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Original Article

Lower circulating levels of dehydroepiandrosterone, independent of insulin resistance, is an important determinant of severity of non-alcoholic steatohepatitis in Japanese patients

Yoshio Sumida,[1] Yoshikazu Yonei,[2] Kazuyuki Kanemasa,[1] Tasuku Hara,[1] Yutaka Inada,[1] Kyoko Sakai,[1] Shunsuke Imai,[3] Sawako Hibino,[4] Kanji Yamaguchi,[5] Hironori Mitsuyoshi,[5] Kohichiroh Yasui,[5] Masahito Minami,[5] Yoshito Itoh,[5] Yuji Naito,[5] Toshikazu Yoshikawa[5] and Takeshi Okanoue[6]

[1]Center for Digestive and Liver Diseases, [3]Department of Pathology, Nara City Hospital, Nara, [2]Anti-Aging Medical Research Center, Graduate School of Life and Medical Science, Doshisha University, Kyotanabe, [4]Anti-Aging Medical Science, Division of Basic Research, Louis Pasteur Center for Medical Research, [5]Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, and [6]Hepatology Center, Saiseikai Suita Hospital, Suita, Japan

Aim: The biological basis of variability in histological progression of non-alcoholic fatty liver disease (NAFLD) remains unknown. Dehydroepiandrosterone (DHEA), the most abundant steroid hormone, has been shown to influence sensitivity to reactive oxygen species, insulin sensitivity and expression of peroxisome proliferator-activated receptor-α. Our aim was to determine whether more histologically advanced NAFLD is associated with low circulating levels of DHEA in Japanese patients.

Methods: Serum samples were obtained in 133 Japanese patients with biopsy-proven NAFLD and in 399 sex- and age-matched healthy people undergoing health checkups. Serum levels of sulfated DHEA (DHEA-S) were measured by chemiluminescent enzyme immunoassay.

Results: Serum DHEA-S levels in NAFLD patients were similar to those in the control group. Of 133 patients, 90 patients were diagnosed as non-alcoholic steatohepatitis (NASH): 73 patients had stage 0–2, and 17 had stage 3 or 4. Patients with advanced NAFLD (NASH with fibrosis stage 3 or 4) had lower plasma levels of DHEA-S than patients with mild NAFLD (simple steatosis or NASH with fibrosis stage 0–2). The area under the receiver operating characteristic curve for DHEA in separating patients with and without advanced fibrosis was 0.788. A "dose effect" of lower DHEA-S and incremental fibrosis stage 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, respectively. The association between DHEA-S and severity of NAFLD persisted after adjusting for age, sex and insulin resistance.

Conclusion: Low circulating DHEA-S might have a role in the development of advanced NASH.

Key words: fibrosis, dehydroepiandrosterone, insulin resistance, non-alcoholic fatty liver disease.

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is the most common chronic liver disease in many developed countries and results in a serious public health problem worldwide. NAFLD includes a wide spectrum of liver diseases, ranging from simple steatosis (SS), which is usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in the absence of significant alcohol consumption.[1–3] In Japan, current best estimates make the prevalence of NAFLD approximately 20% and of NASH 2–3% in the general population.[4,5] Although several factors have been associated with more advanced NAFLD, the biological basis of the histological diversity of severity of NAFLD (i.e. why some patients develop

Correspondence: Dr Yoshio Sumida, Center for Digestive and Liver Diseases, Nara City Hospital, 1-50-1 Higashi Kidera-cho, Nara 630-8305, Japan. Email: sumida@nara-jadecom.jp
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SS and others develop NASH with advanced fibrosis) remains unknown. More advanced NAFLD is characterized by insulin resistance,[6,7] oxidative stress[8,9] and advanced fibrosis.

Endocrine hormones control cell metabolism and the distribution of body fat and therefore may contribute to the development of NAFLD or NASH. It has been postulated that dehydroepiandrosterone (DHEA) and its sulfate ester, dehydroepiandrosterone sulfate (DHEA-S), the major secretory products of the human adrenal gland, may be discriminators of life expectancy and aging.[10] DHEA-S concentration is independently and inversely related to death from any cause and death from cardiovascular disease in men over the age of 50 years.[11] DHEA is a potential mediator of reactive oxygen species scavenger synthesis[12] and has also been reported to augment insulin sensitivity[13–16] and peroxisome proliferator activation.[17,18] Recently, Charlton *et al.* observed that levels of DHEA are significantly lower in patients with histologically advanced NASH, as compared with patients with mild NASH or SS.[19] DHEA levels exert a good sensitivity and specificity in discriminating patients with more advanced histological disease, as shown by receiver–operator curve (ROC) analysis.

To validate their results, we determined circulating DHEA levels in Japanese patients with biopsy-proven NAFLD.

METHODS

Patients

A TOTAL OF 133 patients with well-characterized and liver biopsy-confirmed NAFLD were included in this study. They were consecutively biopsied patients seen at the Center for Digestive and Liver Diseases, Nara City Hospital during 2007–2009. The diagnosis of NAFLD was based on the following criteria: (i) persistent elevations of transaminase activities for more than 6 months; (ii) liver biopsy showing steatosis in at least 5% of hepatocytes;[20] and (iii) appropriate exclusion of liver diseases of other etiology including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis (PBC), biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin-deficiency-associated liver disease. Patients consuming more than 20 g alcohol/day and patients with evidence of decompensated LC or HCC were excluded from the present study. Written informed consent was obtained from all patients at the time of their liver biopsy, and the study was conducted in conformance with the Helsinki Declaration. In addition, 399 sex- and age-matched healthy people participating in health checkups who showed normal levels of alanine aminotransferase (ALT) levels (≤30 IU/L) were also enrolled as the control group.

Clinical laboratory parameters

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation in all patients included a blood cell count and the measurement of aspartate aminotransferase (AST), ALT, γ-glutamyltransferase (γGT), cholinesterase (ChE), total cholesterol, triglyceride, albumin, fasting plasma glucose (FPG), immunoreactive insulin (IRI), free fatty acid (FFA), ferritin levels, hyaluronic acid and type IV collagen 7S. These parameters were measured using the standard techniques of clinical chemistry laboratories. Body mass index (BMI) was calculated using the following formula: weight in kilograms / (height in meters).[2] Obesity was defined as a BMI greater than 25, according to the criteria of the Japan Society for the Study of Obesity.[21] Patients were assigned a diagnosis of diabetes mellitus (DM) if a documented use of oral hypoglycemic medication, a random glucose level in excess of 200 mg/dL or an FPG greater than 126 mg/dL was present.[22] Dyslipidemia was diagnosed if the cholesterol level was higher than 220 mg/dL and/or the triglyceride level was over 160 mg/dL. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure of greater or equal to 140/90 mmHg on at least two occasions.

Sulfated DHEA concentrations were measured by chemiluminescent enzyme immunoassay (CLEIA). Serum DHEA-S levels of the control group were determined in the Anti-Aging Medical Research Center, Graduate School of Life and Medical Science, Doshisha University, Kyoto, Japan. The Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR) was calculated on the basis of fasting values of plasma glucose and insulin according to the HOMA model formula: HOMA-IR = IRI (μU/mL) × FPG (mg/dL) / 405.[23] Quantitative insulin sensitivity check index (QUICKI) = 1 / (log fasting IRI [μU/mL] + log FPG [mg/dL]).[24]

Histological evaluation

All patients enrolled in this study underwent a percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin–eosin, Masson trichrome and reticulin

Table 1 Characteristics of NAFLD patients and control group

Parameters	NAFLD	Control	*P*-value
n	133	399	
Sex (female)	70 (53%)	210 (53%)	Matched
Age (year)	55.2 (15.4)	55.6 (12.1)	0.7990
BMI (kg/m^2)	27.9 (4.9)	23.4 (3.4)	<0.0001
Obesity (BMI > 25 kg/m^2)	98 (74%)	109 (27%)	<0.0001
AST (IU/L)	58.0 (33.0)	21.7 (4.9)	<0.0001
ALT (IU/L)	85.6 (51.7)	19.4 (5.4)	<0.0001
γGT (IU/L)	82.8 (73.0)	33.1 (28.8)	<0.0001
Cholesterol (mg/dL)	207.9 (41.2)	215.6 (34.9)	0.0572
Triglyceride (mg/dL)	179.1 (96.3)	109.0 (87.8)	<0.0001
HDL-C (mg/dL)	52.0 (24.7)	63.8 (16.6)	<0.0001
FPG (mg/dL)	103.5 (38.9)	97.5 (15.7)	0.0131
IRI (μU/mL)	14.70 (9.46)	5.57 (4.17)	<0.0001
HOMA-IR	3.93 (3.83)	1.37 (1.09)	<0.0001
QUICKI	0.33 (0.03)	0.38 (0.04)	<0.0001
DHEA-S (μg/dL)	128.7 (111.2)	113.6 (91.8)	0.1578

P-values were calculated by Student's *t*-test or χ^2-test analysis.
Results are presented as numbers with percentages in parenthesis for qualitative data or as means with standard deviation in parenthesis for quantitative data.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DHEA-S, dehydroepiandrosterone sulfate; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; IRI, immunoreactive insulin; NAFLD, non-alcoholic fatty liver disease; QUICKI, Quantitative insulin sensitivity check index; γGT, gamma glutamyl transpeptidase.

silver stain. A pathologist (S. I.) who was blinded to the clinical data reviewed the liver biopsy specimens. Adequate liver biopsy sample was defined as biopsy specimen length greater than 1.5 cm and/or having more than six portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration with or without Mallory–Denk body or fibrosis.[2,3] Patients whose liver biopsy specimens showed steatosis, or steatosis with non-specific inflammation, were identified as the SS cohort.[2,3] The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis.[25] Scoring of steatosis included both microvesicular and macrovesicular steatosis and was based on the percentage area of the parenchyma that was fatty. Mild was considered less than 33%, moderate 33–65% and advanced if greater than 66% was observed.[20]

Statistical analysis

Results are presented as the means and standard deviation (SD) for quantitative data or as numbers with percentages in parentheses for qualitative data. Statistical differences in quantitative data were determined using the Student's *t*-test (Table 1). Statistical differences among three groups for quantitative data were determined by one-way ANOVA with Scheffe's post-hoc test (Table 3). Fisher's exact probability test or χ^2-test analysis was used for qualitative data (Tables 1,3). Correlation coefficients were calculated by using Spearman's rank correlation analysis (Table 2). Multivariate analysis was performed by logistic regression analysis to identify variables independently associated with advanced stage of NASH (Table 4). To assess the accuracy of clinical scoring system in differentiating NASH from SS or advanced NAFLD from mild NAFLD, we calculated the sensitivity and the specificity for each value of each test and then constructed ROC by plotting the Se against the reverse Sp (1 – Sp) at each value (Fig. 1). The diagnostic performance of scoring systems was assessed by analysis of ROC. The most commonly used index of accuracy is the area under the ROC (AUC), with values close to 1.0 indicating high diagnostic accuracy (Table 4). The Youden index was used to identify the optimal cut-off points. Differences were considered statistically significant at all $P < 0.05$.

Table 2 Correlation between serum DHEA-S and clinical parameters in 133 patients with biopsy-proven NAFLD

Variables	Correlation coefficient	*P*-value
Age	−0.6982	<0.0001
Hemoglobin	0.4859	<0.0001
Platelet	0.3475	<0.0001
AST	−0.1988	0.0218
ALT	0.1733	0.0460
AST : ALT ratio	−0.5847	<0.0001
γGT	−0.0580	0.5092
Cholinesterase	0.3827	<0.0001
Albumin	0.4165	<0.0001
Prothrombin time	0.0767	0.4029
Cholesterol	0.1525	0.0820
Triglyceride	0.2037	0.0206
HDL-C	−0.2016	0.0033
FPG	−0.1386	0.1158
IRI	−0.0208	0.8138
HOMA-IR	−0.0545	0.5379
QUICKI	0.0545	0.5379
Free fatty acid ($n = 121$)	−0.1023	0.2644
Ferritin	0.0037	0.9666
Hyaluronic acid	−0.6408	<0.0001
Type IV collagen 7 s	−0.4477	<0.0001

P-values are based on Spearman's non-parametric correlation analysis.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; DHEA-S, dehydroepiandrosterone sulfate; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; IRI, immunoreactive insulin; NAFLD, non-alcoholic fatty liver disease; QUICKI, Quantitative insulin sensitivity check index; γGT, gamma glutamyl transpeptidase.

RESULTS

Patient demographics

TABLE 1 SUMMARIZES the clinical, laboratory and liver biopsy data of the patient population and the control group. NAFLD patients were predominantly obese, had higher levels of transaminase activities, γGT, triglyceride, FPG, IRI and insulin resistance, and had lower levels of high-density lipoprotein cholesterol (HDL-C). Serum levels of DHEA-S in NAFLD patients were not different from those in sex- and age-matched controls. In both groups, there were significant sex differences in serum levels of DHEA-S (control group, male 154.4 ± 102.1 vs female 76.8 ± 59.6 μg/dL, $P < 0.0001$; NAFLD group, male 186.7 ± 129. 2 vs female 76.5 ± 53.1 μg/dL, $P < 0.0001$).

Of 133 NAFLD patients involved in this study, 90 patients (68%) were histologically diagnosed as NASH, and 43 patients (32%) were SS. NASH patients were significantly older, predominantly female, hypertensive, more likely to have DM, had lower levels of hemoglobin (Hb), platelet count, albumin, cholinesterase and QUICKI, and had higher levels of AST, ALT, IRI, hyaluronic acid, type IV collagen 7S and HOMA-IR. Patients with NASH had lower levels of DHEA-S (108.8 ± 96.1 μg/dL) than those with SS (170.4 ± 129.2 μg/dL, $P = 0.003$). The AUC for DHEA in separating patients with and without NASH was 0.678 (Fig. 1a). The sensitivity of a DHEA-S-value of 99 μg/dL or less for the presence of NASH was 62.2% (56/90) and specificity was 67.4% (29/43).

Correlation between DHEA-S and other clinical variables in NAFLD patients

Levels of DHEA-S were positively correlated with Hb, platelet, ALT, cholinesterase, albumin and triglyceride, and negatively correlated with age, AST, AST/ALT ratio, ALP, HDL-C, hyaluronic acid and type IV collagen 7S. They had no associations with markers of insulin resistance such as HOMA-IR and QUICKI (Table 2). Serum DHEA-S levels were not different between patients with HOMA-IR of more than 2.5 ($n = 73$, 125.6 ± 116.0 μg/dL) and with HOMA-IR of less than 2.5 ($n = 57$, 134.4 ± 107.9 μg/dL, $P = 0.660$). Similarly, serum DHEA-S levels were not different between patients with QUICKI of more than 0.3 ($n = 102$, 123.7 ± 114.4 μg/dL) and with QUICKI of less than 0.3 ($n = 26$, 114.8 ± 108.2 μg/dL, $P = 0.448$).

Comparison between participants with simple steatosis, and mild and advanced NASH

Patients with NAFLD were divided into three groups, including SS, mild NASH (NASH with fibrosis stage 0–2) and advanced NASH (NASH with fibrosis stage 3–4). Female sex was more prevalent in patients with advanced NASH than in those with SS and mild NASH. Participants in the SS group were younger than participants with mild and advanced NASH. The prevalence of obesity and lifestyle-related diseases did not differ among three groups. Platelet count decreased in accordance with the incremental fibrosis of NAFLD. The AST/ALT ratio, fibrosis markers (hyaluronic acid, type IV collagen 7S) and insulin resistance were elevated in the advanced stage of NAFLD. Participants with advanced NASH had significantly lower levels of DHEA-S compared with participants with SS, and tended to have low

Table 3 Comparison between participants with simple steatosis, and mild and advanced NASH

Parameters	Simple steatosis (SS)	Mild NASH (mNASH)	Advanced NASH (aNASH)	*P*-value
n	43	73	17	
Sex (female)	16 (37%)	40 (55%)	14 (82%)	0.0020
Age (year)	48.7 (15.9)[a]	56.8 (14.9)	65.1 (7.9)	[a]0.0007 vs aNASH, 0.0169 vs. mNASH
BMI (kg/m^2)	27.0 (4.8)	28.5 (5.0)	27.2 (4.0)	NS
obesity (BMI > 25 kg/m^2)	30 (70%)	56 (77%)	12 (71%)	NS
Diabetes	10 (23%)	38 (52%)	9 (53%)	NS
Hypertension	8 (19%)	30 (41%)	5 (29%)	NS
Dyslipidemia	12 (43%)	26 (36%)	9 (53%)	NS
Hemoglobin (g/dL)	14.8 (1.5)	14.1 (1.4)[b]	13.9 (1.3)	[b]0.046 vs SS
Platelet (10^4/μL)	24.4 (5.5)	22.2 (6.3)	17.7 (5.6)[c]	[c]0.0007 vs SS, 0.0210 vs mNASH
AST (IU/L)	40.8 (24.0)[d]	64.1 (31.8)	75.6 (40.5)	[d]0.0001 vs mNASH, aNASH
ALT (IU/L)	72.2 (56.5)	93.8 (48.0)	84.4 (50.2)	NS
AST/ALT ratio	0.64 (0.20)	0.75 (0.31)	0.96 (0.28)[e]	[e]0.0003 vs SS, 0.0160 vs mNASH
γGT (IU/L)	75.6 (66.9)	84.4 (81.4)	93.4 (45.8)	NS
Cholinesterase (IU/L)	397.8 (69.2)	378.9 (67.6)[f]	324.9 (101.3)	[f]0.0039 vs SS, 0.0283 vs aNASH
Albumin(g/dL)	4.51 (0.32)	4.37 (0.30)	4.29 (0.38)	NS
Cholesterol (mg/dL)	214.5 (43.7)	208.3 (39.6)	189.9 (38.5)	NS
Triglyceride (mg/dL)	172.9 (83.6)	189.1 (105.0)	153.4 (86.7)	NS
HDL-C (mg/dL)	50.8 (14.7)	52.8 (31.5)	51.8 (10.7)	NS
Prothrombin time (%)	106.9 (16.3)	99.0 (17.8)	90.3 (17.7)[g]	[g]0.0061 vs SS
Ferritin (ng/mL)	179.0 (182.6)	241.0 (182.1)	278.1 (246.2)	NS
Hyaluronic acid (ng/mL)	26.1 (21.8)	69.8 (104.7)	169.1 (172.4)[h]	[h]<0.0001 vs SS, 0.0014 vs mNASH
Type IV collagen 7 s (ng/mL)	3.67 (0.61)	4.99 (1.51)[i]	6.86 (1.68)[j]	[i]<0.0001 vs SS, [j]<0.0001 vs SS, mNASH
FPG (mg/dL)	97.8 (17.8)	107.8 (49.2)	99.1 (24.7)	NS
IRI (μU/mL)	9.0 (5.6)[k]	16.6 (8.7)	21.0 (12.9)	[k]*P* < 0.0001 vs aNASH, 0.001 vs mNASH
HOMA-IR	2.15 (1.34)[l]	4.68 (4.51)	5.21 (3.45)	[l]0.0023 vs mNASH, 0.0160 vs aNASH
QUICKI	0.35 (0.03)[m]	0.32 (0.03)	0.31 (0.03)	[m]<0.0001 vs mNASH, 0.001 vs aNASH
Free fatty acid (mEq/L)	0.56 (0.18)	0.58 (0.20)	0.58 (0.16)	NS
DHEA-S (μg/dL)	170.4 (129.2)	121.2 (100.7)	55.7 (45.0)[n]	[n]0.0012 vs SS

Results are presented as numbers with percentages in parenthesis for qualitative data or as means with standard deviation in parenthesis for quantitative data.
P-values were calculated by Scheffe's method or χ^2-test analysis.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DHEA-S, dehydroepiandrosterone sulfate; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; IRI, immuno-reactive insulin; QUICKI, quantitative insulin sensitivity check index; γGT, γ-glutamyl transpeptidase.

levels of DHEA-S compared with mild NASH (Table 3). Though patients with SS had significantly higher levels of serum DHEA-S (170.4 ± 129.2 μg/dL) than the control group (113.6 ± 91.8 μg/dL, $P < 0.001$), the former was younger (48.7 ± 15.9 years) than the latter (55.6 ± 12.1 years, $P < 0.001$) (Tables 1,3). Thus, we selected 129 sex- and age-matched healthy people out of the control group to clarify whether the real difference exists. Serum DHEA-S levels of 129 sex- and age-matched healthy people (145.0 ± 114.8 μg/dL) were not different from patients with SS ($P = 0.225$).

Participants with advanced NAFLD (NASH with stage 3–4 fibrosis) had significantly lower levels of DHEA-S compared with participants with mild NAFLD (SS and NASH with stage 0–2 fibrosis) (55.7 ± 45.0 vs 139.4 ± 114.1 μg/dL, $P = 0.003$). None of the younger patients (<40 years, $n = 21$) had advanced NASH. We compared DHEA-S levels in patients with more advanced NASH, aged 40–65 years (mean 57.9 ± 4.2 years), with patients with mild NAFLD, aged 40–65 years (mean 53.8 ± 7.5 years, $P = 0.139$). DHEA-S levels tended to be lower in patients with advanced NASH than in patients with mild NAFLD (76.1 ± 55.9 vs 117.1 ± 66.2 μg/dL, $P = 0.098$) without reaching significant difference. Next, we compared DHEA-S levels in patients with more advanced NASH,

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,

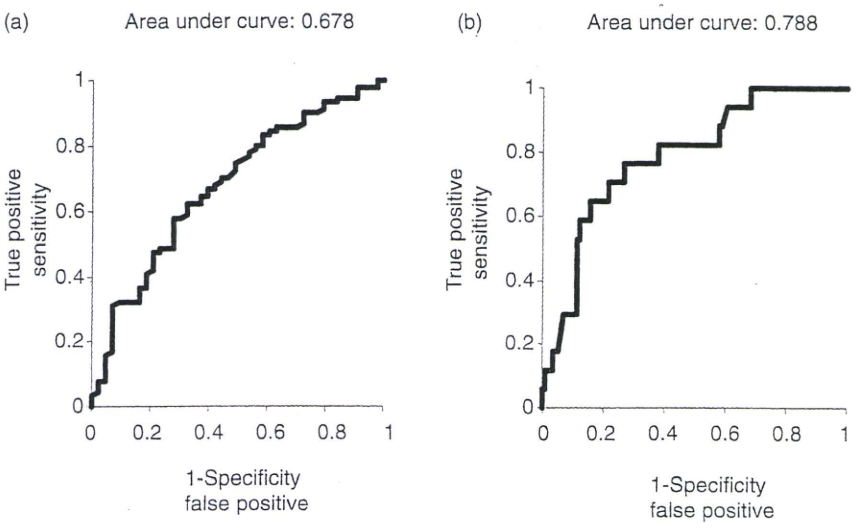

Figure 1 The area under the receiver-operator curve for dehydroepiandrosterone in separating patients with and without non-alcoholic steatohepatitis (a) or separating patients with and without advanced fibrosis (b).

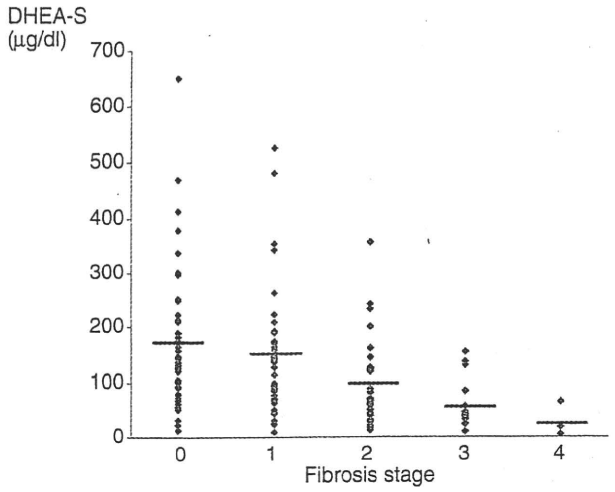

Figure 2 Variation in dehydroepiandrosterone sulfate (DHEA-S) levels with fibrosis stage for participants with non-alcoholic fatty liver disease. Mean DHEA-S levels are indicated by horizontal lines. A "dose effect" of lower DHEA-S and advanced fibrosis was observed, with a mean DHEA-S of 170.4 ± 129.2, 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.

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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

Kohichiroh Yasui · Yuichi Harano · Hironori Mitsuyoshi · Kazuhiro Tsuji · Mio Endo · Tomoaki Nakajima · Masahito Minami · Yoshito Itoh · Yoh Zen · Yasuni Nakanuma · Toshikazu Yoshikawa · Takeshi Okanoue

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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

K. Yasui (✉) · Y. Harano · H. Mitsuyoshi · K. Tsuji · M. Endo · T. Nakajima · M. Minami · Y. Itoh · T. Yoshikawa · T. Okanoue
Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan
e-mail: yasuik@koto.kpu-m.ac.jp

Y. Zen
Institute of Liver Studies, King's College Hospital, London, UK

Y. Nakanuma
Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan

T. Okanoue
Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, 1-2 Kawazono-cho, Suita 564-0013, Japan

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

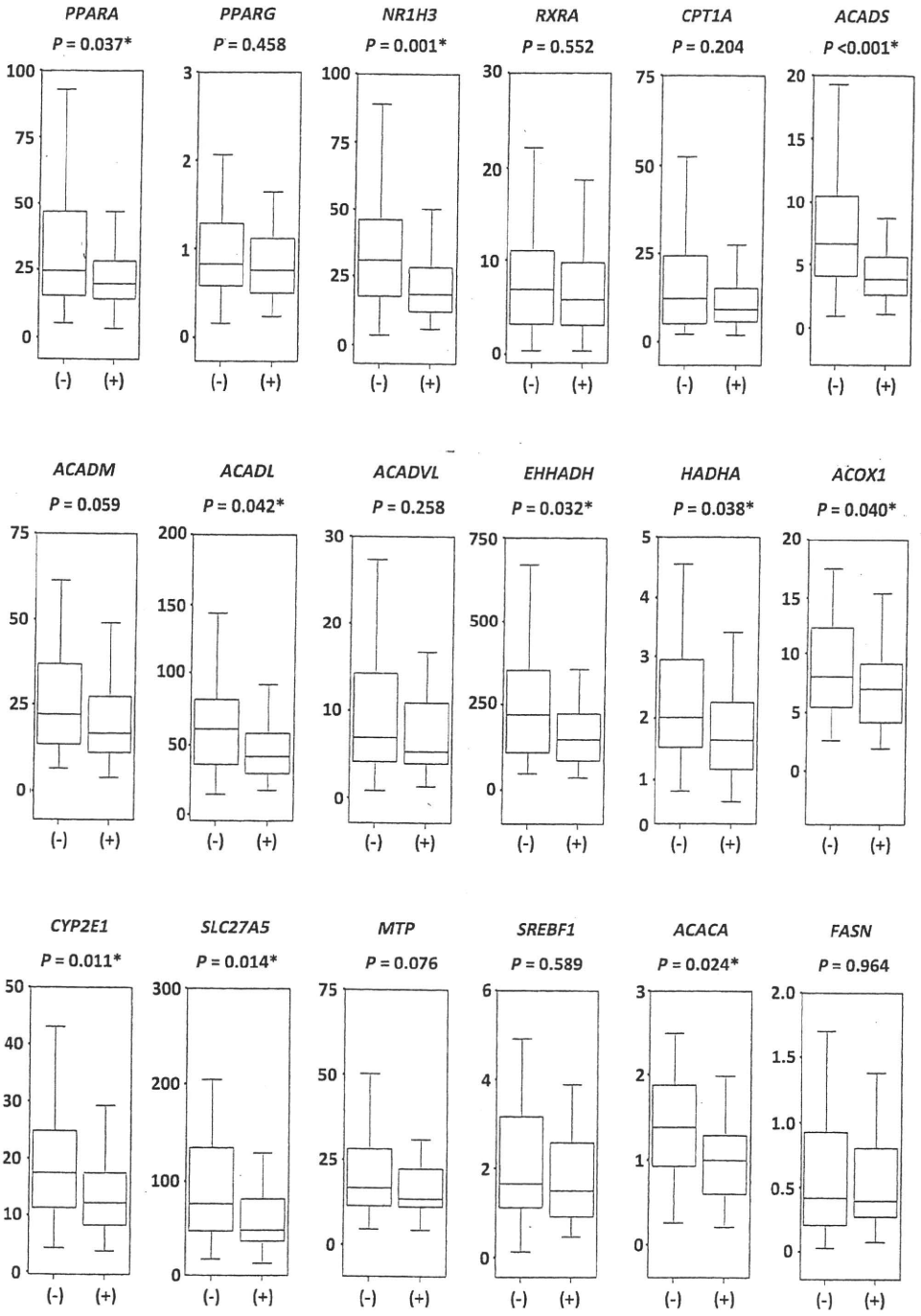

Fig. 1 Relative expression levels of 18 genes (see Table 1) in liver tissues from 57 patients without steatosis (−) and 43 patients with steatosis (+). Gene expression was evaluated by real-time quantitative RT-PCR. Results are presented relative to the expression of a reference gene (*ACTB*) to correct 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

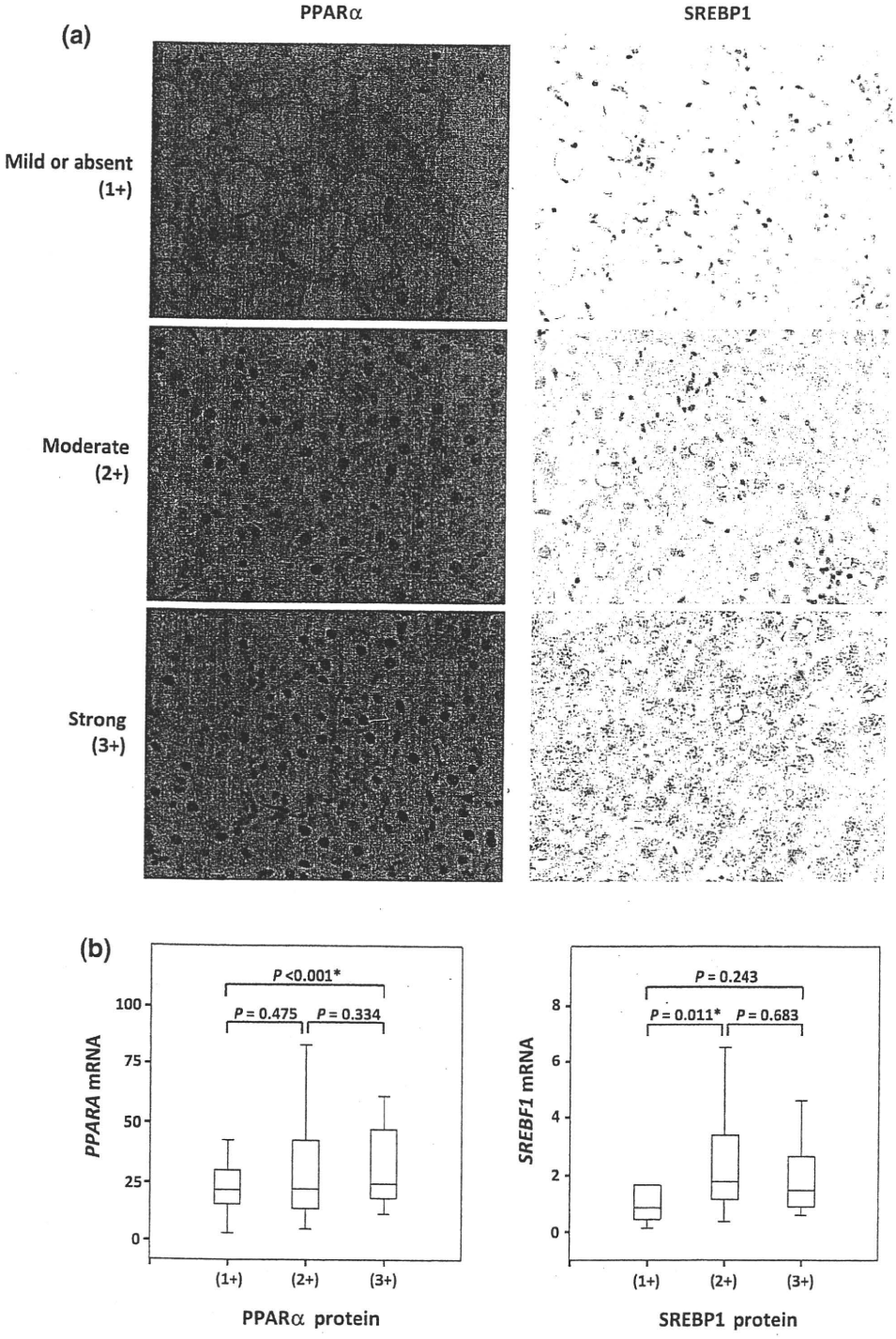

Fig. 2 Immunohistochemistry for PPARα and SREBP1 proteins. **a** Representative images from immunostaining for PPARα and SREBP1 proteins in liver tissues from patients with chronic hepatitis C. Shown are weak or absent staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, ×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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