Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Down-regulation of SREBP-1c is associated with the development of burned-out NASH

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Background & Aims: It is well-known that hepatic triglycerides (TG) diminish with the progression of non-alcoholic steatohepatitis (NASH), which has been designated as burned-out NASH, but its mechanism remains unclear. We aimed to explore the changes in hepatic fatty acid (FA) and TG metabolism with disease progression.

Methods: Hepatic expression of key genes in healthy individuals ($n = 6$) and patients with simple steatosis (SS, $n = 10$), mild NASH (fibrosis stage 1–2, $n = 20$), and advanced NASH (fibrosis stage 3–4, $n = 20$) were assessed by quantitative polymerase chain reaction.

Results: Hepatic expression of genes related to FA uptake and oxidation and very-low-density lipoprotein synthesis/export did not differ among the groups. However, the mRNA levels of sterol regulatory element-binding protein (SREBP)-1c and its downstream genes FA synthase, acetyl-coenzyme A carboxylase 1, and diacylglycerol acyltransferase 1 were inversely correlated with fibrosis stage. Immunoblot analysis revealed a remarkable reduction in mature SREBP-1c levels in advanced NASH. Furthermore, hepatic expression of tumor necrosis factor-α increased in accordance with fibrosis progression, which was possibly related to the decrease in hepatic SREBP-1c expression.

Conclusions: Down-regulation of SREBP-1c and lipogenic enzymes may be associated with the development of burned-out NASH.

Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and is estimated to afflict approximately 20% of the general population in developed countries [1,2]. NAFLD can be divided into simple steatosis (SS) and non-alcoholic steatohepatitis (NASH) by histological findings. NASH is characterized by the presence of ballooning and lobular inflammation in addition to macrovesicular steatosis [3,4] and may progress to cirrhosis, hepatocellular carcinoma, and ultimately death [4,5]. Lipotoxicity, oxidative stress, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, bacterial lipopolysaccharides, and iron accumulation in the liver are presumed to trigger the progression of steatosis to steatohepatitis [1,6].

The hallmark feature of NAFLD/NASH is the accumulation of triglycerides (TG) in the liver. Sources of intrahepatic TG include non-esterified fatty acids (FAs) released from adipose tissue and taken up from the blood, as well as those newly synthesized from citrate. FAs are later metabolized mainly by β-oxidation or by esterification to produce TG, which are either stored in hepatocytes or incorporated into very-low-density lipoprotein (VLDL) particles for export. An imbalance among these metabolic pathways may lead to hepatic steatosis [1,6].

Keywords: Burned-out NASH; SREBP-1c; TNF-α.

Received 11 January 2010; received in revised form 16 March 2010; accepted 11 April 2010; available online 1 July 2010

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Abbreviations: ACC, acetyl-coenzyme A carboxylase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; ANGPTL4, angiopoietin-like protein 4; AOX, acyl-coenzyme A oxidase; apo, apolipoprotein; AST, aspartate aminotransferase; BMI, body mass index; ChREBP, carbohydrate regulatory element-binding protein; CoA, coenzyme A; CPT, carnitine palmitoyl-coenzyme A transferase; CYP, cytochrome P450; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FABP, fatty acid-binding protein; FAS, fatty acid synthase; FAT, fatty acid translocase; FSP, fat-specific protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; γGT, γ-glutamyltransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment for insulin resistance; IL, interleukin; LXR, liver X receptor; MCAD, medium-chain acyl-coenzyme A dehydrogenase; MTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD histological activity score; NASH, non-alcoholic steatohepatitis; PDK, pyruvate dehydrogenase; PGC, peroxisome proliferator-activated receptor-γ co-activator; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative polymerase chain reaction; RXR, retinoid X receptor; SCD, stearoyl-coenzyme A desaturase; SREBP, sterol regulatory element-binding protein; SS, simple steatosis; TG, triglycerides; TNF, tumor necrosis factor; TNR, TNF receptor; TP, trifunctional protein; US, ultrasonography; VLDL, very-low-density lipoprotein.

In 1990, Powell et al. first reported a NASH patient who progressed to cirrhosis without hepatic fat deposition in 5 years [7]. Other reports have also demonstrated a significant reduction in hepatic TG accumulation in patients with advanced NASH, which is also designated as burned-out NASH [5,8]. This phenomenon implicates altered hepatic FA/TG metabolism with progression of NAFLD, but its precise molecular mechanism remains unclear. To clarify this, we assessed the hepatic expression of key genes involved in FA/TG metabolism in patients having SS, NASH with mild fibrosis, and NASH with severe fibrosis.

Patients and methods

Patients

Fifty NAFLD patients who underwent a liver biopsy at Shinshu University or its affiliated hospitals between April 2006 and March 2008 were examined. Liver samples included those with SS ($n = 10$), mild NASH (steatohepatitis with fibrosis stage 1–2, $n = 20$), and advanced NASH (steatohepatitis with fibrosis stage 3–4, $n = 20$). NAFLD was suspected by the following criteria: (1) the detection of steatosis by abdominal ultrasonography (US) [9]; (2) the absence of regular intake of alcohol or drugs; (3) negative results for hepatitis B virus (HBV) surface antigen and anti-HBV core and anti-hepatitis C virus (HCV) antibodies; and (4) the absence of other types of chronic liver disease, such as autoimmune liver disease, hereditary hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, and citrin deficiency [10]. The diagnosis of NAFLD was confirmed by liver histology.

The presence of obesity was defined as having a body mass index (BMI) of more than 25 kg/m^2 based on criteria released by the Japan Society for the Study of Obesity. Patients were considered to be hypertensive if their systolic/diastolic pressure was greater than 140/90 mmHg. Patients were considered to be diabetic if they had a fasting glucose level equal to or higher than 126 mg/dl. Patients were considered to have hyperlipidemia if their fasting serum levels of cholesterol or TG were equal to or higher than 220 or 150 mg/dl, respectively [11].

Selection of normal controls

Normal livers were obtained from healthy liver transplantation donors ($n = 6$) at the time of pre-operative percutaneous US-guided liver biopsy who satisfied the following criteria: (1) the absence of past history of liver disease and regular intake of alcohol and drugs; (2) the absence of obesity, diabetes, hypertension, and hyperlipidemia; (3) normal liver function tests; and (4) normal liver histology.

Laboratory examination

Blood samples were obtained at the time of liver biopsy following overnight fasting for 8–10 h. Laboratory data, such as aspartate and alanine aminotransferase (AST and ALT, respectively) and γ-glutamyltransferase (γGT), were measured by standard methods using automated analyzers. The homeostasis model assessment for insulin resistance (HOMA-IR) value was calculated as fasting glucose (mg/dl) × immunoreactive insulin (μU/ml)/405.

Histological and immunohistochemical analyses

Percutaneous US-guided liver biopsies were performed as described previously [12]. The average length of the samples was 17.6 ± 2.8 mm, and the average number of portal tracts found in each sample was 11.8 ± 3.5. Fragments of liver tissue (5–7 mm) were immediately frozen with an RNA stabilization solution (RNA*later*® solution, Applied Biosystems, Foster City, CA, USA) in liquid nitrogen and stored at −80 °C until RNA extraction. The remaining tissues were fixed in 10% neutral formalin, embedded in paraffin, cut at 4 μm thickness, and stained with the hematoxylin and eosin or Azan-Mallory method. Histological findings were assessed by an independent experienced pathologist (KS) in a blinded fashion and scored according to the staging/grading system proposed by Kleiner et al. [13]. The NAFLD histological activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). The histological diagnosis of steatohepatitis was made by the presence of macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation [3,4,13]. Patients with macrovesicular steatosis alone were diagnosed as having SS.

Immunohistochemical staining of sterol regulatory element-binding protein (SREBP)-1c was carried out as described elsewhere [14]. The antibody used in this study (sc-367, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) can recognize two SREBP-1 isoforms, SREBP-1c and SREBP-1a. Since the expression of SREBP-1c is predominant in human livers [15,16], the results of this immunohistochemical analysis were regarded to reflect the expression of SREBP-1c. Sections (4 μm thick) were incubated for one hour with the anti-SREBP-1 antibody (1:50 dilution) and immunostained using a Histofine Simple Stain MAX-PO (MULTI) kit with 3,3′-diaminobenzidine as a substrate (Nichirei Biosciences Inc., Tokyo, Japan). The stained sections were viewed with an Olympus DP-70 microscope (Olympus, Tokyo, Japan). The number of SREBP-1c-positive hepatocyte nuclei was counted for each section and expressed as a percentage of all hepatocytes.

Analysis of mRNA expression

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and cDNA was prepared with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Quantitative polymerase chain reaction (qPCR) was performed using a SYBR Green PCR kit and ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA). The primer sequences are shown in Supplementary Table 1, whose specificity has been confirmed by nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All mRNA levels were determined using the ΔΔCt method as described previously [17]. The mRNA levels of target genes were normalized to those of 18S ribosomal RNA and expressed as fold changes relative to those of normal livers.

Immunoblot analysis

Preparation of whole liver lysates was carried out as described previously [18]. Protein concentration was measured colorimetrically with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). Whole liver lysates (100 μg of protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [19]. Three samples from each group were loaded into each electrophoresis assay and all samples were examined. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with primary antibodies (1:200 dilution) against SREBP-1 (sc-367), peroxisome proliferator-activated receptor (PPAR) α (sc-9000), PPARδ (sc-7197), PPARγ (sc-7196), or retinoid X receptor (RXR) α (sc-553), all purchased from Santa Cruz Biotechnology Inc., followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The positions of precursor and mature SREBP-1c bands were determined by molecular weight (125 and 68 kDa, respectively). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Band intensities were measured densitometrically, normalized to those of GAPDH, and subsequently expressed as fold changes relative to those of normal livers.

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration and was approved by each hospital's respective human ethics committee. Informed consent was obtained from all patients.

Statistical analysis

Statistical analyses were performed using SPSS software version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Clinical parameters were expressed as a number (percentage) or mean ± SEM, and mRNA and protein levels were presented as mean ± SEM. Comparisons between groups were made using the Kruskal–Wallis test with Bonferroni's correction. Correlation coefficients were calculated using Spearman's rank correlation analysis. All p values were based on a two-sided test for statistical significance. A p value of less than 0.05 was considered to be statistically significant.

Results

Clinical and histological findings

The clinical and histological findings of 56 participants are summarized in Tables 1 and 2, respectively. In 50 NAFLD patients, BMI, serum levels of AST, ALT, and γGT, and HOMA-IR values

Genetic and Metabolic

Table 1. Clinical features of subjects enrolled in this study.

	Normal controls (n = 6)	Simple steatosis (n = 10)	Mild NASH (n = 20)	Advanced NASH (n = 20)
Age (yrs)	43 ± 11	41 ± 16	57 ± 14	63 ± 12
Female	2 (30%)	3 (33%)	12 (60%)	12 (67%)
Obesity	0 (0%)	6 (67%)	12 (60%)	14 (78%)
Type 2 Diabetes	0 (0%)	0 (0%)	4 (20%)	6 (33%)
Hypertension	0 (0%)	0 (0%)	6 (30%)	12 (67%)
Hyperlipidemia	0 (0%)	1 (11%)	8 (40%)	6 (33%)
BMI (kg/m^2)	22.3 ± 1.6	26.2 ± 3.4	26.9 ± 4.3	28.6 ± 4.7
Platelet count ($\times 10^3/\mu l$)	221 ± 40	263 ± 56	206 ± 57	169 ± 62
C-reactive protein (mg/dl)	0.1 ± 0.1	0.1 ± 0.1	0.5 ± 1.0	0.3 ± 0.4
Albumin (g/dl)	4.3 ± 0.3	4.6 ± 0.2	4.6 ± 0.3	4.4 ± 0.5
AST (U/L)	17 ± 6	31 ± 9	62 ± 33	74 ± 36
ALT (U/L)	17 ± 8	56 ± 21	96 ± 71	94 ± 73
γGT (U/L)	18 ± 5	66 ± 60	71 ± 44	77 ± 43
Total cholesterol (mg/dl)	164 ± 17	195 ± 40	221 ± 33	193 ± 38
Triglycerides (mg/dl)	81 ± 35	167 ± 86	157 ± 89	139 ± 73
HDL-cholesterol (mg/dl)	52 ± 12	45 ± 8	50 ± 10	53 ± 16
LDL-cholesterol (mg/dl)	108 ± 25	149 ± 12	129 ± 29	109 ± 23
Glucose (mg/dl)	95 ± 12	103 ± 19	118 ± 36	119 ± 31
Glycohemoglobin (%)	5.1 ± 0.7	5.3 ± 0.6	5.9 ± 0.9	5.9 ± 1.2
Insulin (μU/ml)	7.9 ± 10.3	18.3 ± 11.7	19.6 ± 17.2	26.1 ± 11.5
HOMA-IR	1.8 ± 1.3	4.8 ± 3.2	5.9 ± 6.2	7.9 ± 4.2
Ferritin (ng/ml)	162 ± 102	228 ± 235	177 ± 118	292 ± 217

Results are expressed as a number (percentage) or mean ± SEM. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; HDL; high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance.

were significantly greater in the advanced NASH group than in the other groups and increased with disease progression (r = 0.332, p = 0.017 for BMI; r = 0.664, p <0.001 for serum AST; r = 0.398, p = 0.002 for serum ALT; r = 0.480, p <0.001 for serum γGT; and r = 0.509, p <0.001 for HOMA-IR), but platelet count and serum albumin concentrations were inversely correlated with fibrosis stage (r = –0.502, p <0.001 and r = –0.421, p = 0.008, respectively). Lobular inflammation and ballooning scores were both positively correlated with fibrosis stage (r = 0.495 and 0.559, respectively, both p <0.001). The degree of steatosis was the greatest in the mild NASH group, which was significantly different from that in the advanced NASH group (p = 0.008) (Table 2, Fig. 1). Steatosis scores also demonstrated a significant inverse correlation with fibrosis stage (r = –0.380, p = 0.010). These results indicate that steatosis decreased with fibrosis progression in our cohort, which is consistent with previous reports [5,8].

Hepatic expression of genes associated with FA oxidation and VLDL synthesis/export

The mRNA levels of genes encoding FA-metabolizing enzymes were measured next, but revealed no differences in the expression of mitochondrial β-oxidation enzymes [carnitine palmitoyl-coenzyme A (CoA) transferase (CPT) 1, medium-chain acyl-CoA dehydrogenase (MCAD), and trifunctional protein (TP)], peroxisomal β-oxidation enzyme [acyl-CoA oxidase (AOX)], or microsomal ω-oxidation enzymes [cytochrome P450 (CYP) 4A11 and CYP2E1] among the groups (Fig. 2A). Similarly, there were no significant differences in the mRNA levels of microsomal TG transfer protein (MTP) or apolipoprotein (apo) B (Fig. 2B). Based on these results, enhancement of FA oxidation and VLDL synthesis/export is not likely to be responsible for attenuation of hepatic steatosis in advanced NASH.

Table 2. Histological features of the subjects.

		Normal controls (n = 6)	Simple steatosis (n = 10)	Mild NASH (n = 20)	Advanced NASH (n = 20)
Steatosis	0/1/2/3	6/0/0/0	0/7/3/0	0/1/10/9	0/17/3/0
	Percentage	0	23 ± 11	35 ± 16	16 ± 14
Lobular inflammation	0/1/2/3	6/0/0/0	0/9/1/0	0/1/14/5	0/1/11/8
Ballooning	0/1/2	6/0/0	10/0/0	0/17/3	0/13/7
Fibrosis	0/1/2/3/4	6/0/0/0/0	10/0/0/0/0	0/11/9/0/0	0/0/0/12/8
NAS		0	2.3 ± 0.5	5.8 ± 1.0	5.4 ± 1.0

Fifty biopsy-proven NAFLD patients were classified as having simple steatosis, mild NASH (fibrosis stage 1–2), or advanced NASH (fibrosis stage 3–4). Results are expressed as a number or mean ± SEM. NAS, NAFLD activity score.

Diseases

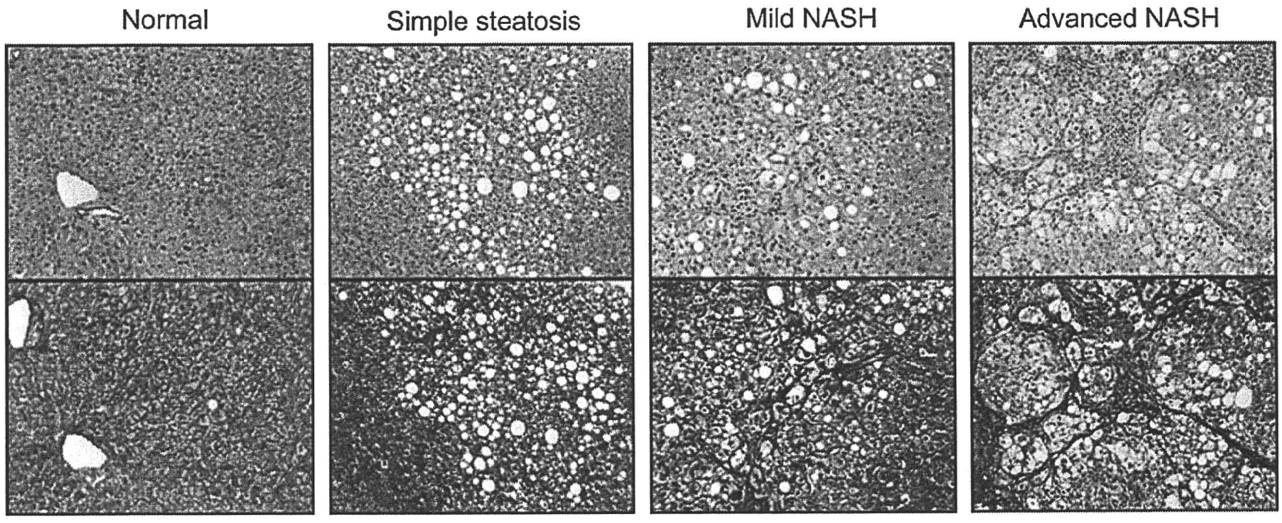

Fig. 1. Representative histological findings of the liver stained with hematoxylin and eosin (upper panels) or the Azan–Mallory method (lower panels). Original magnification, ×100.

Hepatic expression of genes associated with FA uptake from the blood

The expression of FA translocase (FAT), FA-binding protein (FABP) 1, and FABP4 was examined, but no significant differences in the mRNA levels of these FA transporters were found (Fig. 2C).

Decreased hepatic expression of genes associated with de novo lipogenesis in advanced NASH

We next assessed the expression of genes that were associated with *de novo* FA/TG synthesis. The mRNA levels of FA synthase (FAS) were significantly lower in the mild NASH and advanced NASH groups than in the SS group ($p = 0.045$ and 0.007, respectively) (Fig. 3). The expression of acetyl-CoA carboxylase (ACC) 1 was also significantly decreased in the advanced NASH group compared with the SS group ($p = 0.015$), as was the expression of diacylglycerol acyltransferase (DGAT) 1 ($p = 0.008$) (Fig. 3). Furthermore, the mRNA levels of these enzymes demonstrated significant inverse correlations with fibrosis stage ($r = -0.427$, $p < 0.001$ for FAS; $r = -0.338$, $p = 0.015$ for ACC1; and $r = -0.367$, $p = 0.010$ for DGAT1). On the other hand, the mRNA levels of other lipogenic enzymes, including ACC2, stearoyl-CoA desaturase (SCD), and DGAT2 did not differ among the groups (Fig. 3).

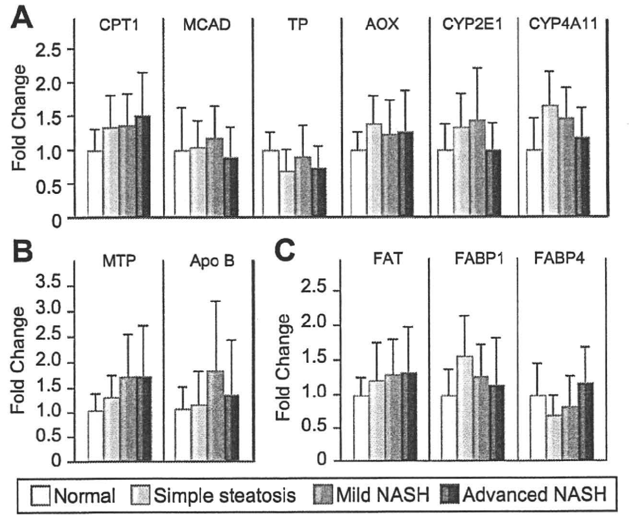

Fig. 2. Hepatic mRNA levels of genes involved in (A) FA oxidation, (B) VLDL synthesis/export, and (C) FA uptake. Samples were obtained from normal livers ($n = 6$) or livers with SS ($n = 10$), mild NASH ($n = 20$), or advanced NASH ($n = 20$). Results are expressed as mean ± SEM.

Decreased SREBP-1c mRNA levels in advanced NASH

Since the mRNA levels of the above lipogenic enzymes are known to be regulated by SREBP-1c [6,20], its expression was evaluated next. The mRNA levels of SREBP-1c were positively correlated with those of FAS ($r = 0.570$, $p < 0.001$), ACC1 ($r = 0.572$, $p < 0.001$), and DGAT1 ($r = 0.516$, $p < 0.001$). In addition to meaningful differences between the SS group and mild NASH or advanced NASH group ($p = 0.049$ and 0.010, respectively) (Fig. 4A), a significant inverse correlation was evident between SREBP-1c mRNA levels and fibrosis stage ($r = -0.392$, $p = 0.009$). On the other hand, there were no statistical differences between NAFLD stages in the expression of liver X receptor (LXR) α or

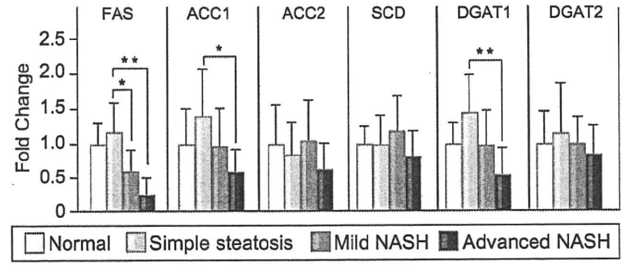

Fig. 3. Hepatic mRNA levels of genes involved in *de novo* lipogenesis. Samples were obtained from normal livers ($n = 6$) or livers with SS ($n = 10$), mild NASH ($n = 20$), or advanced NASH ($n = 20$). Results are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

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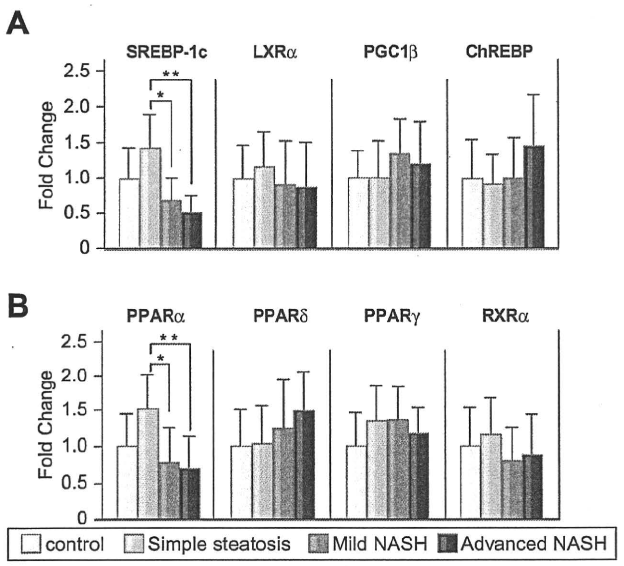

Fig. 4. Hepatic mRNA levels of genes encoding transcription factors associated with (A) SREBP-1c and (B) PPARs. Samples were obtained from normal livers ($n = 6$) or livers with SS ($n = 10$), mild NASH ($n = 20$), or advanced NASH ($n = 20$). Results are expressed as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$.

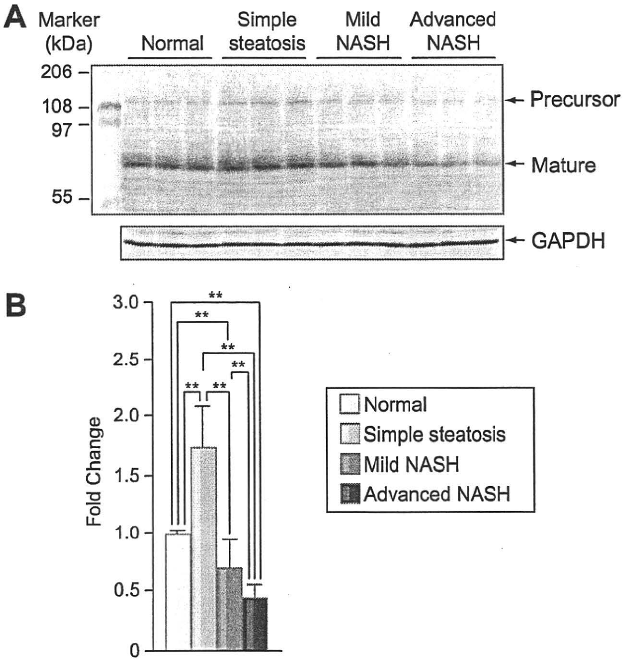

Fig. 5. Immunoblot analysis of SREBP-1c (A) and quantification of mature SREBP-1c levels (B). Samples were obtained from normal livers ($n = 6$) or livers with SS ($n = 10$), mild NASH ($n = 20$), or advanced NASH ($n = 20$). Three samples from each group were loaded into each electrophoresis assay and all samples were examined. (A) A representative blot is shown in panel. The mature form of SREBP-1c is detected as a 68 kDa band. Band intensities of mature SREBP-1c were quantified densitometrically, normalized to those of GAPDH, and subsequently expressed as fold changes relative to those of normal livers. (B) Results are expressed as mean ± SEM. $^{**}p < 0.01$.

PPARγ co-activator (PGC) 1β, both co-regulators of the SREBP-1c-mediated pathway, or in carbohydrate regulatory element-binding protein (ChREBP) among the groups (Fig. 4A).

Decreased mature SREBP-1c levels in advanced NASH

The activity of SREBP-1c is influenced by several post-transcriptional modification steps, i.e., processing of precursor SREBP-1c proteins (maturation) and translocation of mature SREBP-1c proteins into the nucleus [6,20]. To confirm whether mature SREBP-1c levels were also reduced in advanced NASH, immunoblot analysis was performed. Hepatic levels of mature SREBP-1c were significantly increased in the SS group compared with normal controls ($p < 0.001$), but were significantly decreased in the mild and advanced NASH groups compared with the SS group (both $p < 0.001$) (Fig. 5). Mature SREBP-1c levels were positively correlated with those of FAS ($r = 0.403$, $p = 0.004$), ACC1 ($r = 0.359$, $p = 0.014$), and DGAT1 ($r = 0.355$, $p = 0.021$), and were inversely correlated with fibrosis stage ($r = -0.702$, $p < 0.001$).

The degree of nuclear translocation of SREBP-1c was also determined by immunohistochemical analysis. Hepatocyte nuclei intensely stained with anti-SREBP-1 antibody were observed preferentially in the SS group, but were extremely rare in the advanced NASH group (black arrowheads in Fig. 6A). Furthermore, although hepatocytes showing cytoplasmic staining for SREBP-1c were detected mainly in both NASH subgroups, their intensity was comparatively weaker in the advanced NASH group (black arrows in Fig. 6A). Lastly, the number of SREBP-1c-positive hepatocyte nuclei was significantly increased in the SS group compared with normal controls ($p < 0.001$), but was significantly decreased in the mild and advanced NASH groups compared with the SS group (both $p < 0.001$) (Fig. 6B).

Taken together, these results demonstrate that down-regulation of SREBP-1c and its downstream genes is associated with the attenuation of hepatic steatosis in advanced NASH, namely, the development of burned-out NASH.

Hepatic expression of PPARs and RXRα

The expression of PPARs, another group of transcriptional factors involved in hepatic FA/TG metabolism, were also addressed in this study. The mRNA levels of PPARα were decreased in the mild NASH and advanced NASH groups compared with the SS group ($p = 0.038$ and 0.007, respectively) (Fig. 4B), and were inversely correlated with the stage of fibrosis ($r = -0.432$, $p = 0.003$). However, immunoblot analysis revealed no significant differences in PPARα protein levels among the groups (Fig. 7). Furthermore, the levels of PPARδ, PPARγ, and a heterodimeric partner of PPARs, RXRα [21], remained unchanged in qPCR and immunoblot analyses (Figs. 4B and 7). Consistent with the immunoblot results, there were no meaningful differences in the mRNA levels of the PPARα target genes, CPT1, MCAD, AOX and FABP1 [22–25], the PPARδ/α target genes, pyruvate dehydrogenase kinase (PDK) 4 and angiopoietin-like protein 4 (ANGPTL4) [26,27], or the PPARγ target genes, FABP4 and fat-specific protein 27 (FSP27) [28,29], among the groups (Fig. 2 and Supplementary Fig. 1).

Increased expression of pro-inflammatory cytokines in advanced NASH

The mRNA levels of SREBP-1c are reported to be down-regulated by several pro-inflammatory cytokines [30]. We observed that the expression of TNF-α and its receptors, TNR1 and TNR2, was increased in parallel with fibrosis progression in NAFLD patients ($r = 0.442$, $p = 0.008$ for TNF-α; $r = 0.377$, $p = 0.033$ for TNR1; and

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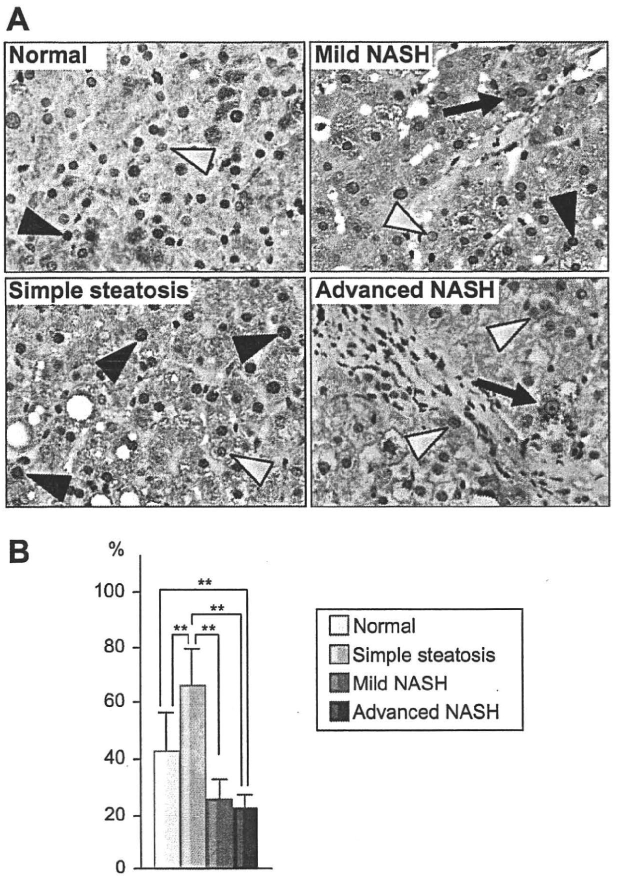

Fig. 6. Immunohistochemistry of SREBP-1c (A) and quantification of SREBP-1c-positive hepatocyte nuclei. (B) Samples were obtained from normal livers (n = 6) or livers with SS (n = 10), mild NASH (n = 20), or advanced NASH (n = 20). SREBP-1c-positive hepatocyte nuclei (black arrowheads) were frequent in SS livers, but rare in advanced NASH livers. Rather, hepatocytes showing cytoplasmic staining for SREBP-1c (arrows) were detected in NASH livers. Yellow arrowheads indicate SREBP-1c-negative hepatocyte nuclei. The number of SREBP-1c-positive nuclei was counted in each section and expressed as a percentage of all hepatocyte nuclei. (B) Results are expressed as mean ± SEM. **p <0.01.

r = 0.383, p = 0.037 for TNR2) (Fig. 8). The mRNA levels of TNF-α and TNR1 were significantly higher in the advanced NASH group than those in the normal group (p = 0.041 and 0.026, respectively). The mRNA levels of interleukin (IL)-1β and its receptor (IL-1R) tended to increase with disease progression, but not significantly (Fig. 8). There was also no statistical difference in hepatic IL-6 mRNA levels among the NAFLD subgroups (Fig. 8). Of these pro-inflammatory cytokines, only the expression of TNF-α showed significant inverse correlations with SREBP-1c mRNA levels (r = −0.329, p = 0.031) and mature SREBP-1c levels (r = −0.396, p = 0.041), suggesting a possible relationship among NAFLD progression, down-regulation of SREBP-1c, and increased expression of hepatic TNF-α.

Discussion

A novel and striking finding in this study was the down-regulation of the hepatic SREBP-1c-mediated lipogenic pathway in advanced NASH. Although a close relationship between increased

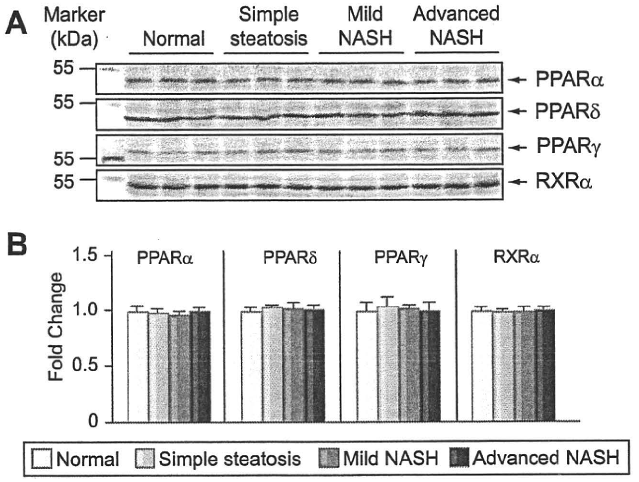

Fig. 7. Immunoblot analysis of PPARs and RXRα. The same samples used in Fig. 5 were adopted. Three samples from each group were loaded into each electrophoresis assay and all samples were examined. (A) A representative blot is shown. Band intensities were quantified densitometrically, normalized to those of GAPDH, and subsequently expressed as fold changes relative to those of normal livers. (B) Results are expressed as mean ± SEM.

expression of SREBP-1c and hepatic steatosis was shown in rodent models [31,32], there have been few studies to assess hepatic SREBP-1c expression in human livers with NAFLD/NASH. In one report, Higuchi et al. reported an increase in hepatic SREBP-1c mRNA levels in NAFLD patients [33], but they evaluated neither histological findings nor protein levels or intracellular localization of SREBP-1c. Here, we demonstrated by qPCR, immunoblot analysis, and immunohistochemistry that hepatic expression of SREBP-1c is increased in SS, but gradually decreases with fibrosis progression. To our knowledge, this is the first study to explore the changes in hepatic SREBP-1c expression throughout the advancement of NAFLD.

It is conceivable that the down-regulation of SREBP-1c in advanced NASH stems from a reduction in the relative number of hepatocytes, but immunoblot analysis demonstrated a significant reduction in functional SREBP-1c levels with NAFLD progression. Furthermore, the ratio of SREBP-1c-positive hepatocyte nuclei to all hepatocyte nuclei was decreased in advanced NASH, confirming that this possibility is considered to be extremely low.

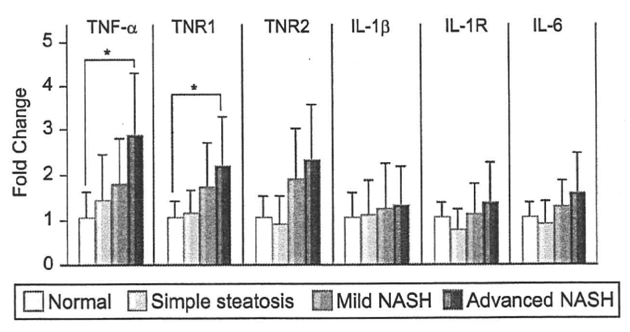

Fig. 8. Hepatic mRNA levels of genes encoding pro-inflammatory cytokines and their receptors. Samples were obtained from normal livers (n = 6) or livers with SS (n = 10), mild NASH (n = 20), or advanced NASH (n = 20). Results are expressed as mean ± SEM. *p <0.05.

It is reported that LXRα, RXRα, insulin, and pro-inflammatory cytokines, such as TNF-α and IL-1β, can modulate the mRNA levels of SREBP-1c [20,30]. However, there were no significant differences in the expression of LXRα, RXRα, or serum insulin in our cohort. On the other hand, we found that hepatic mRNA levels of TNF-α and its receptors increased with fibrosis progression and were inversely related to those of SREBP-1c. Such a relationship is consistent with the result in a previous study that TNF-α and IL-1, but not IL-6, reduced the mRNA levels of SREBP-1c in Hep3B cells [30]. Furthermore in that *in vitro* experiment, the ability of TNF-α to reduce SREBP-1c mRNA levels was more marked than that of IL-1 [30]. Based on these findings and our own, we can speculate that hepatic overexpression of TNF-α is associated with down-regulation of SREBP-1c and the development of burned-out NASH. Adenosine monophosphate-activated protein kinase (AMPK) is also known to be activated in response to oxidative stress and inhibit *de novo* lipogenesis through suppressing the transcriptional activity of SREBP-1c [6]. Further study is necessary to investigate the contribution of AMPK activation to the pathogenesis of burned-out NASH.

Although the mRNA levels of PPARα were lower in the advanced NASH group, PPARα protein levels and the mRNA levels of CPT1, a typical PPARα target gene in humans possessing a functional PPAR response element in its promoter region [34], remained unchanged. Such a discrepancy may have stemmed from post-transcriptional PPARα modifications, such as phosphorylation or stabilization through binding with natural ligands, RXRα and/or various proteins [21,35–38]. Similarly to the case of PPARα, the protein levels of PPARδ and PPARγ and the mRNA levels of their target genes did not vary among the NAFLD subgroups, suggesting a negligible direct contribution of PPARs to the progression of NAFLD.

In conclusion, down-regulation of SREBP-1c and lipogenic enzymes is associated with the development of burned-out NASH. Elucidating the hepatic expression of key genes involved in the development of NAFLD/NASH at every stage of fibrosis may lead to the establishment of novel step-wise therapeutic strategies against NAFLD/NASH.

Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgment

We thank Trevor Ralph for his editorial assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.04.033.

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Dig Dis Sci (2010) 55:2095–2101
DOI 10.1007/s10620-009-0954-6

ORIGINAL ARTICLE

Fucosylated Fraction of Alpha-Fetoprotein as a Predictor of Prognosis in Patients with Hepatocellular Carcinoma After Curative Treatment

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Received: 6 April 2009 / Accepted: 10 August 2009 / Published online: 3 September 2009

Abstract
Aim The aim of this study was to evaluate the clinical usefulness of measuring the *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) for prognostic predictor in patients with hepatocellular carcinoma (HCC).
Methods A total of 477 HCC patients who underwent percutaneous ablative therapy or hepatectomy were enrolled. Overall survival and recurrence-free survival were respectively evaluated retrospectively and prospectively. Multivariate analyses of clinical prognostic factors were performed by Cox's stepwise proportional hazard model.
Results AFP-L3 status was a statistically significant independent prognostic factor of long-term survival ($P = 0.013$) and recurrence-free survival ($P = 0.006$) in patients who underwent percutaneous ablative therapy. In contrast, AFP-L3 did not affect prognosis in patients who underwent hepatectomy.
Conclusions AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Our results suggest that AFP-L3 positivity ($\geq$15%) might be a promising indicator for choosing therapeutic modalities in HCC patients.

Keywords Alpha-fetoprotein · AFP-L3 · DCP (des-γ-carboxy prothrombin) · Hepatocellular carcinoma · Prognostic factor

Introduction

Hepatectomy is a generally accepted method that improves the long-term outcome in patients with hepatocellular carcinoma (HCC) [1]. However, patients with HCC frequently have coexisting liver cirrhosis with impaired hepatic functional reserve, and this may prevent surgical intervention. On the other hand, percutaneous ablative therapies, including percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT), and percutaneous radiofrequency ablation (RFA), have been developed and applied as alternative therapeutic options in cases of small HCC [2–8]. Recently, RFA has been performed as a first-line therapeutic option for early stage HCC; its survival outcomes are similar to those of hepatectomy [6–8]. However, a method for making the correct choice among therapeutic modalities to suit individual patients with early stage HCC remains to be determined.

The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been reported to be a specific marker for HCC [9–11]. Moreover, its level predicts the

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malignant potential of HCC with subsequent unfavorable prognosis after treatment [12–16]. However, there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities, such as hepatectomy and percutaneous ablative therapy.

The aim of this collaborative retrospective and prospective study was to evaluate the clinical usefulness of measuring AFP-L3 for prognostic predictor in patients with HCC after curative treatment.

Patients and Methods

Study Design

A total of 336 HCC patients underwent curative treatment at four participating hospitals (Niigata University Hospital, Ehime University Hospital, Shinsyu University Hospital, and Gunma University Hospital) from January 1998 to March 2005 and were investigated retrospectively. Of these patients, 232 underwent percutaneous ablative therapy and 104 underwent hepatectomy. Percutaneous ablative therapy comprised PEI in 90 patients, MCT in four patients, and RFA in 138 patients. Long-term survival data on these patients were confirmed as of the end of March 2005.

To evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we prospectively investigated 189 patients diagnosed with early stage HCC initially at four hospitals from April 2005 to October 2007. We considered patients who had multiple (up to three) tumors measuring 3 cm or less in diameter as having early stage HCC. Forty-eight of 189 patients were excluded in this study, as they were received transcatheter treatment. As a result, 141 HCC patients, 99 who underwent percutaneous ablative therapy and 42 who underwent hepatectomy, were enrolled in the prospective study. Percutaneous ablative therapy comprised PEI in ten patients, MCT in two patients, and RFA in 87 patients. In these 141 patients, HCC recurrence was assessed by imaging modalities every 3 or 4 months after treatment and recurrence free survival was evaluated as of the end of December 2007. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval by our institution's human research committee.

Diagnosis of HCC and Laboratory Examination

In our study, the diagnosis was based essentially on imaging findings together with increments of tumor marker levels. We employed methods such as computed tomography (CT), magnetic resonance imaging, and CT during hepatic arteriography, considering hyperattenuation in the arterial phase with washout in the late phase to be a typical feature of HCC. In nine cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Hepatic functional reserve was ranked by the criteria of the Child-Pugh scoring system. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were determined at each hospital by using commercially available kits. AFP-L3 percentage was measured at each hospital by liquid-binding assay (Wako Pure Chemical Industries Ltd, Osaka, Japan) [17]. AFP, AFP-L3, and DCP were measured in the same serum before treatment. Cut-off values for positivity for AFP, AFP-L3, and DCP were set at 20 ng/ml, 15%, and 40 mAU/ml, respectively, based on previous studies [18–20].

Treatment

Therapeutic modalities for individual patients were chosen according to hepatic functional reserve, tumor multiplicity, and tumor size. Percutaneous local ablative therapies were performed under a US-guided procedure, and its efficacy was evaluated with dynamic CT within a few days after treatment. Complete ablation of HCC was defined as nonenhancement of the lesion with surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete. During the study, a Cool-tip RF System attached to a 200-W power generator (Radionics, Burlington, Massachusetts, USA) was the main device used for RFA treatment and Microtaze OT-110M (Alfresa-Pharma Co., Inc., Osaka, Japan) was used for MCT.

Statistical Analysis

Differences in the proportions of the independent binary variables were determined by Fisher's exact test. Continuous variables were compared by Student's t-test. Univariate survival and recurrence-free survival were determined by the Kaplan–Meier method. Log-rank test was used to test for equality of long-term survival and recurrence-free survival between the groups. Multivariate analyses of prognostic factors in the clinical features were performed by using Cox's stepwise proportional hazard model. The factors included for multivariate analyses were patient age, gender (female/male), HBsAg (negative/positive), Anti-HCV (negative/positive), Child-Pugh class (A/B, C), AFP (ng/ml) ($<20/\geq20$), DCP (mAU/ml) ($<40/\geq40$), AFP-L3 (%) ($<15/\geq15$), tumor size (cm) ($<3/\geq3$ or $\leq2/>2$), and number of tumors (single/multiple). Statistical analyses were performed with SPSS 15.0 software (SPSS Japan Inc. Tokyo, Japan). A P-value of less than 0.05 was considered as statistically significant.

Results

Retrospective Study

Clinical Features of Patients Classified by Therapeutic Modality

A total of 336 HCC patients who underwent hepatectomy and percutaneous ablative therapy were investigated retrospectively. Patients who underwent percutaneous ablative therapy were characterized by older age ($P < 0.05$), positivity for antibody to hepatitis C virus (anti-HCV) ($P < 0.05$), and advanced Child-Pugh classification ($P < 0.05$). In contrast, patients who underwent hepatectomy were characterized by positivity for hepatitis B surface antigen (HBsAg) ($P < 0.05$), AFP-L3 ($P < 0.05$), and DCP ($P < 0.05$) elevation, as well as large tumor size ($P < 0.05$). No significant differences were observed between the two groups in terms of gender, AFP level, or number of tumors (Table 1A).

Univariate and Multivariate Analyses of the Factors Predicting Long-Term Patient Survival

The median observation time after treatment was 38.3 months (range, 1.0–146.2 months). Of the 232 patients who underwent percutaneous ablative therapy, 172 were alive and 60 had died from HCC, hepatic failure, and/or complications of cirrhosis. Of the 104 HCC patients who underwent hepatectomy, 68 were alive and 36 had died. The median survival time was 69.0 months in patients who had undergone percutaneous ablative therapy and 114.9 months in those who had undergone hepatectomy.

In the univariate analysis, anti-HCV status ($P = 0.034$), AFP status ($P = 0.007$), AFP-L3 status ($P = 0.001$), tumor size ($P = 0.001$), and number of tumors ($P = 0.045$) were significant prognostic factors of long-term survival in patients who underwent percutaneous ablative therapy. AFP status ($P = 0.011$), tumor size ($P = 0.006$), and number of tumors ($P < 0.001$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Multivariate analysis by Cox's stepwise proportional hazard model revealed that tumor size ($P = 0.018$) and AFP-L3 status ($P = 0.013$) were significant independent prognostic factors for long-term survival in patients who underwent percutaneous ablative therapy. Tumor size ($P = 0.013$) and number of tumors ($P = 0.004$) were significant independent prognostic factors in patients who underwent hepatectomy (Table 3). We showed the long-term survival curves of two groups (with or without AFP-L3 elevation) in patients who underwent percutaneous ablative therapy and in those who underwent hepatectomy (Fig. 1). No significant difference in survival was observed

Table 1 Clinical features of patients with HCC classified by therapeutic modality in the retrospective and prospective studies

Variables	Percutaneous ablation ($n = 232$)	Hepatectomy ($n = 104$)
(A) Retrospective study		
Age (median, range)	68 (39–89)	65 (35–81)*
Gender		
Male	145 (62.5%)	66 (63.5%)
Female	87 (37.5%)	38 (36.5%)
HBsAg		
Negative	209 (90.1%)	73 (70.2%)
Positive	23 (9.9%)	31 (29.8%)*
Anti-HCV		
Negative	28 (12.1%)	45 (43.3%)
Positive	204 (87.9%)	59 (56.7%)*
Child-Pugh class		
A	177 (76.3%)	95 (91.3%)
B and C	55 (23.7%)	9 (8.7%)*
AFP (ng/ml)		
<20	65 (28.0%)	22 (21.2%)
≥20	167 (72.0%)	82 (78.8%)
DCP (mAU/ml)		
<40	149 (67.4%)	48 (51.1%)
≥40	72 (32.6%)	46 (48.9%)*
AFP-L3 (%)		
<15	181 (78.0%)	61 (58.7%)
≥15	51 (22.0%)	43 (41.3%)*
Tumor size (cm)		
<3	185 (79.7%)	33 (31.7%)
≥3	47 (20.3%)	71 (68.3%)*
Tumor number		
Single	148 (63.8%)	75 (72.1%)
Multiple	84 (36.2%)	29 (27.9%)
Variables	**Percutaneous ablation ($n = 99$)**	**Hepatectomy ($n = 42$)**
(B) Prospective study		
Age (median, range)	69 (36–85)	65 (40–80)
Gender		
Male	66 (66.7%)	24 (57.1%)
Female	33 (33.3%)	18 (42.9%)
HBsAg		
Negative	85 (85.9%)	29 (69.0%)
Positive	14 (14.1%)	13 (31.0%)*
Anti-HCV		
Negative	27 (27.3%)	15 (35.7%)
Positive	72 (72.7%)	27 (64.3%)
Child-Pugh class		
A	79 (79.8%)	39 (92.9%)
B and C	20 (20.2%)	3 (7.1%)

Table 1 continued

Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
AFP (ng/ml)		
<20	64 (64.6%)	22 (52.40%)
≥20	35 (35.4%)	20 (47.6%)
DCP (mAU/ml)		
<40	63 (63.6%)	27 (64.3%)
≥40	35 (35.4%)	15 (35.7%)
AFP-L3 (%)		
<15	85 (85.9%)	33 (78.6%)
≥15	14 (14.1%)	9 (21.4%)
Tumor size (cm)		
≤2	63 (63.6%)	27 (64.3%)
>2	36 (36.4%)	15 (35.7%)
Tumor number		
Single	78 (78.8%)	34 (81.0%)
Multiple	21 (21.2%)	8 (19.0%)

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. Percentages are shown in parentheses

* $P < 0.05$ between groups by Fisher's exact test and Student's *t*-test

between the two AFP-L3 groups in patients who underwent hepatectomy ($P = 0.308$). In contrast, patients in the ablative therapy group whose AFP-L3 levels were below 15% lived significantly longer than those whose values were more than 15% ($P = 0.001$).

Prospective Study

Clinical Features of Patients with Early Stage HCC Classified by Therapeutic Modality

A total of 141 patients with early stage HCC were evaluated prospectively. Patients who underwent hepatectomy were characterized by positive for hepatitis B surface antigen (HBsAg) ($P < 0.05$). No significant differences were observed in age, gender, anti-HCV positivity, AFP status, AFP-L3 status, DCP status tumor size, and number of tumors between the two groups. Patients who underwent percutaneous ablative therapies tended to have an advanced Child-Pugh classification ($P = 0.055$) (Table 1B).

Univariate and Multivariate Analysis of the Factors Predicting Recurrence-Free Survival in Patients with Early Stage HCC

The median follow-up time after treatment was 12.0 months (range, 1.0–30.5 months). Among the 99 patients who underwent percutaneous ablation, recurrences were observed in 36 (36.4%). Among the 42 patients who underwent hepatectomy, recurrences were observed in six (14.3%).

In the univariate analysis, we found no significant difference in recurrence-free survival rates by pretreatment variables in patients who underwent percutaneous ablation, although AFP-L3 elevation ($P = 0.054$) tended to decrease recurrence-free survival. In contrast, tumor size ($P = 0.038$) and number of tumors ($P = 0.034$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Although this prospective study was conducted over a short period of time, multivariate analysis of prognostic factors among the clinical features was performed and Cox's stepwise proportional hazard model revealed that HBsAg status ($P = 0.033$), DCP status ($P = 0.011$), and AFP-L3 status ($P = 0.006$) were significant independent prognostic factors of recurrence-free survival in patients who underwent percutaneous ablative therapies. On the other hand, we found no significant independent prognostic factors in patients who underwent hepatectomy (Table 3).

We showed recurrence-free survival rates between two groups—with or without AFP-L3 elevation—among

Table 2 Univariate analysis of the factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Variables	Long-term survival		Recurrence-free survival	
	Percutaneous ablation *P*-value	Hepatectomy *P*-value	Percutaneous ablation *P*-value	Hepatectomy *P*-value
Gender (female/male)	0.907	0.525	0.225	0.194
HBsAg (negative/positive)	0.139	0.801	0.151	0.314
Anti-HCV (negative/positive)	0.034	0.963	0.194	0.171
Child-Pugh class (A/B,C)	0.083	0.235	0.293	0.487
AFP (ng/ml) (<20/≥20)	0.007	0.011	0.117	0.994
DCP (mAU/ml) (<40/≥40)	0.328	0.153	0.075	0.059
AFP-L3 (%) (<15/≥15)	0.001	0.308	0.054	0.530
Tumor size (cm) (<3/≥3)	0.001	0.006	0.063	0.038
Tumor number (single/multiple)	0.045	<0.001	0.667	0.034

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. *P*-value was calculated using Log-rank test

Table 3 Multivariate analysis of factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Long-term survival			Recurrence-free survival		
Variables	Hazard ratio (95% CI)	*P*-value	Variables	Hazard ratio (95% CI)	*P*-value
Percutaneous ablation			Percutaneous ablation		
AFP-L3 (%)			HBsAg		
<15	1		Negative	1	
≥15	2.098 (1.169–3.765)	0.013	Positive	2.823 (1.090–7.310)	0.033
Tumor size (cm)			DCP		
<3	1		<40 (mAU/ml)	1	
≥3	1.998 (1.123–3.553)	0.018	≥40 (mAU/ml)	2.767 (1.267–6.046)	0.011
			AFP-L3		
			<15 (%)	1	
			≥15 (%)	3.463 (1.437–8.347)	0.006
Hepatectomy			Hepatectomy		
Tumor size (cm)			Tumor number		
<3	1		Single	1	
≥3	6.162 (1.457–26.064)	0.013	Multiple	4.654 (0.936–23.149)	0.060
Tumor number					
Single	1				
Multiple	3.170 (1.442–6.921)	0.004			

Hazard ratio and *P*-value were calculated using Cox's stepwise proportional hazard model

CI confidence interval, *AFP* alpha-fetoprotein, *HBsAg* hepatitis B surface antigen, *DCP* des-gamma-carboxy prothrombin

patients with early stage HCC who underwent percutaneous ablation and patients who underwent hepatectomy (Fig. 1). No significant difference was observed between groups with or without AFP-L3 elevation ($P = 0.53$) in patients who underwent hepatectomy. In contrast, a close-to-significant ($P = 0.054$) difference was observed between the groups of patients with and without AFP-L3 elevation who underwent percutaneous ablative therapy.

In summary, the results of the retrospective and prospective studies demonstrated that AFP-L3 status was a statistically significant prognostic factor of long-term survival and recurrence-free survival in patients who underwent percutaneous ablative therapy, but did not affect prognosis in patients who underwent hepatectomy.

Discussion

AFP-L3, a fucosylated species of AFP, is the product of alpha 1-6 fucosyltransferase (FUT8) in the presence of GDP-fucose. Our previous result revealed that FUT8 levels in HCC tissue were higher than those in the surrounding non-cancerous tissues and that FUT8 levels of HCC tissue increased in accordance with tumor dedifferentiation [21]. Several reports have shown the relationship between AFP-L3 status and histologic grade in HCC. Miyaaki et al. [16] showed that the frequency of poorly differentiated HCC was significantly higher in AFP-L3-positive patients than in AFP-L3-negative patients. Oka et al. [14] reported that AFP-L3-positive HCC was characterized by portal vein invasion and poorer differentiation, and that tumors in AFP-L3-positive HCC were advanced, even if they were small and the patient had a low serum AFP concentration. These results indicate the relationship between increased AFP-L3 level and increased degree of malignant behavior of HCC tissue.

Recurrence after treatment is an important factor affecting prognosis. Vascular invasion is an established adverse prognostic indicator of recurrence of HCC [22, 23]. Yamashita et al. [24] suggested that portal vein invasion is associated with AFP-L3 positivity, and that there is a strong possibility of intrahepatic invasion when there is positive conversion of this marker. Hayashi et al. [13] reported the relationship between AFP-L3 status and pattern of recurrence in patients with HCC. In their report, intrahepatic metastasis was significantly more common in AFP-L3-positive patients than in negative patients, although the recurrence rate of multicentric tumors did not differ significantly between the two groups with or without AFP-L3 elevation. From this point of view, hepatectomy—especially anatomical resection, which can remove venous tumor thrombi together with the primary lesion—is more suitable than local ablative therapies for the treatment of AFP-L3-positive patients.

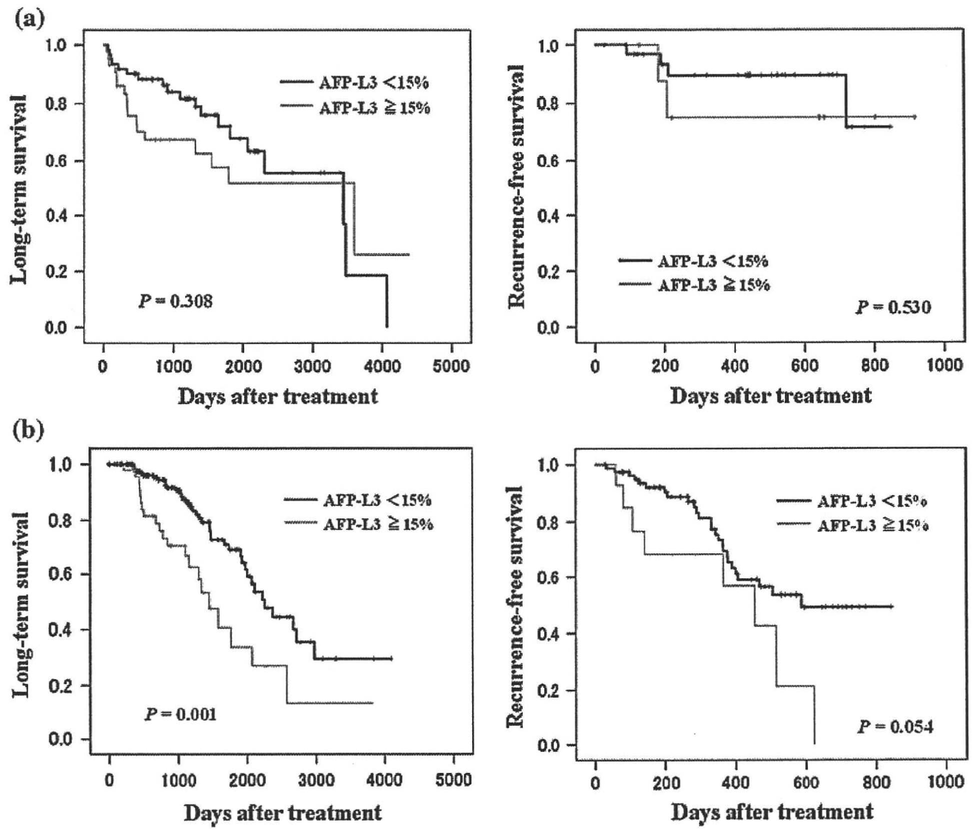

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (**a**) and who underwent percutaneous ablation (**b**)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3, 7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

Acknowledgments This study was supported in part by a Grant-in-Aid (16590582) from the Japan Society for the Promotion Science and a Grant-in-Aid from Health, Labor and Welfare, Japan.

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Biochemical Pharmacology 80 (2010) 1601–1612

Contents lists available at ScienceDirect

Biochemical Pharmacology

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Eicosapentaenoic acid improves hepatic steatosis independent of PPARα activation through inhibition of SREBP-1 maturation in mice

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ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form 20 July 2010
Accepted 23 July 2010

Keywords:
β-Oxidation
Fatty acid uptake
SCAP
S1P
Superoxide dismutase

ABSTRACT

Eicosapentaenoic acid (EPA) in fish oil is known to improve hepatic steatosis. However, it remains unclear whether such action of EPA is actually caused by peroxisome proliferator-activated receptor α (PPARα) activation. To explore the contribution of PPARα to the effects of EPA itself, male wild-type and *Ppara*-null mice were fed a saturated fat diet for 16 weeks, and highly (>98%)-purified EPA was administered in the last 12 weeks. Furthermore, the changes caused by EPA treatment were compared to those elicited by fenofibrate (FF), a typical PPARα activator. A saturated fat diet caused macrovesicular steatosis in both genotypes. However, EPA ameliorated steatosis only in wild-type mice without PPARα activation, which was evidently different from numerous previous observations. Instead, EPA inhibited maturation of sterol-responsive element-binding protein (SREBP)-1 in the presence of PPARα through down-regulation of SREBP cleavage-activating protein and site-1 protease. Additionally, EPA suppressed fatty acid uptake and promoted hydrolysis of intrahepatic triglycerides in a PPARα-independent manner. These effects were distinct from those of fenofibrate. Although fenofibrate induced NAPDH oxidase and acyl-coenzyme A oxidase and significantly increased hepatic lipid peroxides, EPA caused PPARα-dependent induction of superoxide dismutases, probably contributing to a decrease in the lipid peroxides. These results firstly demonstrate detailed mechanisms of steatosis-ameliorating effects of EPA without PPARα activation and ensuing augmentation of hepatic oxidative stress.

Abbreviations: ACC, acetyl-CoA carboxylase; ALT, alanine aminotransferase; apo, apolipoprotein; AOX, acyl-CoA oxidase; AST, aspartate aminotransferase; CoA, coenzyme A; CPT-I, carnitine palmitoyl-CoA transferase-I; EPA, eicosapentaenoic acid; FA, fatty acid; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; FF, fenofibrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; GPx, glutathione peroxidase; 4-HNE, 4-hydroxynonenal; HTGL, hepatic triglyceride lipase; Insig, insulin-induced gene product; LACS, long-chain acyl-CoA synthase; L-FABP, liver fatty acid-binding protein; LXR, liver X receptor; MCAD, medium-chain acyl-CoA dehydrogenase; MDA, malondialdehyde; mRNA, messenger RNA; MTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, non-esterified fatty acid; NL, neutral lipase; Nrf2, nuclear factor-E2-related factor 2; PGC, PPARγ coactivator; PMP, peroxisomal membrane protein; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SCAP, SREBP cleavage-activating protein; S1P, site-1 protease; SD, standard deviation; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TG, triglyceride; TNF, tumor necrosis factor.

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doi:10.1016/j.bcp.2010.07.031

1. Introduction

Recent lifestyle alterations, such as increased consumption of saturated fats and decreased physical activity, have raised the prevalence of obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD) [1,2]. Nonalcoholic steatohepatitis (NASH) is the progressive type of NAFLD and may develop into cirrhosis, liver cancer, and ultimately death [1–4]. Since NAFLD is also associated with a high susceptibility to atherosclerosis and ischemic heart disease [3,5], the increased prevalence of NAFLD is becoming a pressing issue worldwide. Thus, establishment of strategies to treat and prevent NAFLD and related metabolic disturbances is required.

Eicosapentaenoic acid (EPA) is one of the major components of n-3 polyunsaturated fatty acids (PUFA) preferentially contained in fish oil. From the first report of high EPA levels in the diet and blood of the Greenland Inuit [6], who rarely exhibit atherosclerotic diseases, numerous epidemiological and clinical studies have been

Table 1
Changes in anthropometric and biochemical parameters from a 16-week saturated fat diet.

Genotype	*Ppara* (+/+)		*Ppara* (−/−)	
Diet	Con (n=6)	Sat (n=6)	Con (n=6)	Sat (n=6)
Body weight (g)	23.9 ± 1.9	$28.3 \pm 1.5^{*}$	26.5 ± 2.7	$41.0 \pm 5.2^{**,\#\#}$
Liver/body weight (%)	3.8 ± 0.2	$4.6 \pm 0.4^{*}$	4.4 ± 0.2	$5.2 \pm 0.6^{*}$
Epididymal fat/body weight (%)	2.5 ± 0.3	$3.7 \pm 0.6^{*}$	3.0 ± 1.5	$6.1 \pm 0.5^{**,\#\#}$
Serum TG (mg/dL)	61 ± 1	$123 \pm 41^{*}$	124 ± 50	$233 \pm 49^{**,\#\#}$
Serum NEFA (mEq/L)	0.75 ± 0.30	$1.33 \pm 0.3^{*}$	1.19 ± 0.25	$1.54 \pm 0.15^{\#}$
Serum glucose (mg/dL)	92 ± 23	89 ± 24	98 ± 14	103 ± 22
Serum insulin (ng/mL)	0.51 ± 0.09	1.21 ± 0.58	0.48 ± 0.06	$2.24 \pm 0.46^{**}$
Serum AST (U/L)	129 ± 66	243 ± 62	149 ± 92	203 ± 46
Serum ALT (U/L)	13 ± 6	$43 \pm 16^{*}$	18 ± 10	$99 \pm 21^{**,\#}$
Liver TG (mg/g)	10 ± 1	$30 \pm 3^{**}$	17 ± 3	$52 \pm 7^{**,\#\#}$

Results are expressed as mean ± SD. Con, control standard diet; Sat, saturated fat diet; TG, triglyceride; NEFA, non-esterified fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
* $P < 0.05$ compared with mice of the same genotype fed a control diet.
** $P < 0.01$ compared with mice of the same genotype fed a control diet.
\# $P < 0.05$ compared with *Ppara* (+/+) mice fed the same diet.
\#\# $P < 0.01$ compared with *Ppara* (+/+) mice fed the same diet.

undertaken to show the efficacy of n-3 PUFA and EPA on reducing serum triglyceride (TG) concentrations and preventing cardiovascular events [7–9]. Some data on the steatosis-ameliorating effect of n-3 PUFA have also been obtained [10,11], creating the intriguing possibility that EPA might be beneficial for the treatment of NAFLD.

It has been considered that n-3 PUFA exhibited TG-reducing effects through regulation of peroxisome proliferator-activated receptor α (PPARα) and sterol regulatory element-binding protein (SREBP)-1, which control hepatic fatty acid (FA) catabolism and synthesis, respectively [12]. PPARα is a nuclear receptor expressed primarily in the liver and is involved in not only FA/TG metabolism,

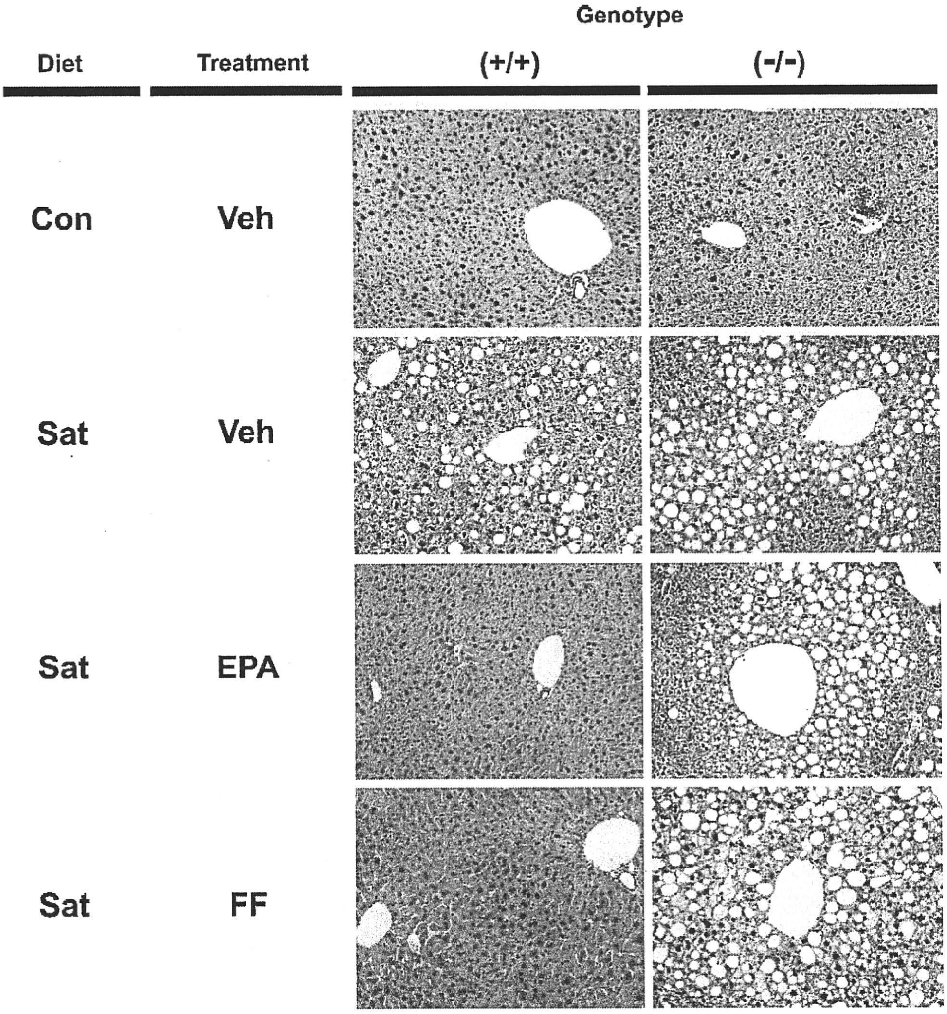

Fig. 1. Histological findings in the livers of wild-type and $Ppara^{-/-}$ mice. Male 8-week-old wild-type (+/+) and *Ppara* (−/−) mice were fed a control standard (Con) or saturated fat diet (Sat) for 16 weeks. After 4 weeks on the saturated fat diet, treatment with highly-purified EPA or FF was initiated and continued for 12 weeks. Liver sections were stained by hematoxylin and eosin method. Original magnification, 200×. Veh, vehicle.

but also cell proliferation and inflammatory response [13]. SREBP is a transcription factor belonging to the basic helix–loop–helix leucine zipper family. Depending on various intracellular signals, SREBP is cleaved by several proteases and the N-terminus region is transferred into the nucleus as a mature protein where it regulates transcription of several target genes. SREBP-1 plays an important role in the development of NAFLD, and insulin and synthetic agonists of liver X receptor α (LXRα) enhance its transcription [14].

Several studies have demonstrated an activating effect of EPA on PPARα [15–20]. However, because most of these studies were *in vitro* experiments [15–17] and used crude fish oil [18–20], it has not been determined whether EPA alone can activate PPARα *in vivo* as well. Additionally, since fish oil is reported to lower serum TG levels even in PPARα-null (*Ppara*$^{-/-}$) mice [21], it remains unclear whether such action occurs via PPARα activation. In order to clarify the contribution of PPARα to the effects of EPA *in vivo*, highly (>98%)-purified EPA was administered to wild-type and *Ppara*$^{-/-}$ mice fed a saturated fat diet, and the expression of genes and proteins involved in hepatic FA/TG metabolism was investigated. Furthermore, the changes caused by EPA treatment were compared to those elicited by fenofibrate (FF), a typical PPARα activator.

2. Materials and methods

2.1. Mice and treatment

Ppara$^{-/-}$ mice on a 129/Sv genetic background (129S4/SvJae) were generated as described previously [22]. Male 8-week-old wild-type and *Ppara*$^{-/-}$ mice weighing 24 ± 5 g (*n* = 30 in each genotype) were maintained in pathogen-free conditions at constant humidity and temperature with a light/dark cycle of 12 h. At the beginning of this study, mice in each genotype were randomly divided into 5 groups (*n* = 6/group) and pair-fed a diet. As a control, one group was treated with a standard diet for 16 weeks composed of 20.0% (per weight basis) casein, 53% corn starch, 10% sucrose, 7% olive oil, 5% cellulose, 3.5% mineral mix, 1% vitamin mix, and 0.25% choline. The other 4 groups were fed a saturated fat diet (Oriental Yeast Co. Ltd., Tokyo, Japan) in which all fat contained in the standard diet was completely hydrogenated to eliminate the effects of naturally contained PUFA. We chose 16 weeks as a saturated fat diet feeding period, since our preliminary experiments demonstrated that this protocol could induce obvious hepatic steatosis not only in *Ppara*$^{-/-}$ mice but also in wild-type mice. Body weight and food intake were recorded every day. After 4 weeks on the saturated fat diet, administration of the test agents was initiated in the 4 groups. One group was given highly (>98%)-purified EPA ethyl ester (ethyl all-cis-5, 8, 11, 14, 17-icosapentaenoate) (Mochida Pharmaceutical Co., Ltd, Tokyo, Japan) (1000 mg/kg of body weight/day) for 1 week, one group was given highly (>98%)-purified EPA ethyl ester at the same dose for 12 weeks, and another group was administered FF (Wako Pure Chemicals Industries, Osaka, Japan) (25 mg/kg of body weight/day) for 12 weeks. EPA and FF were dissolved in Arabic gum, mixed, and administered once a day at 10 a.m. by gastric gavage. The last test group was given the same amount of Arabic gum as a vehicle for 12 weeks. After the administration periods, the mice were anesthesized and sacrificed in a fasting state for collection of livers and blood. All experiments were conducted in accordance with the animal study protocols outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and approved by the Animal Studies Committee at Shinshu University School of Medicine.

2.2. Immunoblot analysis

Preparation of hepatocyte nuclear fractions was carried out as described previously [23,24]. Protein concentration was measured

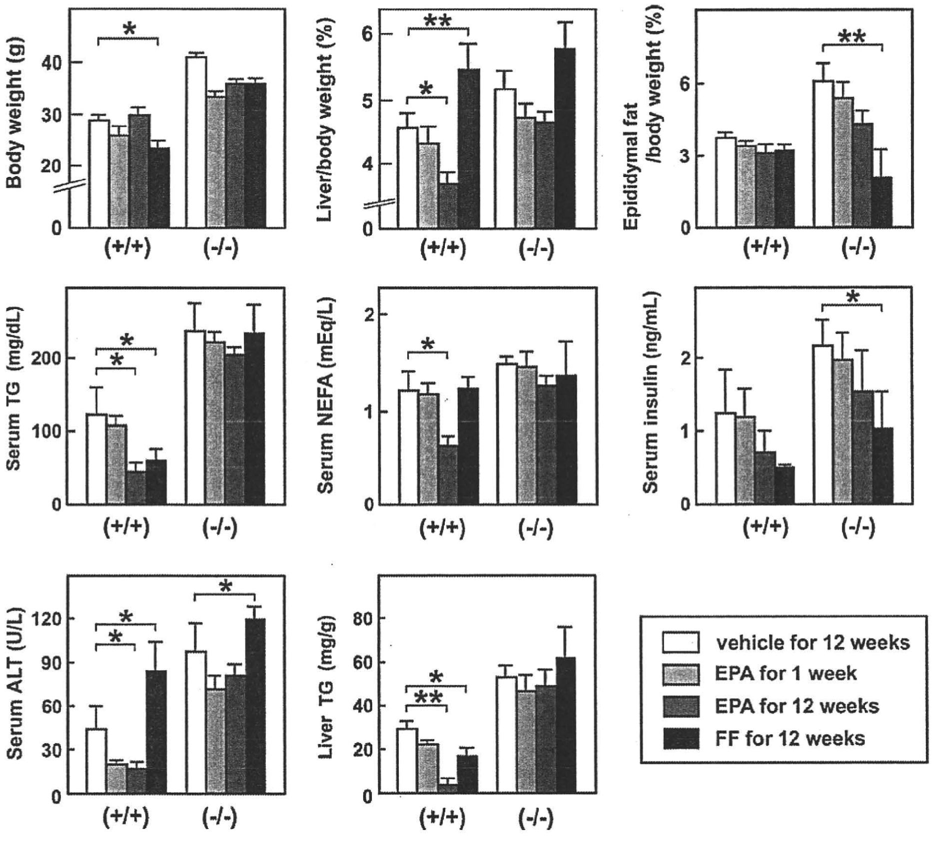

Fig. 2. Effects of EPA and FF on anthropometric and biochemical parameters. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks. Results are expressed as mean ± SD (*n* = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

colorimetrically with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). Whole liver lysates (20–80 μg of protein) or nuclear fractions (80 μg of protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [23–28]. A sample from one mouse in each group was loaded into each electrophoresis assay and all samples were examined ($n = 6$/group). After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with primary antibody followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The antibodies against FA-metabolizing enzymes were described previously [28], and those against other proteins were purchased commercially. Suppliers and dilutions of primary antibodies are summarized in Supplementary Table 1. Actin or histone H1 was used as the loading control. Band intensities were measured densitometrically, normalized to those of actin or histone H1, and subsequently expressed as fold-changes of those of control wild-type mice fed a saturated fat diet. For confirmation of data reproducibility, immunoblot analysis using the same samples was done twice. Overall, the data on 12 band intensities were obtained from each mouse group for each target molecule and subjected to statistical analysis.

2.3. Analysis of mRNA

Total liver RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL, Paisley, Scotland). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as described elsewhere [29,30] with the primer pairs listed in Supplementary Table 2. Measured mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and subjected to statistical analysis.

2.4. Measurement of hepatic lipids and lipid peroxides

Total hepatic lipids were extracted according to the method developed by Folch et al. [31]. Lipid extracts were dissolved in distilled water, and the concentrations of TG and lipid peroxides

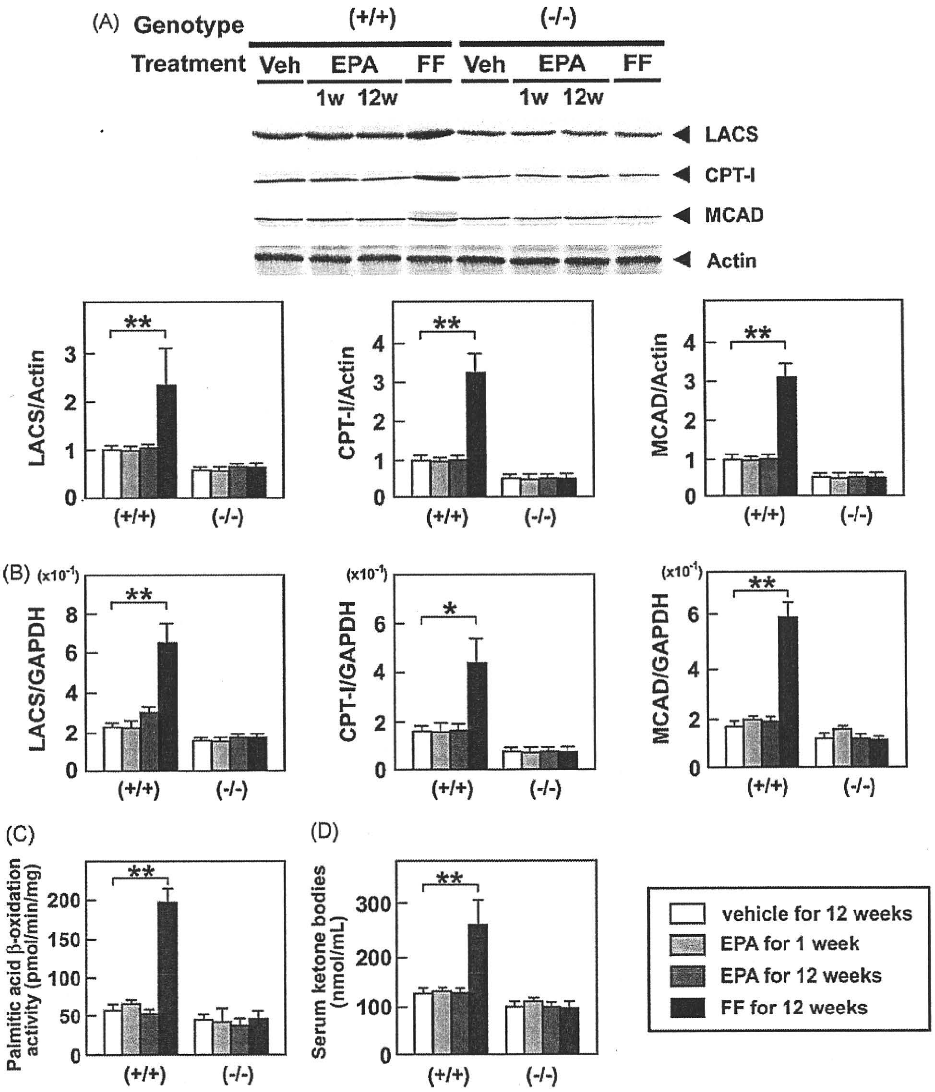

Fig. 3. Immunoblot analysis of LACS, CPT-I, and MCAD. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and whole liver lysates (20 μg of protein) obtained from mice were loaded into 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). $^{**}P < 0.01$. (B) Hepatic mRNA levels of LACS, CPT-I, and MCAD. 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 mRNA levels were determined by quantitative RT-PCR. Measured mRNA levels were normalized to those of GAPDH. Results are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in mitochondrial β-oxidation activity in the liver. 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 β-oxidation activity was measured using palmitic acid as a substrate. Results are expressed as mean ± SD ($n = 6$/group). $^{**}P < 0.01$. (D) Serum concentrations of ketone bodies. Figure presentation is identical to that in Fig. 2.

[the sum of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)] were colorimetrically measured using a TG C-test (Wako Pure Chemicals Industries) and LPO-586 kit (OXIS International, Portland, OR, USA), respectively [32].

2.5. Assessment of FA β-oxidation and uptake abilities

FA β-oxidation activity was measured according to a method described previously with palmitic acid as a substrate, and expressed as pmol/min/mg of liver tissue [28]. FA uptake ability was assessed as described elsewhere and expressed as a fold-change of that in control wild-type mice fed a saturated fat diet [33].

2.6. Assay of hepatic neutral lipase (NL) activity

Fresh liver samples (approximately 100 mg) were homogenized in 20 mM phosphate buffer (pH 7.5) containing 250 mM sucrose and 1 mM EDTA, sonicated, and centrifuged at 20,000 × g for 10 min at 25 °C. NL activity of the supernatant was colorimetrically measured using a MONOTEST (Boehringer Mannheim, Tokyo,

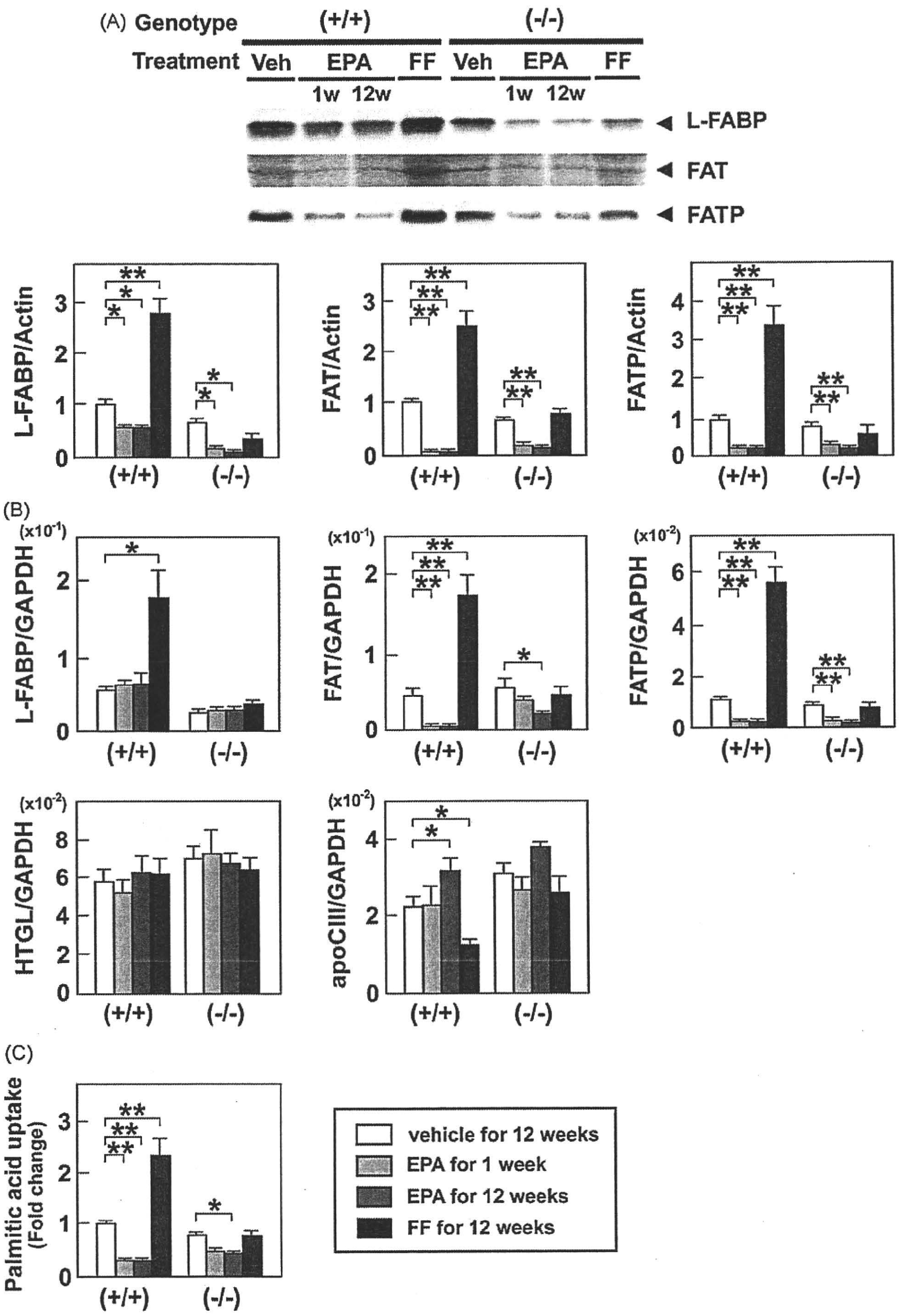

Fig. 4. Effects of EPA and FF on the hepatic FA uptake system. (A) Immunoblot analysis of L-FABP, FAT, and FATP. The same samples used in Fig. 3A were loaded. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of molecules associated with FA uptake. The same samples in Fig. 3B were adopted. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in palmitic acid uptake ability into the liver. 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 FA uptake activity was assessed as described in Methods. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$.