Japan) after adjusting the pH of the substrate-containing buffer to 7.2 and expressed as mU/mg of supernatant protein. Triolein was used as a substrate.

2.7. Histological examination

Small blocks of liver tissue from each mouse were fixed in 10% formalin in phosphate-buffered saline and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin or Azan-Mallory method. The procedure for cytochemical staining for peroxisomal catalase was described elsewhere [24].

2.8. Other assays

Serum levels of TG, non-esterified FA (NEFA), glucose, aspartate and alanine aminotransferases (AST and ALT), and ketone bodies were determined with commercial kits purchased from Wako Pure Chemicals Industries. Serum insulin concentrations were mea-

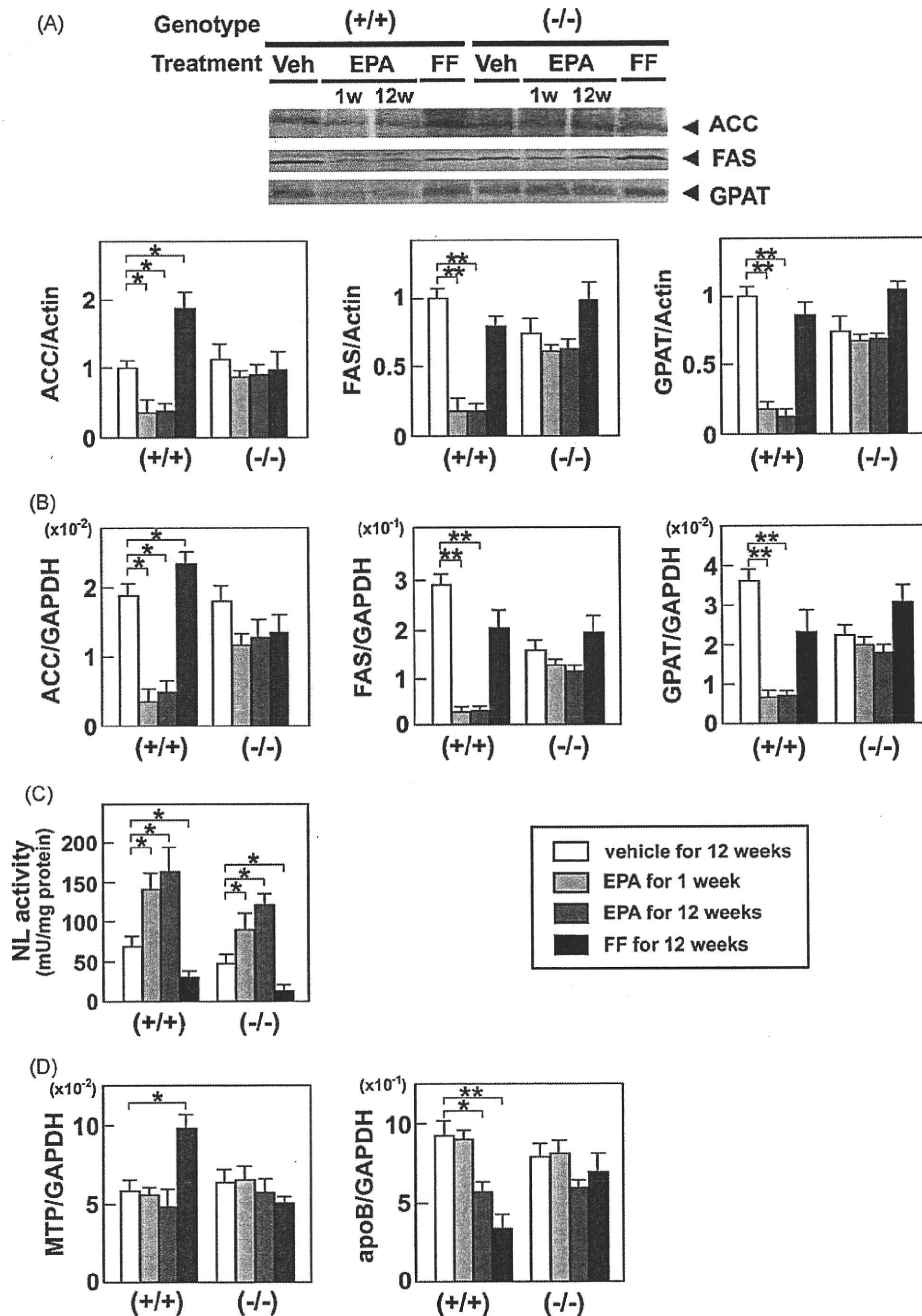

Fig. 5. Effects of EPA and FF on hepatic *de novo* lipogenesis and the TG hydrolysis and secretion pathway. (A) Immunoblot analysis of ACC, FAS, and GPAT. Whole liver lysates (40–80 μg of protein) obtained from wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were loaded into each well. Band intensities were measured densitometrically, normalized to those of actin, and subsequently normalized to those in control wild-type mice [(+/+) Veh]. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of lipogenic enzymes. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in hepatic NL activity. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and hepatic NL activity was determined as described in Methods. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$. (D) Hepatic mRNA levels of molecules associated with TG secretion. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$.

sured using a mouse insulin ELISA KIT (AKRIN-011T, Shibayagi, Gunma, Japan).

2.9. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the two-tailed Student's *t*-test. A probability value of less than 0.05 was considered statistically significant. All calculations were performed with SPSS version 11.0 software for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. A saturated fat diet induces hepatic steatosis in mice

The phenotypic changes caused by a 16-week saturated fat diet are shown in Table 1. At the endpoint, body weight, ratios of liver-to-body weight and epididymal fat-to-body weight, serum TG and ALT concentrations, and hepatic TG contents were all significantly increased by the saturated fat diet. These changes were observed in both genotypes, but were more prominent in *Ppara*$^{-/-}$ mice. Histological analysis revealed that the saturated fat diet induced mild-to-moderate macrovesicular steatosis mainly in zone 3 in both genotypes, but ballooned hepatocytes, lobular inflammation, or Mallory hyaline were not found (Fig. 1).

3.2. EPA ameliorates hepatic steatosis in a PPARα-dependent manner

FF treatment for 12 weeks decreased body weight and serum/liver TG levels in wild-type mice only, but significantly increased serum ALT levels in both genotypes (Fig. 2). When EPA was administered for 1 week, there were no remarkable changes in either genotype. However, EPA treatment for 12 weeks significantly decreased liver-to-body weight ratio, serum levels of TG, NEFA, and ALT, and hepatic TG content in wild-type mice without decreases in body weight or epididymal fat weight (Fig. 2). Histologically, EPA markedly attenuated macrovesicular

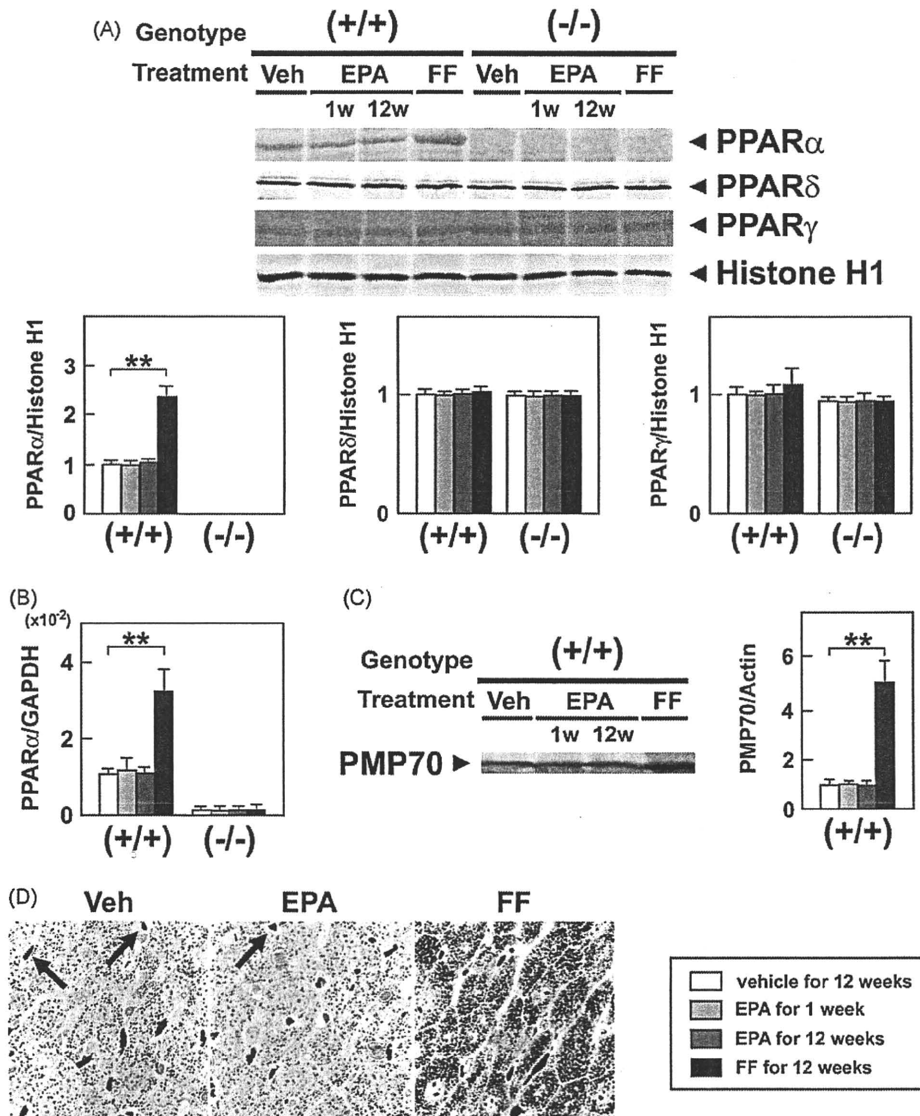

Fig. 6. Effects of EPA and FF on hepatic PPAR. (A) Immunoblot analysis of PPAR. Hepatocyte nuclear fractions (80 μg of protein) obtained from wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were loaded into each well. Band intensities were measured densitometrically, normalized to those of histone H1, and subsequently normalized to those in control wild-type mice [(+/+) Veh]. Results are expressed as mean ± SD (*n* = 6/group). $^{**}P < 0.01$. (B) Hepatic mRNA levels of PPARα. The same samples in Fig. 3B were used. $^{**}P < 0.01$. (C) Immunoblot analysis of PMP70, a typical PPARα target. The same samples used in Fig. 3A were loaded. $^{**}P < 0.01$. (D) Cytochemical staining for hepatic peroxisomes. Liver tissues were obtained from saturated fat diet-fed wild-type mice treated with a vehicle (Veh), highly-purified EPA, or FF for 12 weeks and subjected to cytochemical staining for peroxisomal catalase. Peroxisomes are seen as darkly-stained particles. Arrows indicate erythrocytes. Original magnification, 400×.

steatosis in wild-type mice only (Fig. 1). These results confirmed that EPA ameliorated saturated fat diet-induced steatosis and hypertriglyceridemia preferentially in wild-type mice.

3.3. EPA does not enhance mitochondrial β-oxidation

To investigate the precise mechanism of the PPARα-dependent steatosis-attenuating effect of EPA, we first analyzed hepatic β-oxidation pathway. FF markedly increased the expression of representative mitochondrial β-oxidation enzymes [long-chain acyl-CoA synthase (LACS), carnitine palmitoyl-CoA transferase-1 (CPT-I), and medium-chain acyl-CoA dehydrogenase (MCAD)] at the protein and mRNA levels (Fig. 3A and B), enhanced mitochondrial β-oxidation activity (Fig. 3C), and elevated serum ketone body concentrations (Fig. 3D) in wild-type mice only. However, these changes were not observed in the EPA-treated mice (Fig. 3), suggesting that the effect of EPA on hepatic steatosis was not derived from enhancement of mitochondrial β-oxidation.

3.4. EPA suppresses FA uptake in a PPARα-independent manner

FF markedly increased the levels of liver fatty acid-binding protein (L-FABP), fatty acid translocase (FAT), and fatty acid transport protein (FATP) and lowered apolipoprotein CIII (apoCIII) in wild-type mice only (Fig. 4A and B). In contrast, EPA significantly increased the levels of mRNA encoding apoCIII (Fig. 4B), and strongly decreased the expression of L-FABP, FAT, and FATP in a PPARα-independent manner (Fig. 4A). A reduction in the latter two proteins was also confirmed by quantitative RT-PCR analysis (Fig. 4B). Furthermore, EPA significantly suppressed palmitic acid uptake into hepatocytes (Fig. 4C). Both agents did not affect the mRNA levels of hepatic TG lipase (HTGL) (Fig. 4B).

3.5. EPA suppresses de novo lipogenesis in a PPARα-dependent manner

FF increased the expression of acetyl-CoA carboxylase (ACC) in wild-type mice, but had no impact on the expression of fatty acid

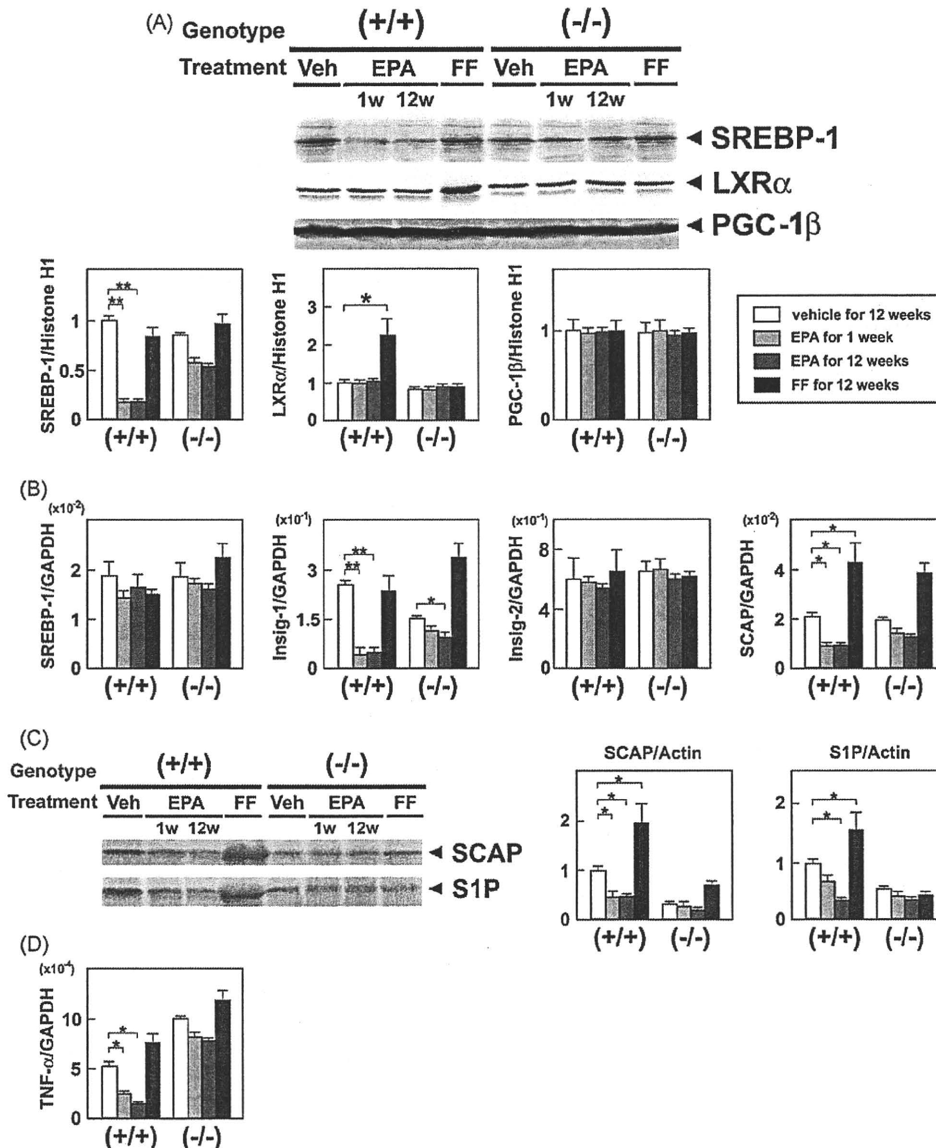

Fig. 7. Effects of EPA and FF on hepatic SREBP-1. (A) Immunoblot analysis of SREBP-1 and its co-regulators, LXRα and PGC-1β. The same samples used in Fig. 6A were loaded. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of SREBP-1 and its activating proteins. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Immunoblot analysis of SCAP and S1P. The same samples in Fig. 3A were loaded. $^{*}P < 0.05$. (D) Hepatic mRNA levels of TNF-α. The same samples in Fig. 3B were used. $^{*}P < 0.05$.

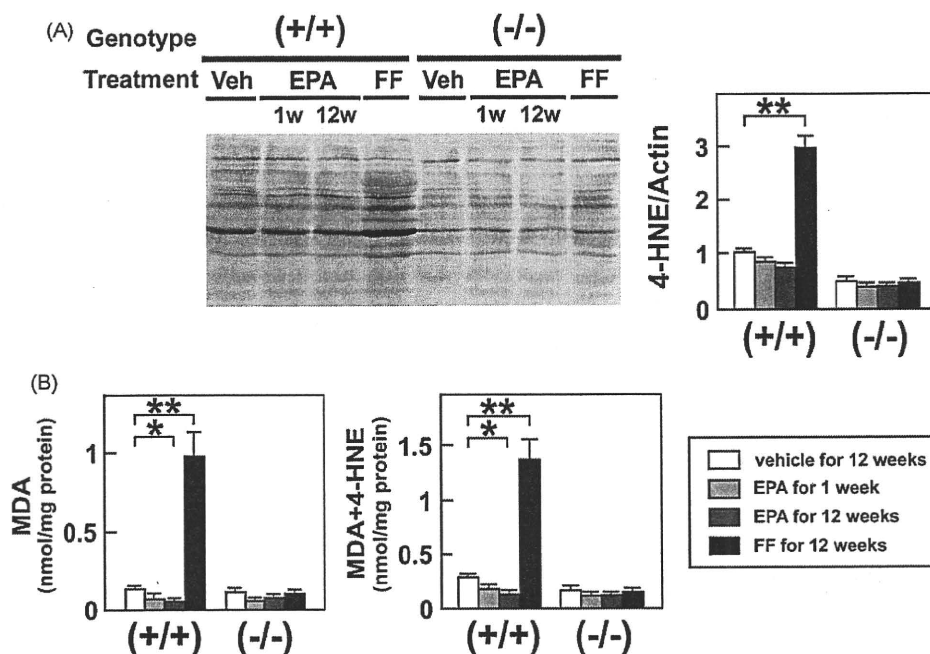

Fig. 8. Analysis of hepatic lipid peroxide content. (A) Immunoblot analysis of 4-HNE. The same samples in Fig. 3A were loaded. $^{**}P < 0.01$. (B) Hepatic levels of MDA and MDA + 4-HNE. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, EPA for 1 week or 12 weeks, or FF for 12 weeks. Total lipids were extracted from mouse liver tissues and the amounts of MDA and MDA + 4-HNE were determined. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

synthase (FAS) or glycerol-3-phosphate acyltransferase (GPAT) (Fig. 5A and B). On the other hand, EPA markedly decreased the expression of these three enzymes in wild-type mice (Fig. 5A and B). These results showed that EPA inhibited *de novo* lipogenesis in a PPARα-dependent fashion.

3.6. *Effects of EPA on the hepatic TG degradation and secretion pathway*

In contrast to FF, EPA significantly enhanced NL activity in both genotypes (Fig. 5C), indicating that it strongly facilitated hydrolysis of intrahepatic TG. EPA did not affect the expression of microsomal TG transfer protein (MTP) or apoB (Fig. 5D).

3.7. *EPA does not activate PPARα*

Although EPA lowered hepatic TG levels in a PPARα-dependent manner, it did not increase the nuclear or mRNA levels of PPARα (Fig. 6A and B), induce expression of representative PPARα target genes [LACS, CPT-I, MCAD (Fig. 3), L-FABP, FAT, FATP (Fig. 4), or peroxisomal membrane protein 70 (PMP70) (Fig. 6C)], or cause hepatic peroxisome proliferation (Fig. 6D), all of which were distinct from the actions of FF. These results provide compelling evidence that the steatosis-ameliorating effect of EPA is not through PPARα activation. In addition, EPA did not influence the nuclear levels of the other PPARs: PPARδ and PPARγ (Fig. 6A).

3.8. *EPA suppresses SREBP-1 maturation in a PPARα-dependent manner*

EPA significantly decreased the mature SREBP-1 levels only in hepatocyte nuclei of wild-type mice, but did not affect the levels of LXRα and PPARγ coactivator-1β (PGC-1β), known to be involved in SREBP-1 regulation [34] (Fig. 7A). Decreases in mature SREBP-1 levels correlated with those in SREBP-1 target genes, including ACC, FAS, and GPAT (Fig. 5A and B). However, EPA did not lower SREBP-1 mRNA levels (Fig. 7B), suggesting that EPA modulated the expression of SREBP-1 at the post-transcriptional level. When factors associated with SREBP-1 maturation were examined, the expression of SREBP cleavage-activating protein (SCAP) and site-1 protease (S1P) were significantly decreased by EPA in a PPARα-dependent manner (Fig. 7B and C). The levels of mRNA encoding insulin-induced gene product (Insig)-2, another SREBP-1-activating molecule expressed exclusively in the liver, remained unchanged by EPA treatment (Fig. 7B). Collectively, these results demonstrated that EPA inhibited maturation of SREBP-1 in the presence of PPARα through down-regulation of SCAP and S1P.

A recent study has shown that the expression of SCAP was induced by pro-inflammatory cytokines [35]. The mRNA levels of tumor necrosis factor-α (TNF-α) were decreased in EPA-treated wild-type mice only (Fig. 7D).

3.9. *EPA reduces hepatic oxidative stress*

Persistent PPARα activation may increase generation of reactive oxygen species (ROS) [33,36]. As expected, FF markedly increased hepatic lipid peroxide content (Fig. 8) and the expression of ROS-generating enzymes, such as NADPH oxidase (gp91^{phox} and p47^{phox}) and acyl-CoA oxidase (AOX), in wild-type mice only (Fig. 9). On the other hand, EPA reduced hepatic lipid peroxides in wild-type mice (Fig. 8), likely due to increased expression of manganese- and copper, zinc-superoxide dismutases (Mn- and Cu, Zn-SOD) (Fig. 9). EPA did not affect the expression of glutathione peroxidase (GPx) (Fig. 9). Thus, EPA can ameliorate fatty liver without activation of PPARα and ensuing augmentation of hepatic oxidative stress.

4. Discussion

The present study demonstrated detailed mechanisms of steatosis-attenuating effects of highly-purified EPA in mice, which were unexpectedly unrelated to PPARα activation. They can be summarized as follows: (1) suppression of SREBP-1 processing, (2) suppression of FA uptake from the blood into hepatocytes, and (3)

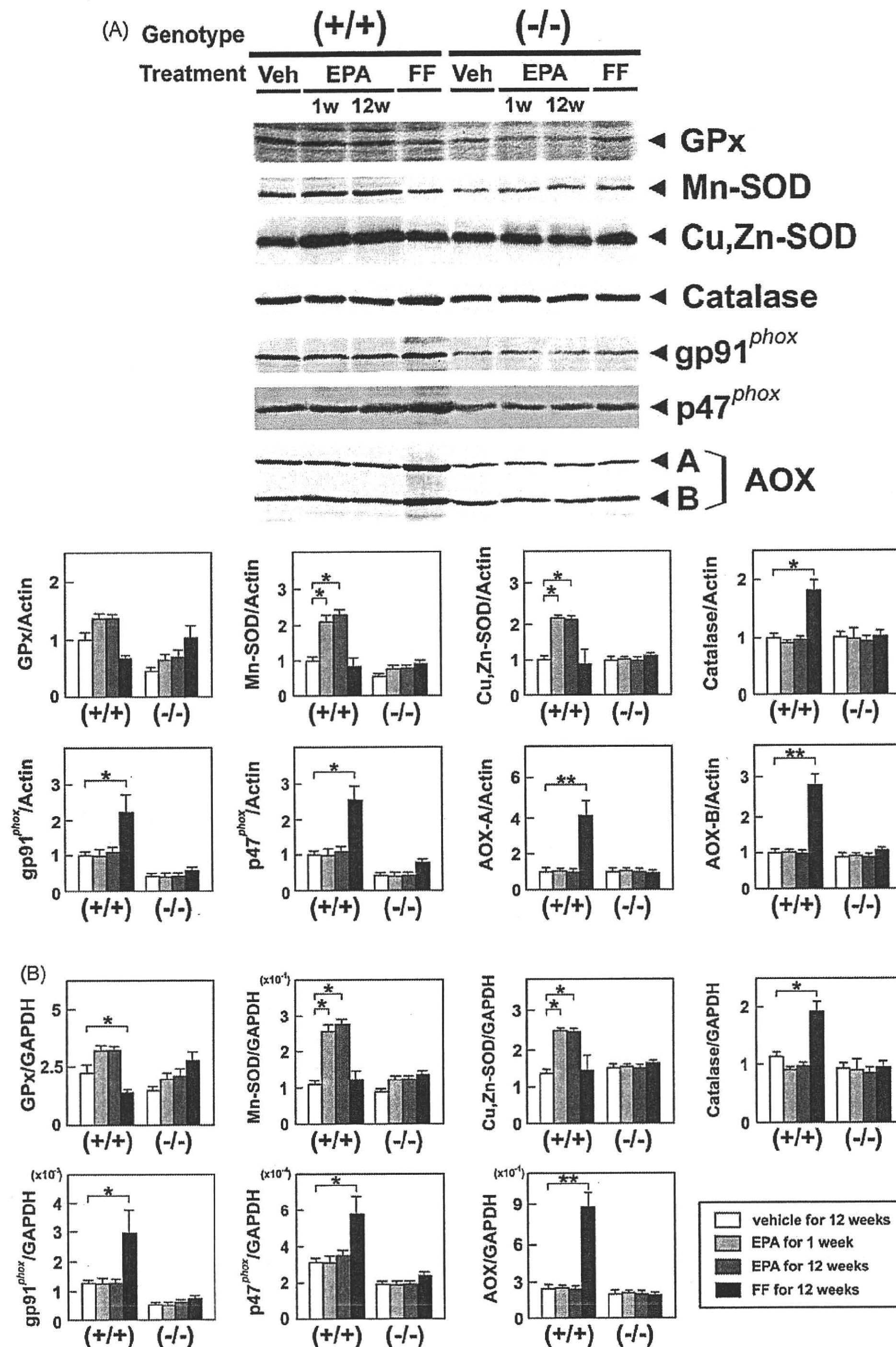

Fig. 9. Effects of EPA and FF on hepatic ROS-scavenging and ROS-generating enzymes. (A) Immunoblot analysis of ROS-scavenging and ROS-generating enzymes. Whole liver lysates (40–80 μg of protein) were loaded into each well. The same samples used in Fig. 3A were loaded. A and B bands of AOX, full-length and truncated AOX, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of ROS-scavenging and ROS-generating enzymes. The same samples in Fig. 3B were adopted. $^{*}P < 0.05$; $^{**}P < 0.01$.

enhancement of intrahepatic TG hydrolysis. Interestingly, down-regulation of SCAP and S1P and the resultant suppression of SREBP-1-mediated pathway by EPA occurred in a PPARα-dependent manner. These actions clearly differed from those of FF, a typical PPARα activator.

We uncovered that highly-purified EPA cannot singularly activate PPARα in mice. This fact is quite different from the results of the previous *in vivo* studies using fish oil [10,18–20]. This discrepancy probably stems from PPARα-activating properties of constituents of fish oil other than EPA. Indeed, tuna oil, in which docosahexaenoic acid is dominant, has been shown to be a much stronger PPARα activator than EPA in mice [37]. Thus, it is conceivable that pure EPA may possess pharmacological actions distinct from crude fish oil.

Furthermore, Ishii et al. [38] have reported that EPA-supplemented diet increased the expression of PPARα in *Pten*-deficient mice, which is inconsistent with the present results. EPA contained in the diet is easily oxidized at room temperature and several types of oxidized EPA derivatives are generated. Sethi et al. [39] have demonstrated that oxidized EPA markedly activated PPARα and its

PPARα-activating properties were approximately 2.5-fold stronger than native non-oxidized EPA and as potent as fenofibric acid in bovine aortic endothelial cells. Based on these observations, we suppose that such a discrepancy in PPARα expression may result from the difference in the amount of oxidized EPA.

In the preliminary experiments, we administered highly-purified EPA at a daily dose of 200, 400, 600, and 1000 mg/kg of body weight to wild-type mice fed a saturated fat diet for 12 weeks, and found that EPA treatment at the latter three doses was safe and effective to reduce hepatic TG contents. Down-regulation of SREBP-1 and the lack of PPARα activation were also detected by EPA administration at each dose. To confirm the absence of PPARα activation by EPA, we adopted the highest dose (1000 mg/kg of body weight/day) in this study.

A novel and unexpected finding in the present study was that EPA-induced decreases in the mature SREBP-1 protein in hepatocyte nuclei occurred by down-regulating the expression of SCAP and S1P, but not by lowering the SREBP-1 mRNA levels. This phenomenon was not observed in *Ppara*$^{-/-}$ mice, suggesting that down-regulation of SCAP and S1P by EPA are related to the presence of PPARα. The detailed molecular mechanism regarding the contribution of PPARα to SREBP-1 processing/activation system still remains unclear. However, it has been reported that TNF-α increased the expression of SCAP in the livers of casein-injected apoE knockout mice [35]. Furthermore, binding motifs for Sp1 have been detected in the promoter region of S1P gene [40]. Indeed, TNF-α- and lipid peroxide-reducing effects were observed only in EPA-treated wild-type mice (Figs. 7D and 8). Therefore, we can speculate that PPARα-dependent alleviation of inflammatory stress by EPA is associated with decreases in the expression of SCAP and S1P.

EPA markedly suppressed the expression of L-FABP, FAT, and FATP and inhibited FA uptake into hepatocytes by a PPARα-independent mechanism. EPA-induced suppression of L-FABP appeared at the post-transcriptional level. L-FABP is prone to S-thiolation, N-acetylation, phosphorylation, and conformational changes, all of which are related to protein stability [41]; EPA might influence such modifications. Since the enhancement of FA uptake has also been reported in the livers of patients with NAFLD [42], its correction caused by EPA may also lead to the improvement of hepatic steatosis.

It is noteworthy that EPA significantly increased the expression of Mn- and Cu, Zn-SODs in a PPARα-dependent fashion. These increases occur via nuclear stabilization of nuclear factor-E2-related factor 2 (Nrf2) [43]. It has been documented that EPA stabilized Nrf2 by reacting directly with Keap1, a negative regulator of Nrf2, and disrupting its function [44]. Thus, EPA might affect the stabilization process of Nrf2 via PPARα. Further studies are needed to address its precise mechanism.

It is well recognized that a saturated fat-rich diet is associated with the development of NAFLD in humans, and that lower levels of hepatic n-3 PUFA predispose livers to steatosis by favoring *de novo* lipogenesis [12,45]. In this study, mice fed a saturated fat diet exhibited elevation of serum ALT levels, hypertriglyceridemia, and macrovesicular steatosis, which resembled the clinical features of patients with NAFLD. Furthermore, EPA treatment in these mice decreased hepatic TG levels without affecting body weight or serum insulin concentrations; these findings were also observed in NASH patients treated with highly-purified EPA [46]. Thus, the molecular mechanism of EPA action found in this study may at least in part be translated to humans.

It is known that lipotoxicity, oxidative stress, and mitochondrial dysfunction play important roles in the pathogenesis of NAFLD/NASH [2,36]. Although FF decreased hepatic TG/FA contents, it enhanced mitochondrial β-oxidation activity and induced the expression of NADPH oxidase and AOX, which may result in production of ROS inside and outside the mitochondria. On the other hand, EPA not only down-regulated two major pathways to increase intrahepatic FA contents, but also elevated the levels of mitochondrial Mn-SOD and extra-mitochondrial Cu, Zn-SOD, which may lead to alleviation of lipotoxicity and oxidative stress. Therefore, we believe that highly-purified EPA may be useful for the treatment of NAFLD/NASH [46].

In conclusion, this study clarified that the main action of EPA in hepatic steatosis improvement was not based on the activation of PPARα. In contrast to FF, EPA significantly inhibited *de novo* lipogenesis and hepatic FA uptake with a reduction in hepatic oxidative stress. These data raise the possibility that EPA may be a promising candidate for various types of liver diseases associated with hepatic fat accumulation and oxidative stress, including NASH, alcoholic liver disease [32,47], and chronic hepatitis C [33]. Further studies are needed to confirm the efficacy of highly-purified EPA against these diseases.

Conflicts of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.031.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Nonalcoholic fatty liver disease in Japanese junior high school students: its prevalence and relationship to lifestyle habits

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Abstract

Background Despite the increase in nonalcoholic fatty liver disease (NAFLD) in Japanese adults, its prevalence in adolescents remains unclear. This prompted us to evaluate the incidence and clinical characteristics of NAFLD among junior high school students.

Methods A population-based cross-sectional study was conducted among students in a single junior high school in Nagano prefecture. Serum alanine aminotransferase (ALT) and γ-glutamyltransferase (γGT) measurements and abdominal ultrasonography were performed in 249 and 288 students in 2004 and 2007, respectively. In the latter survey, student lifestyle habits were also assessed, using questionnaires.

Results The prevalence of NAFLD was 4.4% and 4.5% in 2004 and 2007, respectively, which was lower than that of obesity (10.0% and 5.9%). Body mass index and ALT and γGT levels increased significantly with hepatic steatosis severity. Multivariate logistic regression analysis demonstrated that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD (odds ratio 16.9, $P < 0.001$ and odds ratio 16.6, $P = 0.001$, respectively). The ratios of students commuting to and from school by car and not doing sports outside of school were higher in NAFLD students compared with non-NAFLD ones. Such tendencies were observed independently of the presence of obesity. Additionally, one obese student with severe steatosis and liver dysfunction was diagnosed as having nonalcoholic steatohepatitis (NASH).

Conclusions Approximately 4% of junior high school students had NAFLD that was primarily associated with obesity and reduced daily physical activity. Serum ALT measurement during school check-ups is recommended for the early detection of young adolescent NAFLD/NASH.

Keywords Obesity · ALT · Physical activity · Skipping breakfast · Nonalcoholic steatohepatitis

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Introduction

Due to increasing sedentary lifestyles and the rising prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease. NAFLD encompasses a spectrum of histological findings that range from macrovesicular steatosis alone (simple

steatosis) to macrovesicular steatosis with hepatocyte ballooning and/or lobular inflammation (steatohepatitis). Nonalcoholic steatohepatitis (NASH) is the severe and progressive form of NAFLD and may develop into cirrhosis, hepatocellular carcinoma, and ultimately death [1–3]. Based on current health screening data, the prevalence of NAFLD in Japanese adults is estimated to be around 10% [1].

Of great recent concern is the fact that NAFLD/NASH exists even in children and adolescents and that pediatric NASH can also progress to cirrhosis. Tominaga et al. [4] reported that 2.6% of Japanese children aged 4–12 years had NAFLD. Furthermore, Kinugasa et al. [5] described seven Japanese obese children aged between 9 and 15 years having hepatic steatosis with various degrees of lobular inflammation and/or portal fibrosis and one child having cirrhosis. Therefore, the early detection of NAFLD/NASH and appropriate disease management are now important in the pediatric population as well.

According to data from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the prevalence of obesity among Japanese junior high school students was double in 2003 compared to that in 1977 (from 5% to 10%). Given the strong association of NAFLD with obesity, NAFLD is suspected to affect a substantial, but as yet unidentified, proportion of junior high school students. As such, this population-based cross-sectional study was planned to evaluate the prevalence of NAFLD in young adolescents, along with any lifestyle habits associated with its development.

Materials and methods

Participants

The study population consisted of all students attending a public junior high school in a village located in southern Nagano prefecture. Real-time liver function tests and abdominal ultrasonography (US) were performed in 2004 and 2007 after explaining the significance and protocol of the study to all students and their parents and obtaining written informed consent. This study was approved by the ethics committee of Shinshu University School of Medicine and Showa Inan General Hospital and adheres to the principles of the Declaration of Helsinki. In this study, NAFLD was defined by the existence of hepatic steatosis based on abdominal US, regardless of liver function tests.

Data collection

Anthropometric, biochemical, and ultrasonographic examinations were carried out on the same school-day mornings in June 2004 and July 2007 after an overnight fast. Body height and weight were measured by a school nurse, with subjects barefoot and in light clothing. Body mass index (BMI) and the age-gender-adjusted Japanese standardized weight index for height (JSI) were used as anthropometric parameters. The JSI was calculated as [(body weight − standard body weight)/standard body weight] × 100 (%), where the standard body weight for each subject's age, sex, and height was determined from data on 700 000 Japanese children aged 5–17 years in 1990. The JSI is considered to be more suitable for the evaluation of the physiques of children and adolescents than the BMI in Japan. According to the JSI, subject weight status was classified as lean (≤−20% of JSI), moderately lean (−19.9% to −10.1%), normal (−10% to +10%), overweight (+10.1% to +19.9%), or obese (≥+20%).

Venous blood samples were drawn just after an anthropometric examination. Serum levels of alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), triglycerides (TG), and high-density-lipoprotein cholesterol (HDL-C) were determined using standard automated analyzers. Normal ranges for serum ALT and γGT levels were set as 0–30 U/L each, in accordance with previous studies in pediatric populations [6–8].

Real-time abdominal US was performed by two experienced ultrasonographers (K.H. and C.I.) using a LOGIQ book equipped with a 4.0 MHz convex-type transducer (GE Yokogawa Medical Systems, Tokyo, Japan). Representative US images of each student were kept in the LOGIQ book and evaluated afterwards in a blinded manner by three independent hepatologists (G.T., N.T., and M.K.). The degrees of hepatorenal contrast, profound attenuation of the diaphragm, and blurring of the vascular wall were each scored as 0 (absent), 1 (present), or 2 (marked) [4, 9–11] The sum of these scores from each diagnostician ranged from 0 to 6, and a mean total score of 1 or more was judged as the presence of hepatic steatosis. Total scores of 1–2 and 3 or more were classified as the presence of mild steatosis and moderate-to-severe steatosis, respectively.

Assessment of lifestyle habits

In the 2007 survey, lifestyle habits were assessed in addition to abdominal US and liver function tests. A 15-item questionnaire focusing on personal dietary and exercise habits was prepared and distributed to all students. All questionnaires were completed and submitted by the students themselves, and were then analyzed for any relationships with NAFLD.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS, Chicago, IL, USA). Qualitative

variables were expressed as numbers (percentages) and compared using the χ^2 test. Quantitative data were expressed as means ± SD and compared using the two-tailed Student's *t* test or one-way analysis of variance. Post-hoc comparison was also performed between groups, using the Tukey's or Games–Howell's method. Multivariate logistic regression analysis was conducted to find independent predictors of NAFLD. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Prevalence of obesity and NAFLD

The overall prevalence of obesity was 10.0% and 5.9% in 2004 and 2007, respectively (Table 1). Because all students were presumed to not habitually consume alcohol, the overall prevalence of NAFLD was calculated as 4.4% and 4.5% in 2004 and 2007, respectively (Table 1). Of these, the prevalence of moderate-to-severe steatosis was 0.8% in 2004 and 1.4% in 2007 (Table 1). There was a male preponderance in NAFLD prevalence in the 2004 survey (7.5% in boys vs. 1.6% in girls, $P = 0.022$).

Association between weight status and NAFLD

As shown in Table 2, approximately 90% of students had neither obesity nor NAFLD and 2%–3% had both disorders. In the 2007 survey, 4 of 13 NAFLD students were overweight (Table 2). Although a small number of NAFLD students having normal body weight was found (1.2% in 2004 and 1.0% in 2007), the degree of steatosis was very mild in all cases. On the other hand, all students with moderate-to-severe steatosis were obese (Table 2).

Comparison of clinical data between students with and without NAFLD

In both surveys combined, 24 (4.5%) of the 537 students were judged as having NAFLD. Several parameters were then compared between students with NAFLD ($n = 24$) and those without ($n = 513$) to investigate the clinical features of NAFLD. The prevalence of male gender, obesity, and elevated ALT and γGT levels was significantly higher in the NAFLD group than in the non-NAFLD one (Table 3). JSI, BMI, and serum levels of ALT, γGT, and TG were increased and serum HDL-C levels were decreased in the NAFLD group (Table 3). When these parameters were analyzed in relation to the degree of steatosis, BMI and serum levels of ALT, γGT, and TG all increased with the steatosis severity (Fig. 1). Multivariate logistic regression analysis revealed that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD. The odds ratio was 16.9 for the presence of obesity (95% confidence interval [CI], 6.5–43.9; $P < 0.001$) and 16.6 for an ALT level of of 30 U/L or more (95% CI, 3.1–87.6; $P = 0.001$).

Additionally, when these parameters were compared between boys ($n = 275$) and girls ($n = 262$), JSI and serum levels of ALT and γGT were increased in the former group (2.6 ± 17.2 vs. −1.5 ± 14.2, $P = 0.003$ for JSI;

Table 1 Prevalence of obesity and NAFLD

Year	2004			2007		
	Boys ($n = 120$)	Girls ($n = 129$)	Overall ($n = 249$)	Boys ($n = 155$)	Girls ($n = 133$)	Overall ($n = 288$)
Weight status						
Lean	2 (1.7)	2 (1.5)	4 (1.6)	0 (0)	13 (9.8)	13 (4.5)
Moderately lean	9 (7.5)	21 (16.3)	30 (12.0)	23 (14.8)	38 (28.6)	61 (21.2)
Normal	87 (72.5)	81 (62.8)	168 (67.5)	102 (65.8)	61 (45.9)	163 (56.6)
Overweight	9 (7.5)	13 (10.1)	22 (8.8)	19 (12.3)	15 (11.3)	34 (11.8)
Obese	13 (10.8)	12 (9.3)	25 (10.0)	11 (7.1)	6 (4.5)	17 (5.9)
JSI (%)	2.9 ± 15.1	1.9 ± 14.3	2.4 ± 14.7	2.4 ± 18.7	−4.7 ± 13.4	−0.9 ± 16.8
BMI (kg/m^2)	19.7 ± 3.0	19.9 ± 3.0	19.8 ± 3.0	19.2 ± 2.7	19.2 ± 2.8	19.2 ± 2.7
Hepatic steatosis						
Absent	111 (92.5)	127 (98.4)	238 (95.6)	147 (94.8)	128 (96.2)	275 (95.5)
Present (NAFLD)	9 (7.5)	2 (1.6)	11 (4.4)	8 (5.2)	5 (3.8)	13 (4.5)
Mild	8 (6.7)	1 (0.8)	9 (3.6)	4 (2.6)	5 (3.8)	9 (3.1)
Moderate-to-severe	1 (0.8)	1 (0.8)	2 (0.8)	4 (2.6)	0 (0)	4 (1.4)

Data are expressed as numbers (percentages of students of the same gender or overall in each year) or means ± SD. Body weight status was classified according to the criteria of the age-gender-adjusted Japanese standardized weight index for height (JSI)

BMI body mass index, *NAFLD* nonalcoholic fatty liver disease

Table 2 Association between weight status and NAFLD

Year	2004			2007		
	Lean-to-normal (n = 202)	Overweight (n = 22)	Obese (n = 25)	Lean-to-normal (n = 237)	Overweight (n = 34)	Obese (n = 17)
Hepatic steatosis						
Absent	199 (79.9)	22 (8.8)	17 (6.8)	234 (81.3)	30 (10.4)	11 (3.8)
Present (NAFLD)	3 (1.2)	0 (0)	8 (3.2)	3 (1.0)	4 (1.4)	6 (2.1)
Mild	3 (1.2)	0 (0)	6 (2.4)	3 (1.0)	4 (1.4)	2 (0.7)
Moderate-to-severe	0 (0)	0 (0)	2 (0.8)	0 (0)	0 (0)	4 (1.4)

Data are expressed as numbers (percentages of all students in each year)

NAFLD nonalcoholic fatty liver disease

Table 3 Comparison of clinical data

NAFLD	(−) (n = 513)	(+) (n = 24)	P
Male	258 (50.3)	17 (70.8)	**0.049**
Obesity (JSI ≥ 20%)	28 (5.7)	14 (58.3)	**≤0.001**
ALT ≥30 U/L	4 (0.8)	6 (25.0)	**≤0.001**
γGT ≥30 U/L	6 (1.2)	5 (20.8)	**≤0.001**
JSI (%)	−0.6 ± 12.9	27.0 ± 38.0	**0.002**
BMI (kg/m^2)	19.3 ± 2.7	23.2 ± 4.6	**≤0.001**
ALT (U/L)	13 ± 5	22 ± 15	**0.012**
γGT (U/L)	14 ± 4	20 ± 10	**0.006**
TG (mg/dL)	75 ± 42	99 ± 53	**0.037**
HDL-C (mg/dL)	64 ± 13	57 ± 10	**0.006**

Data were obtained from 249 junior high school students in 2004 and 288 students in 2007 and are expressed as numbers (percentages) or means ± SD. P values of less than 0.05 are in bold and underlined

NAFLD nonalcoholic fatty liver disease, *JSI* age-gender-adjusted Japanese standardized weight index for height, *ALT* alanine aminotransferase, *γGT* γ-glutamyltransferase, *BMI* body mass index, *TG* triglycerides, *HDL-C* high-density-lipoprotein cholesterol

15 ± 6 vs. 12 ± 6 U/L, $P < 0.001$ for ALT; and 16 ± 5 vs. 13 ± 3 U/L, $P < 0.001$ for γGT). The prevalence of elevated ALT and γGT levels tended to be higher in boys, though not significantly so (2.5% vs. 1.1% and 2.9% vs. 1.1%, respectively).

Comparison of lifestyle habits between students with and without NAFLD

To explore lifestyle habits associated with NAFLD development, questionnaires were distributed to all students in 2007 and results were compared between students with NAFLD (n = 13) and those without (n = 275). The clinical features of the students examined in 2007 are shown in Table 4. The ratios of students skipping breakfast (≥2 times/week) and always drinking more than half of the broth that comes with noodles were significantly higher in

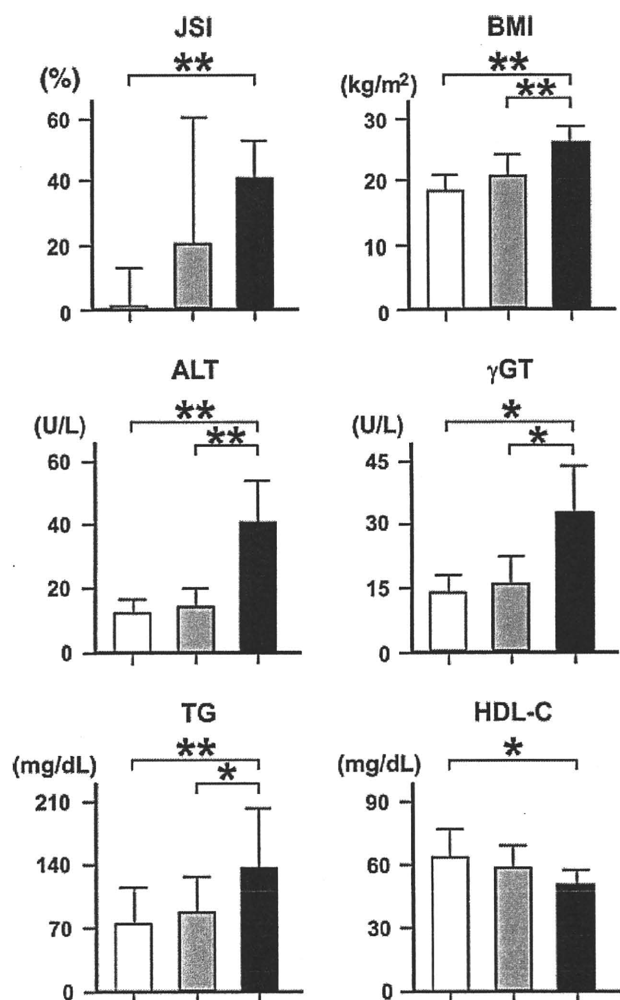

Fig. 1 Comparison of clinical parameters by degree of steatosis. Data were obtained in 2004 and 2007 and were compared among students without steatosis (*white bars*, n = 513), those with mild steatosis (*gray bars*, n = 18), and those with moderate-to-severe steatosis (*black bars*, n = 6). Data are expressed as means ± SD. Statistical analysis was conducted using Tukey's or Games–Howell's analysis. **$P < 0.01$, *$P < 0.05$; *JSI* age-gender-adjusted Japanese standardized weight index for height, *BMI* body mass index, *ALT* alanine aminotransferase, *γGT* γ-glutamyltransferase, *TG* triglycerides, *HDL-C* high-density-lipoprotein cholesterol

NAFLD students than in non-NAFLD ones (Table 5). The ratio of students often commuting to and from school by car was also significantly higher in NAFLD students (Table 5). As expected, the ratios of students liking exercise and doing sports outside of school were significantly lower in the NAFLD group (Table 5). There were no gender differences in the ratios of students having such lifestyle habits.

A similar comparison was performed between obese students with NAFLD ($n = 6$) and those without ($n = 11$). Although there were no remarkable differences in dietary habits, obese NAFLD students showed a higher tendency to commute to and from school by car (80.0% vs. 8.3%, $P = 0.010$) and a lower tendency to do sports outside of school (0 vs. 58.3%, $P = 0.041$) than obese non-NAFLD students. Furthermore, when lifestyle habits were compared between nonobese students with NAFLD ($n = 7$) and those without ($n = 264$), the former group had a higher ratio of drinking more than half of the broth that comes with noodles (87.5% vs. 50.6%, $P = 0.042$) and a lower ratio of doing sports outside of school (0 vs. 31.9%, $P = 0.048$).

Overall, the absence of outside-of-school sports was detected in all students with NAFLD, regardless of obesity, suggesting an important association between the development of NAFLD and reduced physical activity in junior high school students.

Table 4 Comparison of clinical data in 288 junior high school students examined in 2007

NAFLD	(−) ($n = 275$)	(+) ($n = 13$)	P
Male	147 (53.5)	8 (61.5)	0.568
Obesity (JSI ≥ 20%)	11 (4.0)	6 (46.2)	**≤0.001**
ALT ≥30 U/L	3 (1.1)	3 (23.1)	**0.001**
γGT ≥30 U/L	4 (1.5)	3 (23.1)	**0.002**
JSI (%)	−2.2 ± 12.2	26.8 ± 49.9	**0.048**
BMI (kg/m^2)	19.0 ± 2.5	22.5 ± 4.9	**0.025**
ALT (U/L)	14 ± 5	21 ± 14	**0.044**
γGT (U/L)	14 ± 4	20 ± 13	**0.032**
TG (mg/dL)	62 ± 29	83 ± 47	0.136
HDL-C (mg/dL)	68 ± 13	60 ± 8	**0.004**

Data are expressed as numbers (percentages) or means ± SD. P values of less than 0.05 are in bold and underlined. Abbreviations are the same as those in Table 3

Follow up of junior high school students with NAFLD

The 11 junior high school students with NAFLD found in 2004 were advised to come to our hospital for treatment and monitoring. Of these, only two obese students with severe steatosis and elevated ALT and γGT levels received further examination. They were negative for hepatitis B virus surface antigen, anti-hepatitis C virus antibody, and anti-nuclear antibody in sera, had normal levels of serum ceruloplasmin, and had no history of regular intake of drugs, which indicated that their liver dysfunction stemmed from NAFLD. One student reduced dietary calorie intake and started daily walking, and marked attenuation of hepatic steatosis and normalization of serum ALT levels were observed 1 year later. On the other hand, the other student continued to gain weight despite repeated lifestyle instructions, experienced worsened serum ALT levels, and was later diagnosed as having NASH by liver biopsy

Table 5 Lifestyle habits of 288 junior high school students examined in 2007

NAFLD	(−) ($n = 275$)	(+) ($n = 13$)	P
Skipping breakfast (≥2 times/week)	26 (9.5)	4 (30.8)	**0.036**
Eating quickly	118 (42.9)	7 (53.8)	0.444
Drinking more than half of the broth with noodles	140 (50.9)	11 (84.6)	**0.018**
Consuming sweetened drinks every day	131 (47.6)	6 (46.2)	0.907
Eating junk food (≥3 times/week)	209 (76.0)	8 (61.5)	0.186
Eating a midnight snack (≥3 times/week)	115 (41.8)	4 (30.8)	0.423
Watching TV during meals	207 (75.3)	12 (92.3)	0.144
Eating all of the fat around meat	160 (58.2)	9 (69.2)	0.438
Often consuming mayonnaise	152 (55.3)	6 (46.2)	0.509
Eating cakes/sweet rolls (≥3 times/week)	129 (46.9)	5 (38.5)	0.543
Eating convenience store lunches (≥once/week)	35 (12.7)	4 (30.8)	0.084
Often commuting to and from school by car	67 (24.4)	7 (53.8)	**0.026**
Playing computer games (≥1 h/day)	180 (65.5)	10 (76.9)	0.305
Liking exercise	217 (78.9)	8 (61.5)	**0.045**
Doing sports outside of school	82 (29.8)	0 (0)	**0.012**

Data are expressed as numbers (percentages). P values of less than 0.05 are in bold and underlined

NAFLD nonalcoholic fatty liver disease

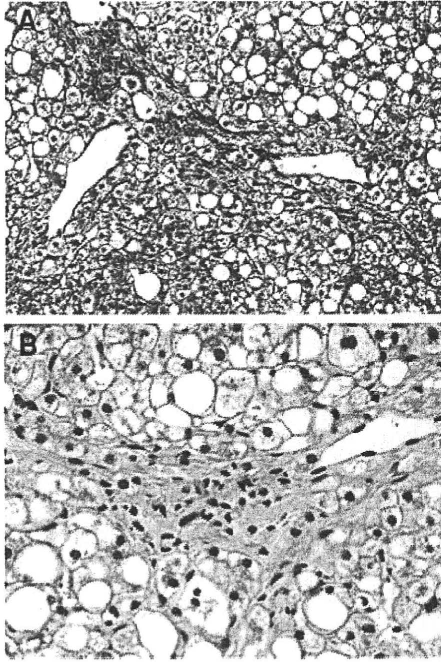

Fig. 2 Histological findings of a liver sample obtained from an obese student with nonalcoholic fatty liver disease (NAFLD). Severe macrovesicular steatosis, hepatocyte ballooning, and pericellular/perivenular fibrosis were evident. The NAFLD activity score was 7. **a** Azan-Mallory staining, ×140; **b** H&E staining, ×300

(Fig. 2). This student is now undergoing treatment for NASH.

Discussion

As far as we know, this is the first epidemiological study on the prevalence and clinical features of NAFLD in Japanese junior high school students. In addition, this study explored for the first time the relationship between the development of young adolescent NAFLD and lifestyle habits. Our results demonstrated that NAFLD in junior high school students occurs in approximately 1 out of 20 students and is primarily associated with obesity and decreased daily physical activity.

Very few population-based epidemiological studies have been conducted on pediatric NAFLD to date. Tominaga et al. [9] reported that the recent prevalence of NAFLD in Japanese children aged 11–15 years was 5.2%. Our findings are similar to these observations.

In the present study, the presence of obesity and an ALT level of 30 U/L or more were identified as independent predictors of NAFLD; anthropometric indices, such as the JSI and BMI, and serum ALT levels all increased with the degree of steatosis. Therefore, inclusion of serum ALT level determination in addition to anthropometric examination may be useful for detecting students with a risk of NAFLD/NASH in annual school-based health checkups. Alavian et al. [12] also described elevation of serum ALT levels to be a superior predictor of NAFLD over several metabolic variables, such as BMI, waist circumference, and serum concentrations of lipid profiles and insulin, in a cross-sectional study of 966 Iranian children aged 7–18 years. Further large-scale population-based prospective studies are needed to verify the usefulness of serum ALT measurement for the early detection of childhood NAFLD.

The most remarkable finding in the present study was that the ratios of students commuting to and from school by car and not doing sports outside of school were significantly higher in students with NAFLD compared with those without. Such tendencies were also detected in nonobese NAFLD students, suggesting that decreased daily physical activity contributes, at least in part, to the occurrence of NAFLD independently of the presence of obesity. This observation is supported by the evidence that regular aerobic exercise reduces hepatic lipids in obese individuals even in the absence of body weight reduction [13]. Thus, strategies to increase daily physical activity might prevent the development of NAFLD/NASH in junior high school students.

Concerning diet, we found a significant relationship between skipping breakfast and the presence of NAFLD. This observation is partially consistent with a previous study that showed skipping breakfast to be strongly associated with childhood obesity [14]. The dietary habit of always drinking most of the broth that comes with noodles might reflect an excess intake of salt, fat, and calories.

There were some NAFLD students without apparent obesity. Because all nonobese NAFLD students had normal concentrations of serum ALT and γGT and mild fatty deposition in the liver, it is inconclusive whether the clinical course of this type of NAFLD is similar to that of typical obesity-related NAFLD. Long-term longitudinal studies are required in such populations to address this issue.

It is well-known that gender differences exist in NAFLD [9, 15]. In the 537 students in our 2004 and 2007 surveys, the prevalence of NAFLD, JSI, and serum ALT and γGT levels were higher in boys. Such differences might result from the gender differences in susceptibility to fat accumulation in the body and insulin resistance [16]. Although gender differences in lifestyle habits could not be found in the present study, further large-scale assessment would enable us to clarify the relationship between gender differences in NAFLD prevalence and lifestyle habits.

Although the natural history of pediatric NAFLD has not been fully explored, some NAFLD cases in children can develop into advanced fibrosis and cirrhosis.

Considering that a high proportion of obese children are likely to become obese adults [17], early detection of pediatric NAFLD and the establishment of appropriate interventions may lead to a decreased incidence of adult NASH. To better predict pediatric NAFLD, we recommend the addition of serum ALT measurement to school-based health screening. More importantly, it remains difficult to convince NAFLD students to receive further examinations and follow up because of child and parent schedules and the lack of any apparent symptoms. To solve this problem, we believe that the establishment of a comprehensive community-based network system to screen for pediatric NAFLD and related lifestyle disorders [18] will be needed in the future.

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Serum Fragmented Cytokeratin 18 Levels Reflect the Histologic Activity Score of Nonalcoholic Fatty Liver Disease More Accurately Than Serum Alanine Aminotransferase Levels

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Background and Goals: Reliable noninvasive biomarkers to assess the histologic activity of nonalcoholic fatty liver disease (NAFLD) have not been established. As the frequency of Mallory bodies is known to be closely associated with the disease severity, we hypothesized that serum levels of Mallory body-related proteins were correlated with NAFLD histologic activity and evaluated this possibility.

Study: Serum levels of total and fragmented cytokeratin (CK) 18, heat shock protein (Hsp) 70, Hsp90α, ubiquitin + 1, and p38α at the time of liver biopsy were measured in 118 NAFLD patients and their association with histologic findings and NAFLD histologic activity score (NAS) was investigated.

Results: Serum levels of both forms of CK18 and Hsp90α were markedly higher in patients having nonalcoholic steatohepatitis (NASH) compared with non-NASH ones. Both forms of CK18 significantly correlated with degree of steatosis, lobular inflammation, and ballooning, and showed stronger positive correlations with NAS than serum aspartate and alanine aminotransferase (AST and ALT). Multiple regression analysis further revealed that fragmented CK18 and AST were effective predictors of NAS, with the former being the more definitive of the two ($P < 0.001$ vs. 0.005). In 20 NAFLD patients who received a follow-up biopsy, changes in fragmented CK18 levels, but not AST or ALT levels, closely paralleled those in NAS.

Conclusions: These results establish the usefulness of fragmented CK18 measurement for assessing and monitoring the histologic activity of NAFLD.

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The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide.[1,2] Nonalcoholic steatohepatitis (NASH) is the severe and progressive form of NAFLD and may develop into cirrhosis, hepatic failure, and hepatocellular carcinoma.[3–5] Several pharmacologic treatments have been developed for NASH, which necessitate the development of appropriate means to monitor disease severity and evaluate therapeutic response.

At present, the activity of NAFLD is measured by typical histologic findings, such as steatosis, hepatocyte ballooning, and lobular inflammation, as well as NAFLD histologic activity score (NAS), all of which are calculated according to the histopathologic criteria designated by the NASH Clinical Research Network.[6] A liver biopsy is considered essential to estimate the activity of NAFLD, but its invasiveness and cost often limit the chances for biopsy. Additionally, as NASH livers are more heterogeneous than those of chronic hepatitis C,[7] sampling errors are prone to occur in percutaneous liver biopsies. Thus, assessment of NAFLD activity using biopsied specimens only may lead to insufficient evaluation.

Serum alanine aminotransferase (ALT) levels are regarded as a reliable biomarker of hepatocyte damage. However, discrepancies in serum ALT concentrations and histologic activity have been documented in chronic hepatitis C patients.[8] It has also been reported that the maintenance of normal ALT levels does not guarantee nonprogression of NASH.[9] Therefore, the development of other noninvasive biomarkers to compensate for the shortcomings of serum ALT measurement is needed for NAFLD/NASH.

Mallory bodies and hepatocyte ballooning, 2 important hallmarks of NASH, are closely associated with disease severity and progression.[3] These pathologies often coexist; it has been demonstrated that 81% of NASH patients with Mallory bodies have ballooned hepatocytes as well.[6] When hepatocytes are chronically exposed to oxidative stress and toxic substances, such as acetaldehyde, they become ballooned, accumulate fat, show a disruption in the keratin

intermediate filament network, and form Mallory bodies.[10,11] A Mallory body is an irregular-shaped aggresome composed of abnormally phosphorylated and cross-linked keratins, such as cytokeratin (CK) 8 and 18, and stress-induced proteins, including ubiquitin and heat shock proteins (Hsp) 70 and 90.[11] These CKs are hyperphosphorylated by p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase, among others. Microarray analysis using primary hepatocytes isolated from mice treated with diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate, an inducer of Mallory body formation in vivo, also uncovered that up-regulation of these proteins was responsible for Mallory body formation.[12] As hepatocytes containing Mallory bodies are susceptible to apoptosis,[13] we hypothesized that levels of Mallory body-associated proteins released from hepatocytes into peripheral blood may be increased in NASH patients and change in accordance with disease activity. To explore this possibility, serum levels of CK18, Hsp90α, Hsp70, p38α, and ubiquitin+1 were determined in 118 biopsy-proven NAFLD patients by enzyme-linked immunosorbent assay (ELISA), and the relationship between the levels of these proteins and histologic findings was examined.

PATIENTS AND METHODS

Patients

In all, 118 Japanese NAFLD patients who had been admitted to Shinshu University Hospital or affiliated hospitals between 2004 and 2006 for liver biopsy were evaluated. The possibility of NAFLD was determined by the following criteria: (1) persistently abnormal ALT levels (>30 U/L) for more than 3 months, (2) no consumption of alcohol or hepatotoxic drugs, (3) negative results for hepatitis B virus surface antigen, high titer of hepatitis B virus core antibody, anti-hepatitis C virus antibody, and autoantibodies, such as antinuclear and antimitochondrial antibodies, (4) the absence of abnormal serum ceruloplasmin levels and transferrin saturation ratios, and (5) the presence of hepatic steatosis in abdominal ultrasonography.[14] The final diagnosis of NAFLD was confirmed by liver histology.

Body height and weight were measured at admission and body mass index (BMI) was calculated. The presence of obesity was defined as having a BMI of more than 25 kg/m^2 based on criteria released by the Japan Society for the Study of Obesity.[15] Patients were considered to be hypertensive if their systolic/diastolic pressure was greater than 140/90 mm Hg or if they were taking antihypertensive drugs. Patients were considered to be diabetic if they had a fasting glucose level equal to or higher than 126 mg/dL or if they were taking insulin or oral hypoglycemic agents. Patients were considered to have hyperlipidemia if their fasting serum levels of cholesterol or triglycerides were equal to or higher than 220 or 150 mg/dL, respectively, or if they were taking lipid-lowering drugs.[16]

Routine Laboratory Examination

Blood samples were obtained at the time of liver biopsy in a fasting state and regular examinations, and complete blood counts and blood chemistries were carried out using standard methods. All remaining sera were immediately frozen and kept at −80°C until use. The homeostasis model assessment for insulin resistance (HOMA-IR) value was calculated as fasting glucose (mg/dL) × fasting insulin (μU/mL)/405.[17]

Measurement of Mallory Body-related Protein Concentrations

Serum concentrations of Mallory body components were determined in duplicate by ELISA from samples obtained on the day of liver biopsy. All measurements were completed within 1 week of sample collection. Serum levels of total and fragmented CK18 were measured by an M65 ELISA kit and M30-Apoptosense ELISA kit, respectively (PEVIVA AB, Bromma, Sweden). Serum Hsp70 levels were analyzed by in-house ELISA as described by Njemini et al.[18] The ELISA kits for Hsp90α and p38α were purchased from Assay Design (Victoria, British Columbia, Canada), and that for ubiquitin+1 was obtained from R&D systems (Minneapolis, MN).

Histopathologic Examination

Liver samples were obtained from 2 different sites in the same lobe using a 14-gauge needle by percutaneous ultrasonography-guided or laparoscopy-assisted biopsy.[19] For patients who underwent a follow-up biopsy, samples were obtained from the same lobe as the initial biopsy. The lengths of the samples obtained were all more than 1.8 cm, and the mean number of portal tracts found in each sample was 11.3 ± 2.9. The specimens were immediately fixed in 10% neutral formalin, and sections were cut at 4 μm thickness and stained by the hematoxylin and eosin or Masson trichrome method. Histologic findings were assessed in a blinded fashion by an independent pathologic expert (K.S.) and scored according to the staging/grading system proposed by Kleiner et al.[6] As a minor modification, Mallory bodies were scored as none to rare (0), few (1), or many (2). NAS was calculated as the unweighted sum of the scores for steatosis (0 to 3), lobular inflammation (0 to 3), and ballooning (0 to 2), and ranged from 0 to 8. The histologic diagnosis of NASH was made according to the presence of hepatocyte ballooning in addition to macrovesicular steatosis.

Ethics

This study was approved by the ethics committee of Shinshu University School of Medicine and adheres to the principles of the Declaration of Helsinki. Informed consent was obtained from all the patients.

Statistical Analysis

Results were expressed as a number (percentage) or mean ± standard deviation (SD). Significance was analyzed by the χ^2 test for categorical variables and the Mann-Whitney U or Wilcoxon signed-rank test for continuous variables. Correlation coefficients were calculated using Spearman rank correlation analysis. To search for independent predictors of NAS, multiple regression analysis using the backward stepwise variable selection method was conducted. All statistical analyses were performed using SPSS software version 15.0 for Windows (SPSS Inc, Chicago, IL). A P value of less than 0.05 was considered to be statistically significant.

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RESULTS

Clinical and Histologic Characteristics of Patients

Clinical and histologic features of 118 NAFLD patients are shown in Tables 1 and 2, respectively. Approximately 60% of cases had obesity and the mean BMI value was 26.5 kg/m^2. According to the histologic findings, patients were divided into those having NASH (n = 105) or not (n = 13). Age, BMI, serum concentrations of cholesterol, glucose, and insulin, glycohemoglobin levels, and HOMA-IR values were significantly increased in NASH patients (Table 1). Mallory bodies were observed in 89 (75%) patients (Table 2).

Serum Concentrations of Mallory Body-related Proteins

Serum concentrations of total CK18, fragmented CK18, and Hsp90α were significantly increased in NASH patients compared with non-NASH ones (Table 1). On the other hand, there were no meaningful differences in the ratio of fragmented to total CK18 levels or serum levels of Hsp70, p38α, and unbiquitin + 1 between the groups (Table 1).

Relationship Between Histologic Findings and Laboratory Data

Correlations between histologic scores and laboratory data were examined. The degree of steatosis positively correlated with serum levels of both forms of CK18, Hsp90α, and ALT, in addition to platelet count (Table 3). The severity of lobular inflammation was correlated with 6 parameters except for platelet count, and especially with serum aspartate aminotransferase (AST) levels (Table 3). The frequency of ballooned hepatocytes was also correlated with both forms of CK18, Hsp90α, and AST levels, as well as with HOMA-IR values (Table 3). Interestingly, total CK18, fragmented CK18, and Hsp90α levels had positive correlations with all NAS components, namely, steatosis, lobular inflammation, and ballooning. Furthermore, Mallory body scores were positively linked to fragmented CK18 and AST levels and HOMA-IR values, but negatively linked to platelet count (Table 3). Lastly, a significant inverse correlation was found between fibrosis stage and platelet count (Table 3).

Relationship Between NAS and Serum Markers

We next evaluated whether our test parameters were correlated with NAS. Of the 6 parameters that were significantly correlated with NAS, serum fragmented CK18 levels showed the strongest positive correlation ($r = 0.485$, $P < 0.001$) (Fig. 1). Multiple regression analysis uncovered that both fragmented CK18 and AST were effective predictors of NAS, with fragmented CK18 being the more definitive of the two (Table 4).

Correlation Between Changes in NAS and Fragmented CK18 Levels

In the 118 NAFLD patients analyzed, 20 underwent follow-up biopsies and were summarized in Table 5. To explore the clinical applicability of fragmented CK18

TABLE 1. Clinical Features of 118 NAFLD Patients

	Normal Values	Non-NASH (n = 13)	NASH (n = 105)	P
Age (y)		40 ± 15	57 ± 16	0.002
Female		3 (23%)	60 (57%)	0.021
Obesity		4 (31%)	63 (60%)	0.046
Type 2 Diabetes		1 (8%)	38 (36%)	0.047
Hypertension		1 (8%)	42 (40%)	0.027
Hyperlipidemia		2 (15%)	41 (39%)	0.113
BMI (kg/m^2)	< 25.0	23.8 ± 2.2	26.9 ± 3.9	0.005
Platelet count (× 10^3/μL)	140-400	222 ± 77	190 ± 64	0.189
C-reactive protein (mg/dL)	< 0.3	0.2 ± 0.2	0.3 ± 0.5	0.596
AST (U/L)	13-33	46 ± 29	61 ± 42	0.072
ALT (U/L)	8-42	77 ± 62	92 ± 76	0.337
γGT(U/L)	10-47	115 ± 61	79 ± 87	0.038
Cholesterol (mg/dL)	130-220	178 ± 33	213 ± 40	0.005
Triglyceride (mg/dL)	30-150	140 ± 55	143 ± 66	0.961
LDL-cholesterol (mg/dL)	< 140	86 ± 0	173 ± 31	0.143
Glucose (mg/dL)	70-110	84 ± 9	115 ± 31	0.001
Glycohemoglobin (%)	4.0-6.0	4.9 ± 0.4	6.0 ± 1.1	0.013
Insulin (μU/mL)	2.0-15.0	7.6 ± 3.4	14.9 ± 10.6	0.010
HOMA-IR	< 2.0	1.6 ± 0.8	4.4 ± 3.6	< 0.001
Ferritin (ng/mL)	20-200	277 ± 203	234 ± 178	0.404
Total CK18 (U/L)		159 ± 34	247 ± 172	0.041
Fragmented CK18 (U/L)		50 ± 45	143 ± 153	0.004
Fragmented CK18/Total CK18		0.4 ± 0.3	0.6 ± 0.5	0.387
Hsp90α(ng/mL)		1.3 ± 2.6	5.9 ± 9.4	0.041
Hsp70 (ng/mL)		0.4 ± 1.5	6.0 ± 16.4	0.117
p38α (ng/mL)		0.3 ± 0.4	0.5 ± 0.7	0.722
Ubiquitin + 1 (ng/mL)		0.9 ± 1.1	0.7 ± 1.4	0.153

In all, 118 biopsy-proven NAFLD patients were classified as those having NASH or not. Results are expressed as a number (percentage) or mean ± SD. Statistical analysis was performed using the χ^2 test or the Mann-Whitney *U* test. The *P* values of less than 0.05 are bold and underlined.

γGT indicates γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CK, cytokeratin; HOMA-IR, homeostasis model assessment for insulin resistance; Hsp, heat shock protein; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

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TABLE 2. Histologic Features of 118 NAFLD Patients

	All (n = 118) (%)	non-NASH (n = 13) (%)	NASH (n = 105) (%)
Steatosis			
0	0 (0)	0 (0)	0 (0)
1	50 (42)	9 (70)	41 (39)
2	41 (35)	2 (15)	39 (37)
3	27 (23)	2 (15)	25 (24)
Lobular inflammation			
0-1	41 (35)	12 (92)	29 (28)
2	55 (47)	0 (0)	55 (52)
3	22 (19)	1 (8)	21 (20)
Ballooning			
0	13 (11)	13 (100)	0 (0)
1	90 (76)	0 (0)	90 (86)
2	15 (13)	0 (0)	15 (14)
Fibrosis			
0	9 (8)	7 (54)	2 (2)
1	62 (53)	4 (31)	58 (55)
2	15 (13)	0 (0)	15 (14)
3	27 (23)	2 (15)	25 (24)
4	5 (4)	0 (0)	5 (5)
Mallory body			
0	29 (25)	11 (85)	18 (17)
1	57 (48)	2 (15)	55 (52)
2	32 (27)	0 (0)	32 (31)
NAS			
1	1 (1)	1 (8)	0 (0)
2	7 (6)	7 (54)	0 (0)
3	16 (14)	2 (15)	14 (13)
4	32 (27)	3 (23)	29 (28)
5	28 (24)	0 (0)	28 (27)
6	22 (19)	0 (0)	22 (21)
7	10 (9)	0 (0)	10 (9)
8	2 (2)	0 (0)	2 (2)

The amount of Mallory bodies was divided into none to rare (0), few (1), or many (2).

Results are expressed as a number (percentage).

NAFLD indicates nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

measurement in the evaluation and monitoring of NAFLD activity, we examined the relationship between alterations in NAS and those in fragmented CK18 levels in these patients and compared them with those in AST and ALT levels. A reduction in NAS was observed in 13 (65%) subjects (from 5.8 ± 1.4 to 3.8 ± 1.3, $P = 0.001$). Of them, a remarkable NAS improvement of 3 points or more was detected in 3 patients; one stemmed from lifestyle correction and the others from treatment with highly purified eicosapentaenoic acid.[20] In the NAS-improved group, decreases in fragmented CK18, AST, and ALT levels were found in 12 (92%), 12 (92%), and 11 (85%) patients, respectively. These decreases were significant for fragmented CK18 (from 305 ± 225 to 150 ± 79 U/L, $P = 0.018$), AST (from 66 ± 29 to 38 ± 11 U/L, $P = 0.001$), and ALT levels (from 86 ± 70 to 39 ± 18 U/L, $P = 0.024$).

Conversely, NAS had deteriorated in 7 (35%) patients at the time of the second biopsy (from 4.4 ± 1.6 to 6.3 ± 1.7, $P = 0.016$). Corresponding increases in fragmented CK18 levels were observed in 6 (86%) patients, but those in AST and ALT levels were found in only 2 (29%) patients each. Although not statistically significant, fragmented CK18 levels tended to increase (from 90 ± 42 to 275 ± 276 U/L, $P = 0.071$) in the NAS-deteriorated group, but not AST (from 74 ± 37 to 52 ± 27 U/L, $P = 0.352$) or ALT levels (from 122 ± 69 to 85 ± 63 U/L, $P = 0.345$).

Lastly, although changes in fragmented CK18 levels were correlated with those in NAS ($r = 0.748$, $P < 0.001$), the same was not true for changes in AST ($r = 0.428$, $P = 0.060$) or ALT levels ($r = 0.440$, $P = 0.052$) (Fig. 2).

DISCUSSION

Previous studies have revealed the usefulness of serum fragmented CK18 assays for differentiating NASH from NAFLD or predicting the presence of NASH.[21–24] Recently, Diab et al[25] showed a significant positive correlation between serum levels of fragmented CK18 and NAS in 65 NAFLD patients who had undergone bariatric surgery. Although they also reported that fragmented CK18 levels were markedly decreased 6 months afterwards, it was inconclusive whether such a decrease actually reflected an improvement in NAS because of the lack of follow-up biopsies. In the current study, we demonstrated that serum fragmented CK18 levels were closely correlated with individual NAS components and overall NAS in 118 patients with biopsy-proven NAFLD. Multiple regression analysis revealed that fragmented CK18 levels were a strong predictor of NAS. Furthermore, we uncovered for the first time that the degree of changes in fragmented CK18 levels corresponded with those in NAS in NAFLD patients using evidence from paired biopsies. Therefore, these results not only corroborate previous observations, but also demonstrate the potential utility of fragmented CK18 as a reliable noninvasive biomarker to monitor disease activity and evaluate the therapeutic response of NAFLD patients.

TABLE 3. Correlations Between Histologic Findings and Laboratory Data Among 118 NAFLD Patients

	Steatosis	Lobular Inflammation	Ballooning	Mallory Body	Fibrosis
Platelet	0.376**	—	—	−0.250**	−0.432**
AST	—	0.471**	0.205*	0.290**	0.361**
ALT	0.213*	0.355**	—	—	—
HOMA-IR	—	0.362**	0.311**	0.353**	0.376**
Total CK18	0.352**	0.264**	0.325**	—	—
Fragmented CK18	0.482**	0.295**	0.215**	0.200*	—
Hsp90α	0.272**	0.194*	0.236*	—	—

Correlation coefficients were calculated by Spearman rank correlation analysis.

*$P < 0.05$; **$P < 0.01$.

AST indicates aspartate aminotransferase; ALT, alanine aminotransferase; CK, cytokeratin; HOMA-IR, homeostasis model assessment for insulin resistance; Hsp, heat shock protein; NAFLD, nonalcoholic fatty liver disease; −, not significant.

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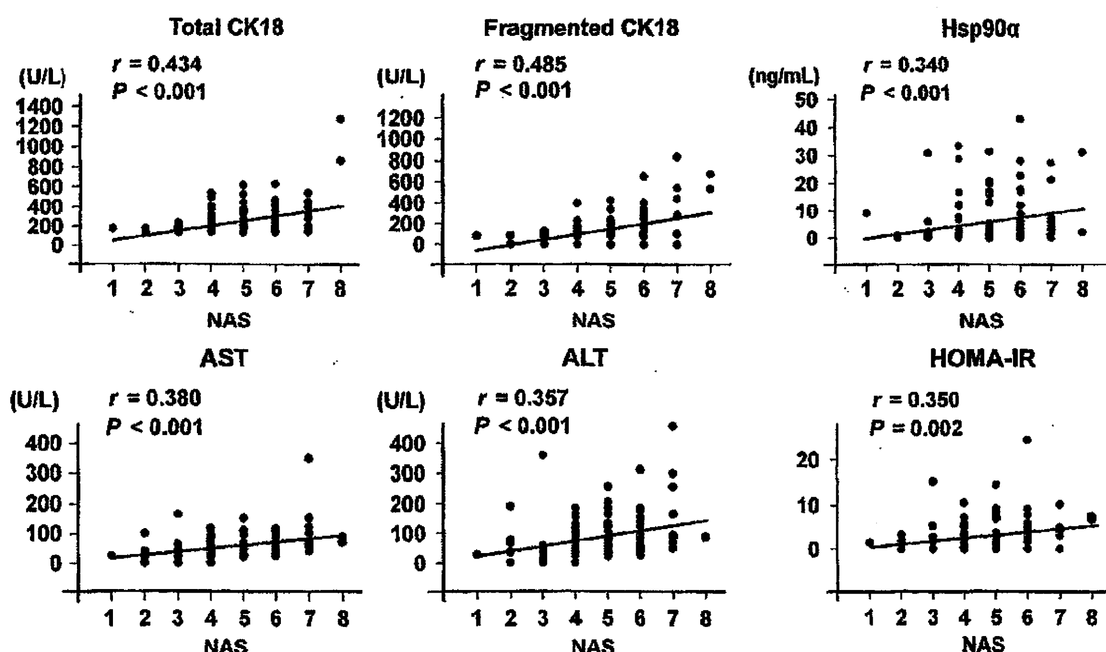

FIGURE 1. Correlations between NAS and laboratory data in 118 biopsy-proven NAFLD patients. Correlation coefficients were calculated using Spearman rank correlation analysis. NAFLD indicates nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Serum fragmented CK18 levels were strikingly higher in NASH patients than in non-NASH ones, which is consistent with previous reports.[21,22] As hepatocyte apoptosis is one of the key components involved in the progression of steatosis to steatohepatitis and caspase 3 reported to be activated in NASH livers,[26] it is conceivable that CK18 fragments, generated mainly by the activation of caspase 3, are increased in the sera of NASH patients. On the other hand, total CK18 is released from cells during necrosis.[27] In this study, serum total CK18 levels were increased with fragmented CK18 levels, but the ratio of fragmented to total CK18 levels, which is an indicator of ascendancy of apoptotic cell death,[27] did not differ between the NAFLD subgroups. Thus, we can speculate that an increase in total CK18 might also contribute to an increase in serum fragmented CK18 levels in patients with NASH. Additionally, as hepatic mRNA expression of CK18 has been reported to be up-regulated by oxidative stress in mice,[28] augmentation of CK18 expression in NASH livers might be associated with the increases in serum levels of both forms of CK18.

An unexpected finding in this study was that serum fragmented CK18 levels showed the strongest correlation with degree of steatosis among the histologic parameters. Overproduction of reactive oxygen species and the ensuing augmentation of oxidative stress may occur in fat-engorged hepatocytes, as reactive oxygen species are generated mainly through mitochondrial fatty acid β-oxidation, whose pathway is enhanced by an excess of fatty acids.[29–32] Accumulation of fatty acids and lipid peroxides in hepatocytes may thus trigger activation of caspase 3 and promote cleavage of total CK18.[29,33] A similar significant association between the extent of steatosis and fragmented CK18 levels has already been elucidated in patients with chronic hepatitis C.[34]

As far as we know, this is the first study to assess the relationship between histologic findings in NAFLD and serum concentrations of Mallory body-related proteins. Not only fragmented CK18 levels, but also total CK18 and Hsp90α levels, were correlated with both NAS and all individual NAS factors. This finding is in agreement with the established fact that the presence of Mallory bodies is an indicator of the severity of NASH.[3,35,36]

TABLE 4. Selected Results of Multiple Regression Analysis

	Partial Regression Coefficient	Standardized Partial Regression Coefficient	P	95% Confidence Interval
Fragmented CK18	0.004	0.442	< 0.001	0.002-0.006
AST	0.010	0.287	0.005	0.003-0.016

Multiple regression analysis was performed using the backward stepwise variable selection method. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase, total and fragmented cytokeratin (CK) 18, and heat shock protein 90α, as well as homeostasis model assessment for insulin resistance values were selected as independent variables. Multiple correlation coefficient and coefficient of determination were 0.550 and 0.302, respectively.

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