51 (29.8%)	
4	3 (2.4%)	8 (4.7%)	0.001

[a] Median (range)

Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a chi-square test for qualitative factors or a Mann–Whitney *U* test on ranks for quantitative factors with non-equal variance. Logistic regression analysis was used for multivariate analysis. *P* values below 0.05 by two-sided test were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for steatosis. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the 297 patients are summarized in Table 2. Steatosis was present in 171 (57.6%) patients. The grade of steatosis was mild in 163 (54.9%) patients, moderate in 7 (2.4%), and severe in 1 (0.3%).

Table 4 Multivariate analysis of factors independently associated with steatosis

Factors	Odds ratio	95% confidence interval	*P*
Age	1.02	1.00–1.05	0.05
Male gender	0.99	0.51–1.93	0.99
BMI	1.19	1.06–1.33	0.002
AST	1.00	0.98–1.02	0.54
ALT	0.99	0.98–1.00	0.37
γ-GTP	1.01	1.00–1.01	0.005
Fasting glucose	0.99	0.97–1.01	0.37
Triglyceride	1.01	1.00–1.01	0.007
Activity grade A2 or A3	1.81	0.94–3.51	0.07
Fibrosis stage F3 or F4	2.59	1.11–6.02	0.02

Data are from a total of 297 patients

Univariate correlations between variables and steatosis are shown in Table 3. Patients with steatosis, as compared to patients without steatosis, were older, more often male, had a higher BMI, higher AST, ALT, γ-GTP, fasting glucose, and triglyceride levels, a higher histological activity grade, and a higher fibrosis stage. Multivariate analysis revealed that the BMI, levels of γ-GTP and triglyceride, and fibrosis stage correlated independently with the presence of steatosis (Table 4).

To determine whether HCV has a direct effect on steatosis, we next analyzed a subgroup of patients lacking known metabolic causes of steatosis. Patients with obesity, diabetes, or ongoing alcohol intake were excluded. From the remaining 173 patients, we selected 100 patients whose liver RNA was available for gene expression analyses. There was no difference in clinicopathological characteristics between these 100 patients and the remaining 73 patients whose liver RNA was not available (data not shown). Steatosis was present in 43 (43%) of these 100 patients (Table 5). The presence of steatosis was associated with higher levels of AST, ALT, and γ-GTP, higher fasting glucose levels, and a higher fibrosis stage (Table 5).

To investigate the molecular mechanisms underlying HCV-related steatosis, we examined the expression of 18 genes regulating lipid metabolism in the liver (Table 1) using liver tissues derived from the 100 patients without obesity, diabetes, or ongoing alcohol intake. Real-time quantitative RT-PCR revealed that the expression of 10 genes (*PPARA*, *NR1H3*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, *CYP2E1*, *SLC27A5*, and *ACACA*) were significantly lower in patients with steatosis than in patients without steatosis (Fig. 1). There was no difference in the expression of the other 8 genes, including *SREBF1*, between the two groups.

To determine whether the protein levels corresponded with the mRNA levels, we performed immunohistochemistry

Table 5 Univariate analysis of factors associated with steatosis in patients without obesity, diabetes, or alcohol intake

Factors	No steatosis ($n = 57$)	Steatosis ($n = 43$)	*P*
Age[a]	56 (30–77)	60 (27–73)	0.12
Male gender (%)	15 (26.3%)	12 (27.9%)	0.86
BMI[a]	21.4 (17.0–24.8)	22.0 (17.8–24.9)	0.34
HCV genotype (%)			
1	39 (68.4%)	30 (69.8%)	
2	18 (31.6%)	13 (30.2%)	
3	0 (0%)	0 (0%)	
Unknown	0 (0%)	0 (0%)	0.89
HCV-RNA level (KIU/mL)[a]	1510 (5–7030)	1110 (5–5100)	0.60
Platelet count ($\times 10^4/\mu L$)[a]	19.8 (9.8–31.1)	17.3 (5.9–32.7)	0.06
AST (IU/L)[a]	31 (15–138)	61 (15–131)	<0.0001
ALT (IU/L)[a]	32 (12–175)	73 (14–290)	<0.0001
γ-GTP (IU/L)[a]	22 (10–137)	47 (12–151)	<0.0001
Fasting glucose (mg/dL)[a]	95 (75–112)	99 (79–121)	0.029
Total cholesterol (mg/dL)[a]	180 (120–281)	171 (119–300)	0.76
Triglyceride (mg/dL)[a]	86 (26–209)	88 (44–178)	0.23
Histological activity (%)			
0	1 (1.7%)	1 (2.3%)	
1	33 (58.0%)	14 (32.6%)	
2	19 (33.3%)	20 (46.5%)	
3	4 (7.0%)	8 (18.6%)	0.06
Fibrosis (%)			
0	1 (1.8%)	1 (2.3%)	
1	30 (52.6%)	10 (23.3%)	
2	20 (35.1%)	18 (41.9%)	
3	6 (10.5%)	13 (30.2%)	
4	0 (0%)	1 (2.3%)	0.018

[a] Median (range)

for PPARα (encoded by *PPARA*) and SREBP1 (*SREBF1*) proteins in liver biopsy tissues from the same 100 patients. We chose these two proteins because they are key regulators of lipid degradation and lipid synthesis, respectively. The results are summarized in Table 6, and representative images are shown in Fig. 2a. PPARα was expressed in hepatocytes. Its expression was mainly observed in the nuclei. SREBP1 was expressed in the cytoplasm of hepatocytes. Levels of PPARα and SREBP1 proteins tended to correlate with levels of *PPARA* and *SREBF1* mRNA, respectively (Fig. 2b). As shown in Table 6, the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis

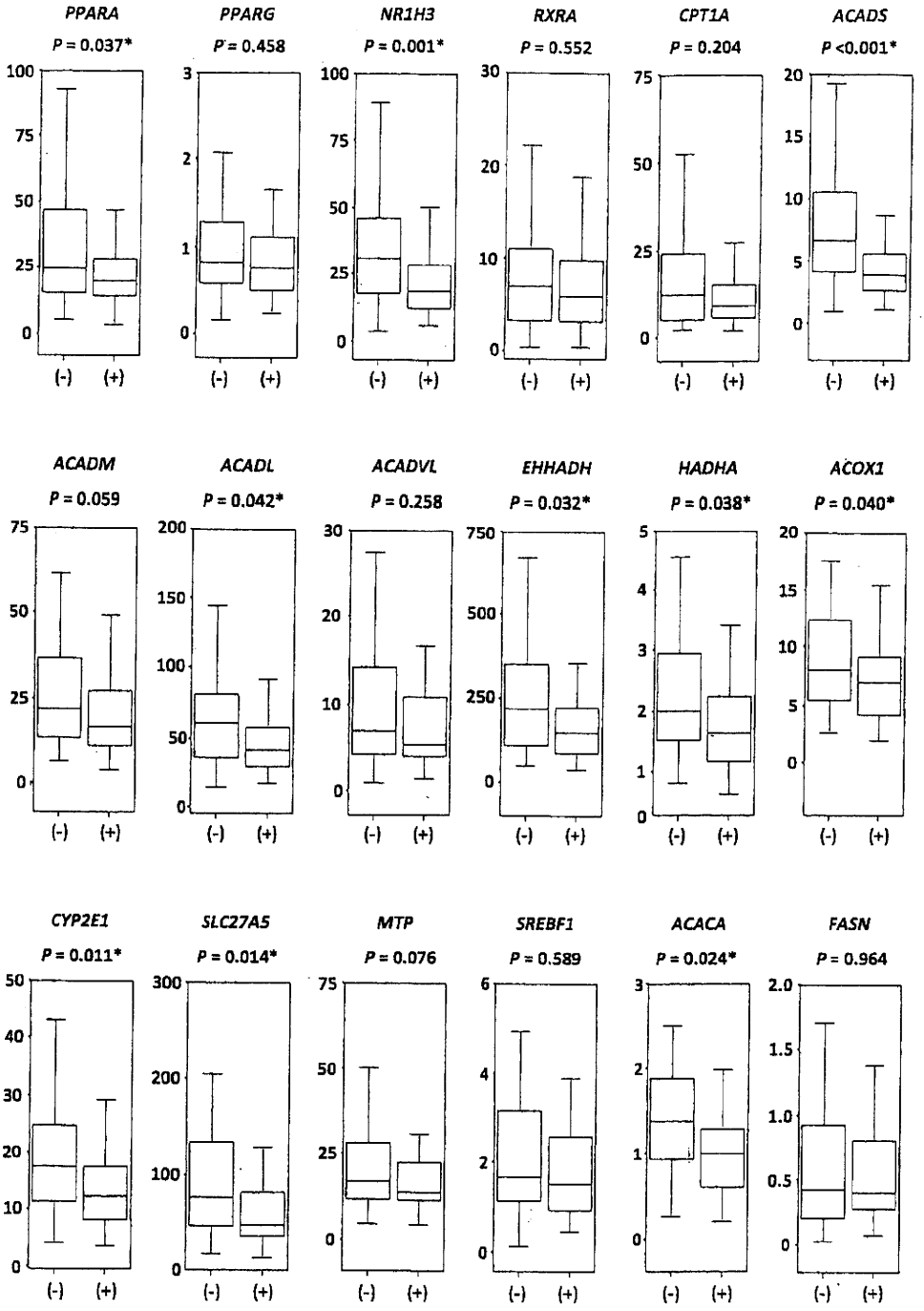

Fig. 1 Relative expression levels of 18 genes (see Table 1) in liver tissues from 57 patients without steatosis (−) and 43 patients with steatosis (+). Gene expression was evaluated by real-time quantitative RT-PCR. Results are presented relative to the expression of a reference gene (*ACTB*) to correct for variation in the amount of RNA in the RT-PCR. The box contains the values between the 25th and 75th percentiles, and the horizontal line is the median; the error bars stretch from the 10th to 90th percentiles. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

($P = 0.017$). On the other hand, the presence of the SREPB1 protein was not associated with the steatosis. These findings agree with those from our analyses of *PPARA* and *SREBF1* mRNA levels. We also examined the relationship between the levels of PPARα and SREBP1 proteins and the degree of fibrosis (Table 6). The level of the PPARα protein was not associated with the degree of fibrosis. The expression of the SREBP1 protein tended to be higher in patients who had a higher fibrosis stage, although the association was not statistically significant.

Discussion

Our results demonstrated a high prevalence (57.6%) of steatosis among patients with chronic HCV infection in Japan, which confirms previous reports in Europe and the United States [1, 25–28]. The prevalence of steatosis was high (43.0%) even when known factors of steatosis, such as obesity, diabetes, or ongoing alcohol intake, were excluded. Consistent with previous reports [1, 29], the grade of steatosis was mild in most cases.

Table 6 Relationship between the presence of steatosis or the degree of fibrosis and levels of PPARα and SREBP1 proteins in liver tissues from patients without obesity, diabetes, or alcohol intake

	Steatosis			Fibrosis		
	Absent ($n = 57$)	Present ($n = 43$)	P	F1/F2 ($n = 80$)	F3/F4 ($n = 20$)	P
PPARα protein expression						
1+; mild or absent	9	17		20	6	
2+; moderate	38	23		49	12	
3+; strong	10	3	0.017	11	2	0.85
SREBP1 protein expression						
1+; mild or absent	16	6		17	5	
2+; moderate	31	29		52	8	
3+; strong	10	8	0.23	11	7	0.055

Multivariate analysis on the 297 patients with steatosis, including those with metabolic cofactors, revealed that a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis. Previous studies have also observed an association between these clinicopathological factors and steatosis [1]. A recent meta-analysis of patients with chronic HCV infection in Europe, Australia, and the United States showed that steatosis is associated independently with HCV genotype 3, the presence of fibrosis, diabetes, hepatic inflammation, ongoing alcohol intake, a higher BMI, and an older age [5]. Although several studies have shown a significant and independent association between HCV genotype 3 and the presence of steatosis [1], we did not observe this association. This is due to the much lower prevalence of genotype 3 in Japan (<1%) than in Europe (24%) [7] and the United States (14%) [9]. There is some controversy with regard to the influence of steatosis on the progression of fibrosis [1, 3]. Some investigators suggest that steatosis accelerates fibrosis only in genotype 3-infected patients [7, 29, 30], whereas others suggest that there is an association in patients infected with genotype 1 [5, 31]. An analysis using paired liver biopsies revealed that steatosis was the only independent factor predictive of progression of fibrosis [32]. In agreement with a previous study [33], we also found that patients with steatosis had a higher γ-GTP. An increase in serum γ-GTP is associated with hepatic steatosis, central obesity and insulin resistance, and is a marker of metabolic and cardiovascular risk [34–36]. Elevated values of γ-GTP are caused by damage to cellular membranes, cellular regeneration or by enhanced synthesis as a result of induction of the biotransformation enzyme system. However, the mechanisms that explain the contribution of γ-GTP to steatosis have not been fully elucidated.

We analyzed the intrahepatic expression of genes that regulate (i) lipid degradation, (ii) lipid secretion, (iii) lipid synthesis, and (iv) lipid uptake. We then investigated the relationship between these levels and the presence of steatosis. Our experiments included more candidate genes than previous studies [13, 18, 19, 37]. The expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, and *CYP2E1* were lower in patients with steatosis. Immunohistochemistry confirmed that the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis. PPARα, one of the proteins involved in lipid degradation, is a nuclear receptor that controls fatty acid metabolism by regulating the expression of genes encoding enzymes involved in mitochondrial and peroxisomal β-oxidation of fatty acids [38]. Short chain acyl-CoA dehydrogenase (encoded by *ACADS*), long-chain acyl-CoA dehydrogenase (*ACADL*), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (*EHHADH*), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit (*HADHA*), and acyl-CoA oxidase (*ACOX1*) are involved in fatty acid β-oxidation. *CYP2E1* encodes a member of the cytochrome P450 superfamily of enzymes that is involved in microsomal ω-oxidation. Acyl-CoA oxidase is the rate-limiting enzymes of peroxisomal β-oxidation. Also, *EHHADH*, *HADHA* and *ACOX1* are known to be a direct transcriptional target of PPARα [38]. The reduced expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, and *ACOX1* may lead to steatosis through down-regulation of fatty acid β-oxidation. However, not all of the genes regulating β-oxidation were down-regulated in patients with steatosis. For example, carnitine palmitoyl transferase 1 (encoded by *CPT1A*) is the rate-limiting enzymes of mitochondrial β-oxidation, and although *CPT1A* is a transcriptional target of PPARα [38], their expression was not significantly reduced in patients with steatosis.

In agreement with a previous study [13], we also found that the expression of *MTP*, a gene involved in lipid secretion, tended to be lower in patients with steatosis, although the association was not statistically significant. *MTP* is a transcriptional target of PPARα [38]. Because

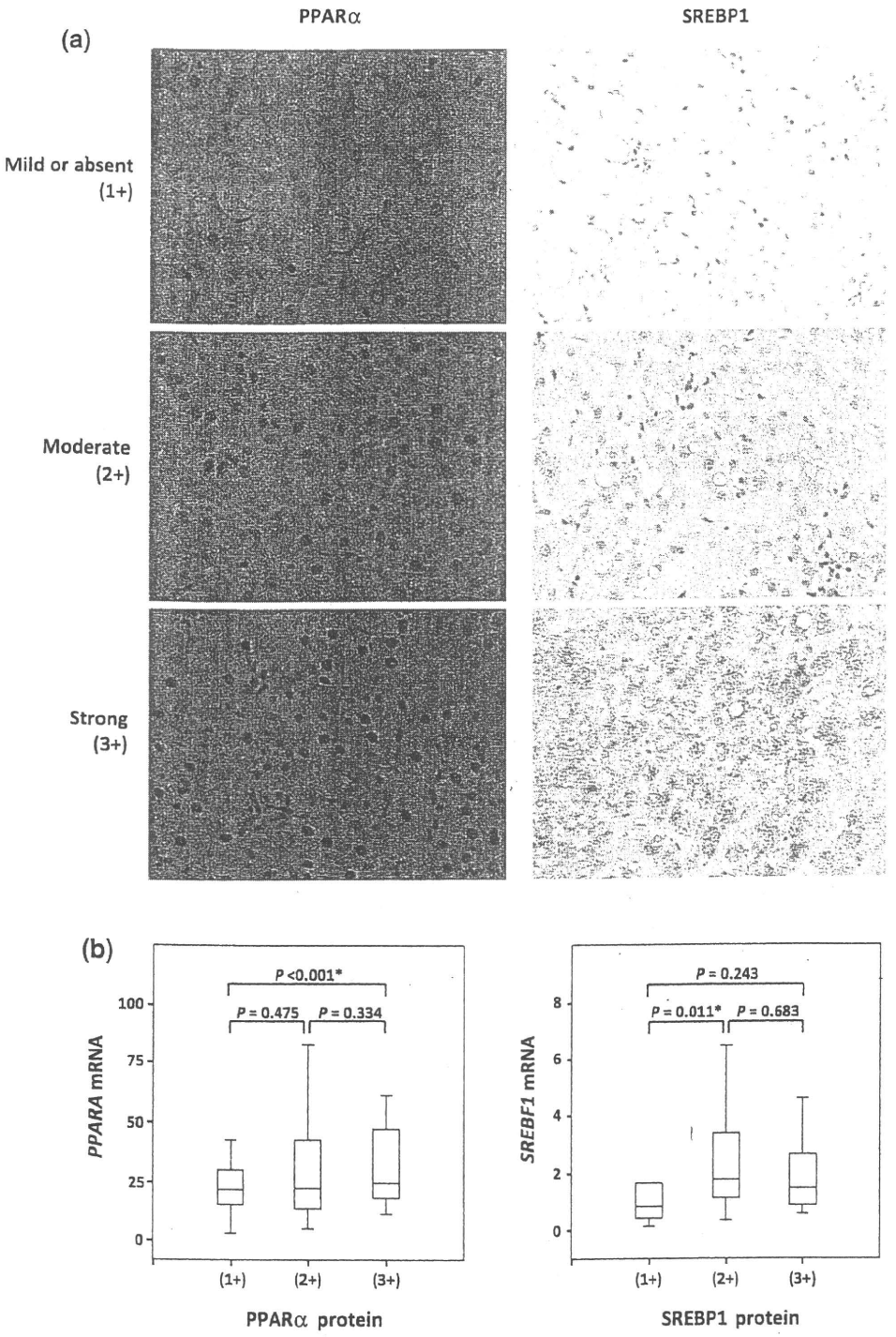

Fig. 2 Immunohistochemistry for PPARα and SREBP1 proteins. **a** Representative images from immunostaining for PPARα and SREBP1 proteins in liver tissues from patients with chronic hepatitis C. Shown are weak or absent staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, ×400. **b** Relationship between relative levels of *PPARA* and *SREBF1* mRNA and proteins. *PPARA* and *SREBF1* mRNA levels were determined as described in Fig. 1. Levels of PPARα and SREBP1 proteins were evaluated as described in **a**. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

microsomal triglyceride transfer protein plays a pivotal role in assembly and secretion of very low density lipoproteins, its reduced expression is expected to result in the increased accumulation of triglycerides (i.e., steatosis).

The nuclear receptor liver X receptor α (encoded by *NR1H3*) is known to promote hepatic lipogenesis by activating SREBP1. SREBP1 increases the transcription of genes involved in hepatic fatty acid synthesis, such as *FASN* (encoding fatty acid synthase) and *ACACA* (acetyl CoA carboxylase), and induces steatosis through increased accumulation of triglyceride. Unexpectedly, the levels of both *SREBF1* mRNA and protein and of *FASN* mRNA were not up-regulated in patients with steatosis. In addition, the expression of *NR1H3* and *ACACA* were lower in patients with steatosis. These findings contradict the idea that the increased expression of genes involved in synthesis of fatty acids leads to steatosis. One possible explanation is that the decreased expression of *NR1H3* and

ACACA compensates for the increased accumulation of triglycerides.

Of the genes involved in lipid uptake, fatty acid transporter protein 5, a liver-specific member of the fatty acid transporter protein family, mediates the uptake of long-chain fatty acids. Unexpectedly, the expression of *SLC27A5* (encoding fatty acid transporter protein 5) was not up-regulated but rather down-regulated in patients with steatosis. Again, this expression could be a compensatory response to increased accumulation of triglyceride.

Further studies are needed to determine the importance of the products of these genes because the limited size of biopsy samples prevented measurement of the enzyme activities. Changes in enzymatic activities of their products are more important for the development of steatosis than changes in their transcriptional levels. Moreover, in vitro studies and mouse models have shown that HCV proteins cause mitochondrial injury, leading to oxidative stress [39–43]. Oxidative stress may inhibit enzymes involved in lipid metabolism, and reactive oxygen species may cause peroxidation of membrane lipids and structural proteins, such as those involved in trafficking and secretion of lipids. Oxidative stress perturbs lipid metabolism, thus contributing to steatosis. It is possible that, instead of a direct effect of HCV proteins on the transcription of genes regulating lipid metabolism, nonspecific inhibition of lipid metabolism through oxidative stress leads to HCV-related steatosis.

In conclusion, a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis in HCV-infected Japanese patients. Thus, the down-regulation of genes involved in fatty acid oxidation may contribute to the development of steatosis in these patients.

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References

1. Asselah T, Bieche I, Narguet S, Sabbagh A, Laurendeau I, Ripault MP, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. Gut. 2008;57:516–24.
2. Clark JM, Brancati FL, Diehl AM. Nonalcoholic fatty liver disease. Gastroenterology. 2002;122:1649–57.
3. Negro F. Mechanisms and significance of liver steatosis in hepatitis C virus infection. World J Gastroenterol. 2006;12:6756–65.
4. Poynard T, McHutchison J, Manns M, Myers RP, Albrecht J. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. Hepatology. 2003;38:481–92.
5. Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, et al. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. Gastroenterology. 2006;130:1636–42.
6. Mihm S, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology. 1997;25:735–9.
7. Rubbia-Brandt L, Quadri R, Abid K, Giostra E, Malé PJ, Mentha G, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol. 2000;33:106–15.
8. Kumar D, Farrell GC, Fung C, George J. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: reversal of hepatic steatosis after sustained therapeutic response. Hepatology. 2002;36:1266–72.
9. Monto A, Alonzo J, Watson JJ, Grunfeld C, Wright TL. Steatosis in chronic hepatitis C: relative contributions of obesity, diabetes mellitus, and alcohol. Hepatology. 2002;36:729–36.
10. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA. 1997;94:1200–5.
11. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol. 1997;78:1527–31.
12. Perlemuter G, Sabile A, Letteron P, Vona G, Topilco A, Chrétien Y, et al. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. FASEB J. 2002;16:185–94.
13. Mirandola S, Realdon S, Iqbal J, Gerotto M, Dal Pero F, Bortoletto G, et al. Liver microsomal triglyceride transfer protein is involved in hepatitis C liver steatosis. Gastroenterology. 2006;130:1661–9.
14. Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA. 2002;99:15669–74.
15. Kim KH, Hong SP, Kim K, Park MJ, Kim KJ, Cheong J. HCV core protein induces hepatic lipid accumulation by activating SREBP1 and PPARgamma. Biochem Biophys Res Commun. 2007;355:883–8.
16. Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, et al. Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. Hepatology. 2002;35:937–46.
17. Yamaguchi A, Tazuma S, Nishioka T, Ohishi W, Hyogo H, Nomura S, et al. Hepatitis C virus core protein modulates fatty acid metabolism and thereby causes lipid accumulation in the liver. Dig Dis Sci. 2005;50:1361–71.
18. Cheng Y, Dharancy S, Malapel M, Desreumaux P. Hepatitis C virus infection down-regulates the expression of peroxisome proliferator-activated receptor alpha and carnitine palmitoyl acyl-CoA transferase 1A. World J Gastroenterol. 2005;11:7591–6.
19. Dharancy S, Malapel M, Perlemuter G, Roskams T, Cheng Y, Dubuquoy L, et al. Impaired expression of the peroxisome proliferator-activated receptor alpha during hepatitis C virus infection. Gastroenterology. 2005;128:334–42.
20. Japan Society for the Study of Obesity. New criteria of obesity (in Japanese). J Jpn Soc Study Obes. 2000;6:18–28.
21. Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, et al. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology. 1994;19:1321–4.
22. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19:1513–20.

23. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94:2467–74.
24. Lefkowitch JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC Jr, et al. Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. The Hepatitis Interventional Therapy Group. Gastroenterology. 1993;104:595–603.
25. Lonardo A, Loria P, Adinolfi LE, Carulli N, Ruggiero G. Hepatitis C and steatosis: a reappraisal. J Viral Hepat. 2006;13:73–80.
26. Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, et al. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol. 2002;37:831–6.
27. Ohata K, Hamasaki K, Toriyama K, Matsumoto K, Saeki A, Yanagi K, et al. Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. Cancer. 2003;97:3036–43.
28. Fujie H, Yotsuyanagi H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, et al. Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. J Med Virol. 1999;59:141–5.
29. Castera L, Chouteau P, Hezode C, Zafrani ES, Dhumeaux D, Pawlotsky JM. Hepatitis C virus-induced hepatocellular steatosis. Am J Gastroenterol. 2005;100:711–5.
30. Westin J, Nordlinder H, Lagging M, Norkrans G, Wejstål R. Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients. J Hepatol. 2002;37:837–42.
31. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallée M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
32. Fartoux L, Chazouillères O, Wendum D, Poupon R, Serfaty L. Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. Hepatology. 2005;41:82–7.
33. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. Hepatology. 2001;33:1358–64.
34. Ikai E, Honda R, Yamada Y. Serum gamma-glutamyl transpeptidase level and blood pressure in nondrinkers: a possible pathogenetic role of fatty liver in obesity-related hypertension. J Hum Hypertens. 1994;8:95–100.
35. Lee DS, Evans JC, Robins SJ, Wilson PW, Albano I, Fox CS, et al. Gamma glutamyl transferase and metabolic syndrome, cardiovascular disease, and mortality risk: the Framingham Heart Study. Arterioscler Thromb Vasc Biol. 2007;27:127–33.
36. Perry IJ, Wannamethee SG, Shaper AG. Prospective study of serum gamma-glutamyltransferase and risk of NIDDM. Diabetes Care. 1998;21:732–7.
37. de Gottardi A, Pazienza V, Pugnale P, Bruttin F, Rubbia-Brandt L, Juge-Aubry CE, et al. Peroxisome proliferator-activated receptor-alpha and -gamma mRNA levels are reduced in chronic hepatitis C with steatosis and genotype 3 infection. Aliment Pharmacol Ther. 2006;23:107–14.
38. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 1999;20:649–88.
39. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res. 2001;61:4365–70.
40. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology. 2002;122:366–75.
41. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology. 2002;122:352–65.
42. Lai MM. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. Gastroenterology. 2002;122:568–71.
43. Asselah T, Rubbia-Brandt L, Marcellin P, Negro F. Steatosis in chronic hepatitis C: why does it really matter? Gut. 2006; 55:123–30.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Hepatic senescence marker protein-30 is involved in the progression of nonalcoholic fatty liver disease

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Abstract
Background Both insulin resistance and increased oxidative stress in the liver are associated with the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Senescence marker protein-30 (SMP30) was initially identified as a novel protein in the rat liver, and acts as an antioxidant and antiapoptotic protein. Our aim was to determine whether hepatic SMP30 levels are associated with the development and progression of NAFLD.
Methods Liver biopsies and blood samples were obtained from patients with an NAFLD activity score (NAS) ≤ 2 ($n = 18$), NAS of 3–4 ($n = 14$), and NAS ≥ 5 ($n = 66$).
Results Patients with NAS ≥ 5 had significantly lower hepatic SMP30 levels (12.5 $\pm$ 8.4 ng/mg protein) than patients with NAS ≤ 2 (30.5 $\pm$ 14.2 ng/mg protein) and patients with NAS = 3–4 (24.6 $\pm$ 12.2 ng/mg protein). Hepatic SMP30 decreased in a fibrosis stage-dependent manner. Hepatic SMP30 levels were correlated positively with the platelet count ($r = 0.291$) and negatively with the homeostasis model assessment of insulin resistance ($r = -0.298$), the net electronegative charge modified-low-density lipoprotein ($r = -0.442$), and type IV collagen 7S ($r = -0.350$). The immunostaining intensity levels of 4-hydroxynonenal in the liver were significantly and inversely correlated with hepatic SMP30 levels. Both serum large very low-density lipoprotein (VLDL) and very small low-density lipoprotein (LDL) levels in patients with NAS ≥ 5 were significantly higher than those seen in patients with NAS ≤ 2, and these lipoprotein fractions were significantly and inversely correlated with hepatic SMP30.
Conclusion These results suggest that hepatic SMP30 is closely associated with the pathogenesis of NAFLD, although it is not known whether decreased hepatic SMP30 is a result or a cause of cirrhosis.

Keywords SMP30 · NAFLD · NASH · Insulin resistance · Oxidative stress

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury throughout the

world [1–3]. It represents a spectrum of conditions characterized histologically by macrovesicular hepatic steatosis, and the diagnosis is made in patients who have not consumed alcohol in amounts sufficient to be considered harmful to the liver.

NAFLD encompasses varying microscopic features that range from simple steatosis, which has a good prognosis, to nonalcoholic steatohepatitis (NASH), which has a poor prognosis. Liver biopsy is recommended as the gold standard for both the diagnosis and staging of fibrosis in patients with NASH [1, 4–6]. Hyperlipidemia, insulin resistance, and oxidative stress can contribute heavily to the initiation and progression of NAFLD [7–9]. However, the exact intricacies of the molecular and cellular mechanisms responsible for the progression from simple steatosis to NASH have not been fully elucidated.

Senescence marker protein-30 (SMP30), a 34-kDa protein originally identified in the rat liver, is a novel molecule that decreases in concentration with aging in an androgen-independent manner [10, 11]. SMP30 transcripts have been detected in a multitude of tissues, and its amino acid alignment reveals a highly conserved structure among humans, rats, and mice [11]. We have reported previously that SMP30 participates in Ca^{2+} efflux by activating the calmodulin-dependent Ca^{2+}-pump in HepG2 cells and renal tubular cells, conferring on these cells resistance to injury caused by high intracellular Ca^{2+} concentrations [12, 13]. Recently, we identified SMP30 as gluconolactonase (GNL), which is involved in L-ascorbic acid biosynthesis in mammals, although human beings are unable to synthesize vitamin C because there are many mutations in the gulonolactone oxidase gene, which catalyzes the conversion from L-gulono-γ-lactone to L-ascorbic acid [14]. To clarify whether a causal relationship exists between a decrease in SMP30/GNL levels and age-associated organ disorders, we established SMP30/GNL knockout (KO) mice [15]. The livers of SMP30/GNL KO mice are highly susceptible to tumor necrosis factor-α (TNF α) and Fas-mediated apoptosis [15]. In addition, they showed mitochondrial damage and abnormal accumulations of triglycerides, cholesterol, and phospholipids [16]. Furthermore, SMP30/GNL in brain and lung tissue appeared to have protective properties against oxidative stress associated with aging [17–20]. Because the SMP30/GNL KO mice showed changes in the liver that mimic the processes of NAFLD, we hypothesized that decreased levels of SMP30 may be linked to the pathogenesis of NAFLD. The purpose of this study was to investigate the role of SMP30 in the pathogenesis of NAFLD.

Patients and methods

Patients

The study protocol was approved by the ethics committee of Saiseikai Suita Hospital and Kyoto Prefectural University of Medicine, and informed consent was obtained from all subjects prior to their enrollment in the study. A total of 98 patients histologically diagnosed as having NAFLD at Saiseikai Suita Hospital or Kyoto Prefectural University Hospital between 2006 and 2008 were enrolled in this study.

All liver biopsy specimens were stained with hematoxylin–eosin and Masson's trichrome stains and examined by two experienced pathologists blinded to the patients' clinical or laboratory data or liver biopsy sequence. Patients with NAFLD were divided into the following groups: simple steatosis and mild NASH (stages 0–1), moderate NASH (stage 2), and advanced NASH (stage 3–4) according to the classification proposed by Brunt et al. [6]. Several liver tissues samples from these groups were embedded in Tissue-Tek OCT (Sakura Finetech, Tokyo, Japan) compound and stained with Oil Red O. The fibrosis staging system was classified as follows: stage 0, no fibrosis; stage 1, zone 3 predominant pericellular fibrosis; stage 2, zone 3 fibrosis plus periportal fibrosis; stage 3, bridging fibrosis; stage 4, cirrhosis. The grade of steatosis was defined as mild ($\leq$33%), moderate (34–65%), or advanced ($\geq$66%). In addition, the NAFLD activity score (NAS) system was used to classify NAFLD into "not NASH" (NAS $\leq$ 2), "borderline NASH" (NAS = 3–4), and "definite NASH" (NAS $\geq$ 5), as shown in Table 1, because the NAS system has been reported as a reliable scoring system for diagnosing NASH [5]. We excluded patients with alcohol intake exceeding 20 g/day and those who reported signs, symptoms, and/or a history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, before evaluation of liver histology.

Immunohistochemistry

Liver biopsy specimens were preserved in 10% formalin and embedded in paraffin. Specimens were serially sectioned onto microscope slides at a thickness of 4 μm and then deparaffinized. After removal of paraffin, the liver sections were heated by microwaving in 0.1 M citrate buffer (pH 7.0), followed by inactivation of endogenous peroxidases by incubation with 1% hydrogen peroxide (H_2O_2) in methanol. The primary antibodies used were monoclonal antibody raised against recombinant human SMP30 (1:2000 dilution) [14] and anti-4-hydroxynoneral

Table 1 Clinical features and laboratory data of three patient groups classified according to NAS scores

	Group A (n = 18) NAS ≤ 2	Group B (n = 14) NAS 3–4	Group C (n = 66) NAS ≥ 5
Male/female	9/9	6/8	34/32
Age (years)	60.9 ± 13.1	53.3 ± 16.8	60.4 ± 12.2
Body mass index (kg/m^2)	26.4 ± 4.8	27.5 ± 4.4	27.5 ± 4.8
Systolic blood pressure (mmHg)	133 ± 18	137 ± 8	140 ± 18
Diastolic blood pressure (mmHg)	77 ± 9	84 ± 12	82 ± 12
HbA1c (%)	6.2 ± 1.6	6.4 ± 1.8	6.3 ± 1.3
Fasting glucose (mg/dL)	116 ± 32	125 ± 41	124 ± 44
Fasting insulin (μU/mL)	8.5 ± 4.9	10.9 ± 4.7	12.7 ± 7.6
HOMA-R	2.9 ± 1.7	3.7 ± 2.1	3.9 ± 2.8*
AST (U/L)	39 ± 12	43 ± 12	44 ± 23
ALT (U/L)	37 ± 11	41 ± 10	53 ± 25
Triglyceride (mg/dL)	160 ± 67	149 ± 72	165 ± 94
Total cholesterol (mg/dL)	221 ± 42	208 ± 36	202 ± 34
HDL cholesterol (mg/dL)	55 ± 22	50 ± 7	49 ± 12
LDL cholesterol (mg/dL)	134 ± 25	119 ± 28	129 ± 30
Oxidized LDL (U/ml)	13.3 ± 2.6	13.8 ± 1.1	14.8 ± 2.2
Electronegative charge modified-LDL (ecd)	3.1 ± 3.0	3.1 ± 3.2	6.4 ± 3.5*
Type IV collagen 7S (ng/dL)	3.9 ± 0.5	4.0 ± 1.2	5.7 ± 1.9*
Platelet count (× 10^4/μL)	21.9 ± 2.9	21.9 ± 4.5	17.1 ± 4.7*
SMP30 in liver tissue (ng/mg protein)	30.5 ± 14.2	24.6 ± 12.2	12.5 ± 8.4**·***
75 g OGTT (NGT/IGT/DM)	4/7/7	3/6/5	13/29/24

Data are expressed as mean ± SD

* $P < 0.05$ versus group A versus group B. ** $P < 0.01$ versus group B. *** $P < 0.001$ versus group A

NAS nonalcoholic fatty liver disease (NAFLD) activity score, *HOMA-R* homeostasis model assessment of insulin resistance, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *ecd* electronegative-charge density, *SMP30* senescence marker protein-30, *OGTT* oral glucose tolerance test, *NGT* normal glucose tolerance, *IGT* impaired glucose tolerance, *DM* diabetes mellitus

(4-HNE) monoclonal antibody (1:100 dilution; Nihon Yushi, Tokyo, Japan). SMP30 and 4-HNE were detected by indirect immunoperoxidase staining using corresponding Histofine Simple Stain MAX-PO kits (Nichirei Biosciences, Tokyo, Japan) and 3, 3-diaminobenzidine (DAB) as a chromogenic substrate. After DAB staining, nuclei were counterstained with Mayer's hematoxylin. Two independent observers evaluated the intensity of immunostaining for 4-HNE as 0, 1, 2, or 3 (negative, weak, moderate, or strong, respectively).

Quantification of hepatic SMP30 content by enzyme-linked immunosorbent assay (ELISA)

A portion of each liver biopsy specimen was immediately frozen and stored at −80°C for hepatic SMP30 measurement. Frozen liver biopsy specimens were suspended in ice-cold phosphate-buffered saline (PBS; pH 7.4). After disruption by homogenization and sonication, samples were centrifuged (15,000 g, 15 min, 4°C) and supernatants were stored at −80°C until assay. SMP30 in supernatant fractions was determined by a sandwich ELISA using a polyclonal anti-SMP30 antibody (Cosmo Bio, Tokyo, Japan) and a monoclonal anti-SMP30 antibody. In brief, microtiter plates were coated with affinity-purified anti-SMP30 rabbit IgG (2.0 μg/ml) diluted with 10 mM carbonate buffer (pH 9.3) for 2 h at room temperature. After washing, nonspecific binding sites in each well were blocked with 10 mM carbonate buffer containing 0.5% bovine serum albumin (BSA). Standard solution (0–2,000 pg/ml recombinant SMP30) and supernatant samples diluted (1:10) with sample buffer (50 mM Tris–HCl buffer, pH 7.0, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, and 1% BSA) were added to the wells, and the plate was incubated for 2 h at room temperature. After a washing with BSA-free sample buffer, biotinylated anti-monoclonal SMP30 antibody was added to each well. The plate was incubated for 2 h at room temperature, washed, and then incubated for an additional 2 h at room temperature with streptavidin-horseradish peroxidase (HRP) diluted 1:10,000 (Vector Laboratories, Burlingame, CA, USA). After a final washing, the plate was treated for 20 min with

a substrate solution of 3,3′,5,5′-tetramethylbenzidine and H_2O_2 was added to each well and allowed to react for 15 min at room temperature. The reaction was stopped by the addition of 1 M phosphoric acid, after which optical density (OD) values at 450 nm were read with an ELISA plate reader. The detection limit of the assay was 20 pg/ml and the intra- and interassay coefficients of variation were 6.4 and 8.2% at 50 pg/ml and 4.6 and 7.0% at 500 pg/ml, respectively. The concentration of SMP30 in liver tissue was expressed based on milligrams of total protein. The protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) with human serum albumin as a standard.

Laboratory investigations

Blood samples were obtained in the morning after an overnight fast. Plasma glucose was measured by the glucose oxidase method and HbA1c was determined by high-performance liquid chromatography (HPLC; Arkray, Kyoto, Japan). Serum insulin (immunoreactive insulin; IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott Japan, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and glucose levels by the following equation: HOMA-R = fasting IRI (mU/ml) × fasting plasma glucose (PG) (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch), and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi, Tokyo, Japan). Serum type IV collagen 7S was measured with a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan). Serum oxidized LDL (oxLDL) was measured with an ELISA kit (Kyowa Medex, Tokyo, Japan).

The net electronegative charge modified-LDL (emLDL) was analyzed using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System; Helena Labs, Saitama, Japan). The percentage frequency of emLDL was calculated on a computer from the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera, according to the following formula: emLDL density = $[b - a/a] \times 100\%$. The intraassay coefficient of variation in this method was <1%. In our preliminary study, the value of emLDL in normal healthy subjects ($n = 45$, mean age 46.5 ± SD 3.9 years) was 0.3 ± 2.6% (unpublished data). Serum lipoproteins were also analyzed by an HPLC system according to the procedure described by Okazaki et al. [21], while lipoprotein particle size was determined based on individual elution times that corresponded to peaks on the chromatographic pattern of cholesterol fractions. In this study, we defined very low-density lipoprotein (VLDL), LDL, and HDL subclasses according to lipoprotein particle size, expressed as diameter [22].

Statistical analysis

All statistical analyses were performed with Statview version 5.0 (Abacus Concepts, Berkeley, CA, USA), with data expressed as mean ± SD. When the data were not normally distributed, logarithmic transformation was performed. Differences between the groups were determined by Student's t test or one-way analysis of variance (ANOVA) with Scheffé's multiple comparison test. Categorical data were assessed by the χ^2 test. The degree of correlation between selected variables was determined by Pearson's correlation analysis or Spearman's correlation analysis. The relationship between hepatic SMP30 levels and other clinical parameters was also analyzed by stepwise multiple regression analysis using forward direction, with the F value for entry set at 4.0. A P value of <0.05 was considered statistically significant.

Results

The clinical, biochemical, and laboratory data of the three patient groups classified by NAS score are summarized in Table 1. Patients with NAS ≥ 5 had significantly lower hepatic SMP30 levels than patients with NAS ≤ 2 ($P < 0.001$) and patients with NAS of 3–4 ($P < 0.01$). Patients with NAS ≥ 5 had significantly higher HOMA-R ($P < 0.05$), serum emLDL ($P < 0.05$), and serum type IV collagen 7S ($P < 0.05$) and had lower platelet counts ($P < 0.05$) than patients with NAS ≤ 2 or patients with NAS of 3–4. There was no significant difference in the other clinical and laboratory data among the three patient groups.

Hepatic SMP30 levels were significantly and positively correlated with platelet count ($P < 0.05$), and were significantly and inversely correlated with HOMA-R ($P < 0.05$), serum emLDL ($P < 0.01$), and serum type IV collagen 7S ($P < 0.01$; Table 2). Stepwise multiple regression analysis also showed that hepatic SMP30 levels were associated with HOMA-R ($F = 4.08$) emLDL ($F = 11.19$), and type IV collagen 7S ($F = 5.23$; Table 2).

As shown in Fig. 1A, immunohistochemical staining showed strong expression of SMP30 protein in parenchymal cells in liver tissue from patients with simple steatosis (Fig. 1A-a) compared with liver tissue from patients with mild NASH (stages 0–1; Fig. 1A-b), moderate NASH (stage 2; Fig. 1A-c), and advanced NASH (stages 3–4; Fig. 1A-d). The level of hepatic SMP30 was significantly higher in patients with simple steatosis (28.5 ± 9.5 ng/mg

Table 2 Pearson's correlation and stepwise multiple regression analysis of the relationship between hepatic SMP-30 and 18 clinical variables

	Pearson's correlation	Stepwise multiple regression	
	r	*β*	*F*
Age	−0.111	–	–
Body mass index	−0.116	–	–
Systolic blood pressure	−0.034	–	–
Diastolic blood pressure	−0.040	–	–
HbA1c (%)	0.026	–	–
Fasting glucose	−0.201	–	–
Fasting insulin	−0.158	–	–
HOMA-R	−0.298*	−0.243	4.08*
AST	−0.127	–	–
ALT	−0.190	–	–
Triglyceride	−0.175	–	–
Total cholesterol	0.026	–	–
HDL cholesterol	0.031	–	–
LDL cholesterol	−0.158	–	–
Oxidized LDL (U/ml)	−0.241	–	–
Electronegative charge modified-LDL	−0.442**	−0.380	11.19**
Type IV collagen 7S	−0.350**	−0.260	5.23*
Platelet count	0.291*	–	–

* $P < 0.05$, ** $P < 0.01$

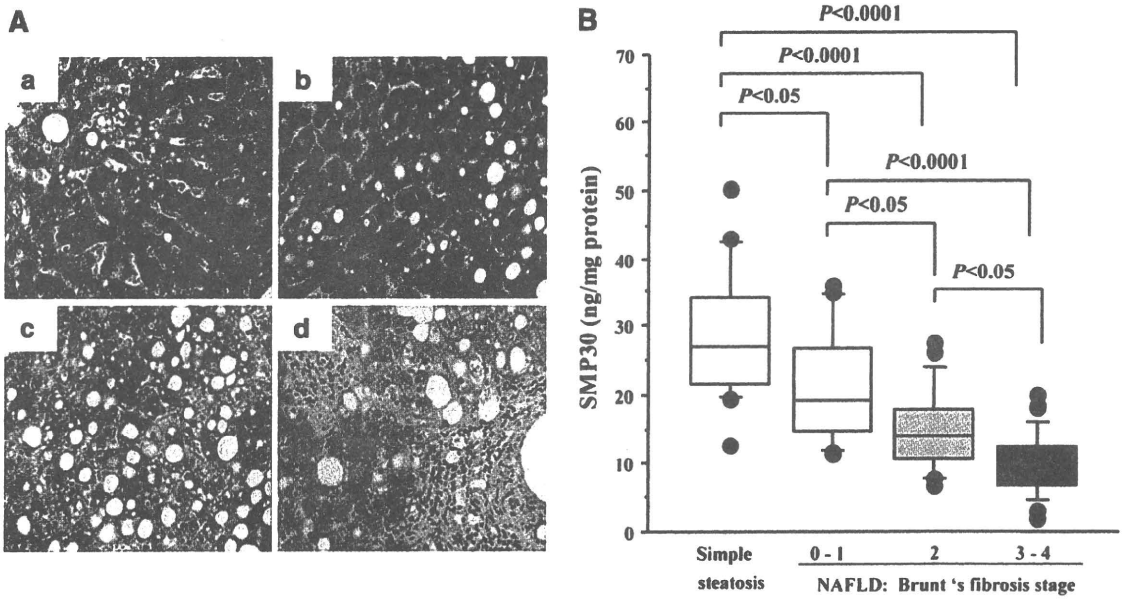

Fig. 1 **A** Immunostaining of senescence marker protein-30 (*SMP30*) in liver tissue from patients with *a* simple steatosis, *b* mild nonalcoholic steatohepatitis (NASH; stages 0–1), *c* moderate NASH (stage 2), and *d* advanced NASH (stage 3–4), ×400. **B** Hepatic SMP30 levels in patients with simple steatosis, mild NASH, moderate NASH, or advanced NASH. The *box plots* include the medians (*horizontal lines*) and interquartile ranges (*boxes*), whereas the *whiskers* represent the 10–90th percentiles, and the *dots* represent the 5–95th percentiles and 1–99th percentiles, respectively. *NAFLD* nonalcoholic fatty liver disease

protein) compared with that in patients with NASH (vs. mild; 21.2 ± 7.9, $P < 0.05$, vs. moderate; 14.9 ± 5.9, $P < 0.001$, and vs. advanced; 9.6 ± 4.6, $P < 0.001$), and was observed as decreasing in a stage-dependent manner (Fig. 1B).

The grade of fatty change evaluated with hematoxylin-eosin staining (Fig. 2a) was correlated well with the intensity of Oil Red O staining (Fig. 2b).

In this study, to estimate oxidative stress in liver tissue, we investigated the expression of 4-HNE, a marker of