Hepatology Research

Hepatology Research 2013; 43: 557–562 doi: 10.1111/j.1872-034X.2012.01091.x

Short Communication

Prediction of a favorable clinical course in hepatitis C virus carriers with persistently normal serum alanine aminotransferase levels: A long-term follow-up study

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Aim: This study examined serum alanine aminotransferase (ALT) levels at first visit and their relationship with long-term normal serum ALT levels in hepatitis C virus (HCV) carriers with persistently normal ALT (PNALT).

Methods: HCV carriers with PNALT were identified as those patients with positivity of serum HCV RNA, ALT levels of 30 IU/L or less over a 12-month period on at least three different occasions, platelet count of more than 15×10^4 μl/mL and body mass index of 30 kg/m^2 or less. Outcome was retrospectively studied in 49 HCV carriers with PNALT, who were followed up for more than 10 years.

Results: During the mean follow-up period of 14.7 ± 2.5 years, ALT levels of 30 IU/L or less were preserved in only eight patients (8/49; 16.3%). Among the 17 patients with initial ALT levels of 19 IU/L or less, nine patients remained with ALT levels of 30 IU/L or less after 10 years (9/17; 52.9%). The probability of ALT levels in PNALT being maintained at 30 IU/L or less was significantly higher ($P = 0.001$) in these patients than in those with initial ALT levels of 20 IU/L or more ($n = 32$). Abnormal ALT levels were more common in female PNALT patients aged 45–55 years, which is usually the time of menopause onset.

Conclusion: Because antiviral therapy in the treatment of chronic hepatitis C is rapidly advancing, waiting for more effective and safer treatments may be an option. The results of this study provide an important insight into this issue.

Key words: alanine aminotransferase threshold, hepatitis C virus carriers with persistently normal alanine aminotransferase, long-term follow up

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major public health concern worldwide. Antiviral therapy to eradicate HCV has progressed.[1,2] Currently, peginterferon (PEG IFN) and ribavirin (RBV) combination therapy is widely used to treat chronic hepatitis C, and triple therapy with a protease inhibitor, telaprevir, is also available.[3–5] However, some physicians are reluctant to treat patients using IFN-based therapy because of the development of new therapies, some of which may be more effective and safer.[6]

Compared with fibrosis progression in patients with elevated transaminase levels, HCV carriers with persistently normal alanine aminotransferase (PNALT) and mild liver fibrosis are unlikely to develop severe fibrosis,[7–10] whereas only some reports presented dissimilar results.[11,12] A report at the consensus meeting of the Japan Society of Hepatology held in 2009 concluded that the progression of hepatic fibrosis in HCV carriers with PNALT is generally slow.[2]

Sustained viral response (SVR) rates of HCV carriers with PNALT are similar to those of patients with elevated transaminase levels.[13,14] The decision to utilize IFN-based therapies should be determined not by ALT

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Received 24 April 2012; revision 15 August 2012; accepted 19 August 2012.

values but by the patient's physical condition, probability of successful therapy or prolonged survival, and likelihood of serious adverse effects.[1,2]

Prediction of ALT abnormality in patients with PNALT may be helpful in determining treatment timing, namely, immediately or 2–3 years later, taking into account the probability of hepatocellular carcinoma (HCC) occurrence.[15] The present retrospective study addressed this issue by evaluating outcome in HCV carriers with PNALT who were followed up for more than 10 years.

METHODS

Patients and follow-up study

WE HAVE REPORTED a follow-up study (>5 years) of 69 patients among the 129 HCV carriers with PNALT.[10] In the present study, 49 HCV carriers with PNALT, in whom follow up was possible every 3–6 months, in principle, at our outpatient clinic for more than 10 years, were retrospectively studied. All 49 patients belonged to the previous study[10] and 16 patients who showed ALT levels of 30 IU/L or more before 10 years follow up were treated with PEG IFN-α-2b and RBV (Shering-Plough, Kenilworth, NJ, USA). Other patients with ALT levels of 30 IU/L or more were followed or treated with ursodeoxycholic acid. The other 80 patients in the previous study[10] were excluded from this study because they were lost to follow up before 10 years or received IFN-based therapy while the ALT levels were 30 IU/L or less. The end-points of follow up in this study are ALT elevation of 30 IU/L or more or last visit to our hospital (≥10 years from the first visit).

Hepatitis C virus carriers with PNALT were identified as those patients with positivity of serum HCV RNA, serum ALT levels of 30 IU/L or less over a 12-month period on at least three different occasions, platelet counts of more than 15×10^4 μl/mL, body mass index (BMI) of 30 kg/m^2 or less, and no evidence of oral contraceptive, co-infection with HIV or known liver disease other than hepatitis C.

Liver biopsy was performed using a Menghini needle guided by ultrasound. Liver biopsy specimens were fixed in 10% formalin and stained with hematoxylin–eosin and Masson-trichrome. Histopathological diagnosis was based on the scoring of the New Inuyama Classification.[16] Evaluation was performed by two expert hepatologists who were blinded to the clinical data of the patients.

This study was a retrospective sub-analysis of the study entitled "Analysis of the pathophysiology of HCV carriers with persistent normal ALT levels", which was approved by the ethics committee of the university and conformed to the provisions of the Declaration of Helsinki.

Statistical analysis

All data analyses were performed using SPSS statistical software (ver. 17.0; SPSS, Chicago, IL, USA). Individual characteristics were presented as means ± standard deviations and compared by Mann–Whitney *U*-test or Pearson's χ^2-test. Receiver–operator curve (ROC) analysis was performed, followed by proper categorization of the data. Probability of PNALT maintenance was determined using the Kaplan–Meier method and analyzed using the log–rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of PNALT

CLINICAL CHARACTERISTICS OF the HCV carriers with PNALT are summarized in Table 1. We inves-

Table 1 Clinical characteristics of the 49 HCV carriers with PNALT at first visit

Follow-up period (years)	14.7 ± 2.5
Age (years)	
Male ($n = 4$)	34.8 ± 5.9
Female ($n = 45$)	48.0 ± 11.2
ALT (IU/L)	
Male ($n = 4$)	16.8 ± 4.7
Female ($n = 45$)	21.9 ± 5.3
PLT ($\times 10^4$/μL)	
Male ($n = 4$)	20.3 ± 4.7
Female ($n = 45$)	21.5 ± 4.7
BMI (kg/m^2)	
Male ($n = 4$)	20.3 ± 1.5
Female ($n = 45$)	21.3 ± 2.5
Genotype (G1/G2/ND)	25/16/8
Liver histology	
Male (F0/F1/F2/F3/F4)	3/1/0/0/0
(A0/A1/A/2/A3)	1/3/0/0
Female (F0/F1/F2/F3/F4)	11/32/2/0/0
(A0/A1/A2/A3)	2/39/4/0

Data are presented as means ± standard deviations.
Liver histology was classified based on New Inuyama Classification.[16]
ALT, alanine aminotransferase; BMI, body mass index; G1, genotype 1; G2, genotype 2; HCV, hepatitis C virus; ND, not determined; PLT, platelets; PNALT, persistently normal alanine aminotransferase.

tigated whether or not the patients who maintained normal ALT levels (≤30 IU/L) for 10 years or more ($n = 8$) are significantly different from those who did not ($n = 41$) in clinical characteristics. We revealed no significant differences in age ($P = 0.109$), platelet count ($P = 0.371$), BMI ($P = 0.989$), hemoglobin concentration ($P = 0.549$), HCV load ($P = 0.712$), HCV genotype (1 or 2; $P = 0.495$), serum ferritin ($P = 0.710$), hepatic fibrosis score (F0/1,2) ($P = 0.588$), hepatic activity score (A0/1,2) ($P = 0.421$) or iron deposition (positive or negative; $P = 0.251$, $n = 20$). Only the initial ALT levels were significantly lower in patients who maintained normal ALT levels (≤30 IU/L) for 10 years or more ($P = 0.003$).

Initial ALT values and clinical outcome of patients with PNALT

To estimate a cut-off initial ALT level predicting the maintenance of ALT of 30 IU/L or less, the ROC analysis was performed (Fig. 1). The result revealed that 19.5 IU/L was an optimal ALT level predicting the maintenance of ALT of 30 IU/L or less, because it achieved the highest sensitivity (0.756%) and specificity (0.875%), yielding an area under the curve of 0.83 and *P*-value of 0.003.

Among the 17 patients with initial ALT levels of 19 IU/L or less, nine patients remained at ALT levels of 30 IU/L or less after 10 years (52.9%) (Fig. 2). The probability of ALT levels being maintained at 30 IU/L or less was significantly higher ($P = 0.001$) in these patients than in those with initial ALT levels of 20 IU/L or more ($n = 32$).

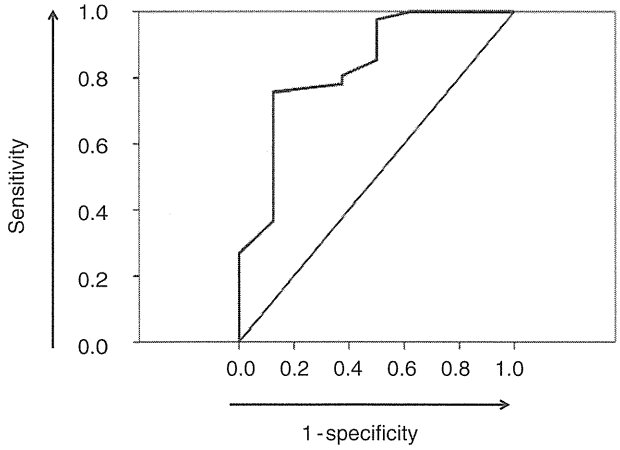

Figure 1 Receiver–operator curve analysis of the relationship between initial alanine aminotransferase (ALT) values and maintenance of normal ALT.

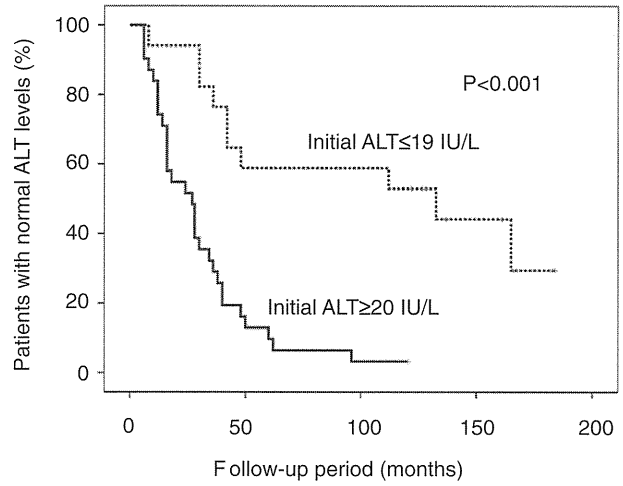

Figure 2 Maintenance of normal alanine aminotransferase (ALT) values (≤30 IU/L) during the follow up. Seventeen patients had initial ALT levels of ≤19 IU/L and 32 of ≥20 IU/L.

Relationship between menopause and ALT elevation

The ages of female PNALT patients at which abnormal ALT first occurred are presented in Figure 3(a). Abnormal ALT levels were most frequently recorded in female patients aged 45–55 years, which is usually the time of menopause onset. We sent a questionnaire to 45 female patients to investigate the relationship between ALT elevation and menopause, but only 16 patients responded. Of the respondents, age of menopause onset varied between 48 and 56 years, except for one patient who underwent hysterectomy at 37 years old and experienced menopause before consulting our hospital. ALT levels were found to be elevated within 3 years of their awareness of menopause in 10 patients (Fig. 3b), but before 3 years of menopause in three patients (Fig. 3c). The remaining three patients experienced menopause before consultation to our outpatient clinic (data not shown).

DISCUSSION

THE COURSE OF illness in HCV carriers with PNALT is not well known. The general consensus in Japan is that most HCV carriers with PNALT exhibit mild liver damage and/or fibrosis.[10] During the follow-up period of 10 years, interestingly, ALT levels remained stable at 30 IU/L or less in 52.9% (9/17) of patients with initial ALT levels of 19 IU/L or less. The probability of PNALT being maintained was significantly higher in patients

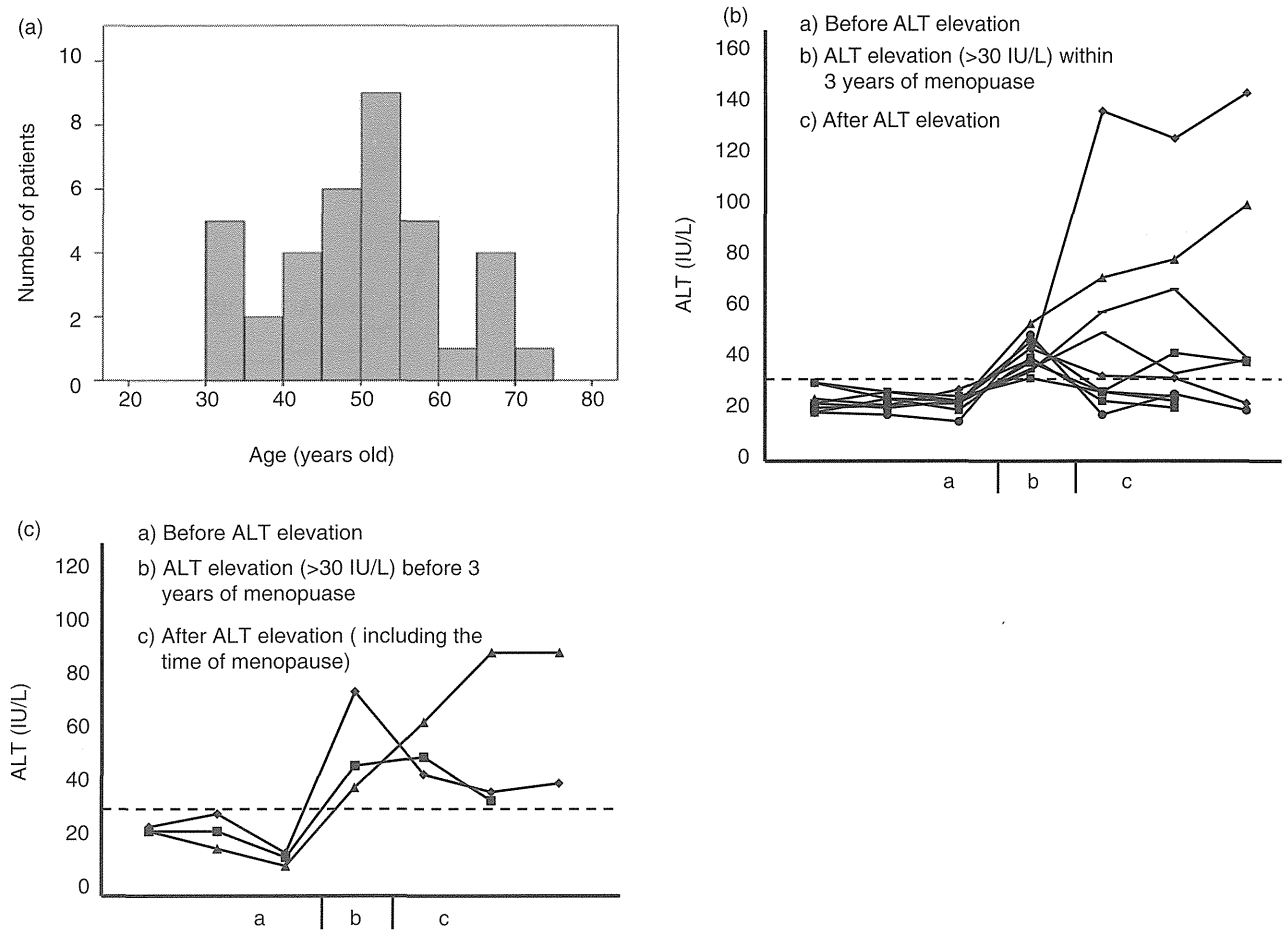

Figure 3 (a) Age of female persistently normal alanine aminotransferase (PNALT) patients at which abnormal ALT first occurred ($n = 37$). (b) PNALT with first ALT levels of >30 IU/L within 3 years of menopause. (c) PNALT with first ALT levels of >30 IU/L before 3 years of menopause.

with initial ALT levels of 19 IU/L or less than in those with initial ALT levels of 20 IU/L or more (Fig. 2, $P < 0.001$). Although the progression of hepatic fibrosis could not be evaluated by repeated liver biopsy during the observation period, this result suggests a benign course in a subgroup of HCV carriers with PNALT, whose ALT levels were 19 IU/L or less at the first visit.

Interestingly, a report from a hyperendemic area in Japan revealed that a basal ALT level of 20 IU/L or more was an important predictive factor of ALT flare-up in HCV carriers with PNALT.[17] This result accords with the favorable ALT levels documented in our study (Fig. 2). Furthermore, 19 IU/L is the updated upper limit of the healthy range for serum ALT level in female patients with chronic HCV infection or non-alcoholic fatty liver disease, as advocated by Prati *et al.*[18]

Concerning the possibility of HCC, one Japanese report demonstrated that HCV carriers with PNALT and ALT levels of more than 20 IU/L were, to some extent, at risk of both hepatocarcinogenesis and ALT elevation.[19] These results reinforce the finding in this study that patients with initial ALT levels of 20 IU/L or more and 30 IU/L or less were at a high risk for ALT elevation during the follow-up period (Fig. 2).

The relationship between menopause and the first abnormal ALT level in female patients was also examined. As shown in Figure 3(a), first abnormal ALT levels in female PNALT patients were frequently observed at 45–55 years of age, which is usually the time of menopause onset.

Although only 16 patients responded to the questionnaire, ALT levels were found to be elevated within

3 years of their awareness of menopause in 10 patients. This finding is interesting because previous studies have reported an association between menopause and progression of hepatic fibrosis[20,21] or resistance to antiviral therapy.[22] Recently, production of the HCV particle has been reported to be inhibited by 17-β-estradiol *in vitro*.[23] Further study in this field will clarify this issue.

Although the mechanism of abnormal ALT is uncertain, we speculate that one of the plausible causes of abnormal ALT levels might be enhanced immunological response against HCV. Recently, Itose *et al.*[24] demonstrated that the frequency of regulatory T cells is higher in PNALT patients and that depletion of $CD25^+$ cells enhanced HCV-specific T-cell response. So, we speculate that some immunological activation may underlie the cause of ALT elevation. Increased BMI during the observation may be another cause of abnormal ALT, although we do not have precise data on that point.

In conclusion, because antiviral therapy for chronic hepatitis C is making rapid and encouraging progress, waiting for more effective and safer treatments may be an option. The results of this study provide an important insight into this issue.

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Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Rosuvastatin ameliorates high-fat and high-cholesterol diet-induced nonalcoholic steatohepatitis in rats

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Keywords
high-fat and high-cholesterol diets – NASH – rosuvastatin

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Received 17 March 2012
Accepted 16 October 2012

DOI:10.1111/liv.12033

Liver Int. 2013: 33: 301–311

Abstract

Background/Aims: Statins, which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase and inhibit endogenous cholesterol synthesis, possess pleiotropic activities, such as anti-inflammatory, anti-oxidative and antifibrotic effects. Here, we investigated whether statins ameliorate steatohepatitis using a high-fat and high-cholesterol (HFHC) diet-induced rat model. *Methods:* Eight-week-old male Sprague–Dawley rats were fed control chow or HFHC diet. Half of the HFHC diet-fed rats were orally administered 2 mg/kg/day rosuvastatin for 12 weeks. Hepatic injury, steatosis, fibrosis and markers of lipid peroxidation/oxidant stress were evaluated. *Results:* As previously reported, HFHC diet induced steatohepatitis in rat livers with hypercholesterolaemia. Rosuvastatin decreased Oil Red O stained-positive areas, liver/body weight ratio, serum total cholesterol levels and hepatic free fatty acid contents in HFHC diet-fed rats. Further study revealed that rosuvastatin significantly decreased hepatic mRNA expression of tumour necrosis factor-α and interleukin-6, serum alanine aminotransferase levels and hepatic lobular inflammation grade. Hepatic fibrosis was also ameliorated by rosuvastatin with decreases in hepatic mRNA expression of transforming growth factor-β, connective tissue growth factor and type-1 procollagen. Similarly, hepatic Sirius red stained or α-smooth muscle actin stained-positive areas and expression of markers of lipid peroxidation/oxidant stress [hepatic 8-hydroxy-oxyguanosine and hepatic 4-hydroxy-2-nonenal] were decreased. Interestingly, whereas the expression of carnitine palmitoyltransferase-1 and long-chain acyl-CoA dehydrogenase was not affected, that of catalase and acyl-coA oxidase was restored. *Conclusions:* These data suggest that rosuvastatin improved not only hepatic steatosis but also hepatic injury and fibrosis via improved peroxisomal β-oxidation in this rat HFHC model.

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases and is significantly associated with features of the metabolic syndrome, including obesity, dyslipidaemia and insulin resistance (1, 2). Furthermore, an increased risk of cardiovascular disease (CVD) has been reported in NAFLD patients with type 2 diabetes (3–5). Patients at risk of CVD are prescribed with statins for down-regulating cholesterol production in the liver and increasing the ability of the liver to excrete low-density lipoprotein cholesterol (LDL-C) in the blood (6, 7). Furthermore, data from multiple randomized trials have revealed that the use of statins to lower increased LDL-C levels can substantially decrease the prevalence of coronary events and death from coronary heart disease, indicating that statins exhibit actions beyond lipid-lowering activity in the prevention of atherosclerosis (8, 9).

Statins have been considered to prevent atherosclerosis by way of improvement of endothelial function, modulation of inflammatory responses and inhibition of thrombus formation. The ASTEROID trial provided direct ultrasonographic evidence of atheroma regression during rosuvastatin therapy (10). The JUPITER study showed that rosuvastatin significantly reduced the incidence of major cardiovascular events in patients with LDL levels below current treatment thresholds, but with elevated high-sensitivity C-reactive protein (CRP) levels. As both LDL and CRP levels were significantly

reduced by rosuvastatin in this trial, it is not possible to attribute the relative contributions of either to the impact of rosuvastatin on cardiovascular events; however, the results leave open a possible anti-inflammatory contribution of statins (11). Furthermore, several studies demonstrated that rosuvastatin suppressed inflammatory responses through inhibition of c-Jun N-terminal kinase and nuclear factor-kappa B in endothelial cells and protected rodents from ischaemic stroke and myocardial reperfusion injury, suggesting that rosuvastatin-mediated benefits are dependent on its anti-oxidant and anti-inflammatory activity in various types of cells (12–16). A recent report demonstrated that rosuvastatin decreased NAFLD prevalence in patients with chronic hepatitis C treated with interferon-α and ribavirin (17); the precise mechanism underlying this remains unclear.

Statins have also been shown to exhibit antifibrotic effects that are independent of their lipid-lowering activity in several organs (18–20). Interestingly, statins are known to inhibit the expression of transforming growth factor (TGF)-β, a key molecule in the angiotensin II/smad pathway. In addition, statins were revealed to inhibit the proliferation of fibrogenic cells and production of extracellular matrix in the kidneys (21–23). Rho A-dependent cyclin D1 and connective tissue growth factor (CTGF) expression in pulmonary fibrosis (24) was also ameliorated. With regard to the liver, several studies have shown that statins decreased the expression of profibrotic cytokines such as TGF-β, CTGF and PDGF, and led to a more quiescent state of hepatic stellate cells (HSCs) with less proliferation and apoptosis (25–27).

Therefore, we investigated the anti-inflammatory, anti-oxidant and antifibrogenic roles of statins using a rat model of high-fat and high-cholesterol (HFHC) diet-induced nonalcoholic steatohepatitis (NASH).

We hypothesize that statins have a protective role in hepatic steatosis, inflammation and fibrosis in human NASH *via* mechanisms involved in the inhibition of endogenous cholesterol synthesis, reduction in pro-inflammatory cytokine production and reactive oxygen species (ROS), and maintenance of the quiescent phenotype in fibrogenic cells. To address this question, we treated a recently described murine NASH model with rosuvastatin (6–17), which is a hydrophilic statin that has a low potential to interact with cytochrome P-450 (28–30). Feeding rodents with an atherogenic diet, which contains high-fat components, has been shown to cause excess oxidative stress in the liver and induce liver steatosis, inflammation and fibrosis. This useful animal model provided us with the opportunity to access the pathophysiology of progressive NAFLD in humans. In this study, rosuvastatin effectively suppressed liver steatosis, inflammation, ROS production and fibrosis through up-regulation of peroxisomal β-oxidation. Thus, we confirmed that statins have a protective role in the development of NASH.

Material and methods

All animal experiments fulfilled the requirements for humane animal care provided in the guidelines of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Animals and treatment

Eight-week-old male Sprague–Dawley rats (n = 15) were purchased from Jackson Laboratories (Japan). They were maintained in a temperature- and light-controlled room and allowed water *ad libitum*. The 15 rats were randomly divided into three groups: control chow-fed rats, HFHC diet-fed rats, and HFHC diet-fed rosuvastatin-treated rats. Rosuvastatin was kindly donated by AstraZeneca (Tokyo, Japan) and administered to rats with food at a daily dose of 2 mg/kg body weight (BW). This dose was selected on the basis of previous reports (31). The HFHC diet was prepared by Oriental Yeast (Tokyo, Japan) and its composition is shown in Table 1. All rats were sacrificed at the end of 12 weeks of treatment.

Two-step real-time PCR

Real-time PCR was performed as described previously (32). Specificity was confirmed for all primer pairs (Table 2) by sequencing the PCR products. Target gene levels are presented as a ratio of levels in treated vs. corresponding control groups according to the ΔΔCt method. Fold changes were determined using point and interval estimates.

Immunohistochemistry and analysis of liver architecture

Serial sections were stained with H&E or Oil Red O using standard techniques. After deparaffinization, microwave antigen retrieval and blocking of endogenous peroxidase activity, other sections were incubated with an anti-4-hydroxy-2-nonenal (4-HNE) (HNEJ-2; Nikken, Shizuoka, Japan) or anti-α-smooth muscle actin (SMA) antibody (Dako Cytomation, Carpinteria, CA, USA). The antigen was demonstrated using secondary anti-mouse polymer HRP and DAB chromogen (Dako) and counterstaining with Gill's haematoxylin. The proportion of α-SMA-positive area was quantified using Image J software in five randomly selected fields per section (magnification ×200).

Table 1. The composition of the high-fat and high-cholesterol diet

Composition	Chow	HFHC
CRF-1 (%)	100	38.25
Lard (%)		58
Cholesterol (%)		1.25
Cholate (%)		0.50
Vitamins and minerals		2

Table 2. RT-PCR primers for analysis

Gene	Direction	Sequence
GAPDH	Forward	TGGGTAGAATCATACTGGAACATGTAG
	Reverse	AGGGCTGCCTTCTCTTGTGAC
SREBP-1c	Forward	GTGGTCTTCCAGAGGCTGAG
	Reverse	GGGTGAGAGCCTTGAGACAG
SREBP-2	Forward	CTGCAGATCCCGCAGTACAG
	Reverse	GGTGGATGAGGGAGAGAAGGT
Fas	Forward	AAGATCCCGGAAAGCAAGAT
	Reverse	TGATACCAGCACTGGAGCAG
ACC1	Forward	CCCAACAGAATAAAGCTACTCTGG
	Reverse	TCCTTTTGTGCAACTAGGAACGT
LDL-R	Forward	ACCGCCATGAGGTACGTAAG
	Reverse	CGGCGCTGTAGATCTTTCTC
MTP-1	Forward	CCTCCCATCCTGATGAAGAA
	Reverse	TGCAGCCTTCATTCTGACAC
TNF-α	Forward	ACTGAACTTCGGGGTGATTG
	Reverse	GCTTGGTGGTTTGCTACGAC
IL-6	Forward	TCCTACCCCAACTTCCAATGCTC
	Reverse	TTGGATGGTCTTGGTCCTTAGCC
TGF-β	Forward	TGGTTGTAGAGGGCAAGGAC
	Reverse	TGCTTCAGCTCCACAGAGAA
Pro Col1α1	Forward	TGAACGTGACCAAAAACCAA
	Reverse	AAGGAACAGAAAAGGCAGCA
CTGF	Forward	CTGAAAGAATAGCTGGCTTCA
	Reverse	CTGGTACTAGCTGAGGTCAT
Catalase	Forward	GAGAACATTGCCAACCACCT
	Reverse	GAGGGATCTCCTCAGTGCAG
CPT1	Forward	TATGTGAGGATGCTGCTTCC
	Reverse	CTCGGAGAGCTAAGCTTGTC
LCAD	Forward	ACAAATGCCAAAAGGTCTGG
	Reverse	CTGTGTCCTGGGCTTTCATT
ACO	Forward	CACGCAATAGTTCTGGCTCA
	Reverse	ACCTGGGCGTATTTCATCAG

Quantification of collagen levels in the liver

Liver sections were stained with picrosirius red and counterstained with fast green (Sigma–Aldrich Japan). Sirius red staining was quantified using Image J software in five randomly selected fields per section (magnification ×200).

Biochemical measurements in tissue and serum

Serum alanine aminotransferase (ALT), total cholesterol (T-CHO) and triglyceride levels were measured as described previously (32). Serum levels of tumour necrosis factor (TNF)-α and interleukin (IL)-6 were measured by Quantikine ELISA kit (R & D Systems, Minneapolis, MN, USA). Tissue triglyceride, free fatty acid (FFA) and cholesterol levels were measured by colorimetric analysis using cholesterol/cholesteryl Ester Quantitation kit (Bio vision, CA, USA). Hepatic 8-hydroxyoxyguanosine (8-OHdG) levels and catalase activity were measured using the DNA Extractor TIS Kit, 8-OHdG Assay Preparation Reagent Set (Wako: Wako, Osaka, Japan) and Highly Sensitive 8-OHdG Check Enzyme-Linked Immunosorbent assay (ELISA; Nikken, Shizuoka, Japan), and Catalase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturers' instructions.

Statistical analysis

Results are presented as mean ± SEM. Significance was established using the Student's *t*-test and analysis of variance if appropriate. Differences were considered significant if $P < 0.05$.

Results

The effects of rosuvastatin on hepatic steatosis and serum total cholesterol levels

We assessed hepatic steatosis with regard to H&E and Oil Red O staining, the liver weight/BW ratio and liver triglyceride levels at the end of 12 weeks of treatment. HFHC diet induced intrahepatic lipid accumulation in rats, but rosuvastatin treatment successfully decreased this accumulation (Fig. 1A). BW decreased in both HFHC diet-fed groups, but the HFHC diet-induced increase in the liver/BW ratio significantly decreased with rosuvastatin treatment (Fig. 1B). Rosuvastatin treatment marginally, but nonsignificantly, decreased liver triglyceride levels ($P = 0.09$) and free cholesterol levels ($P = 0.10$) despite no effect on T-CHO levels (Fig. 1C). However, liver weights in HFHC diet-fed rosuvastatin-treated rats were significantly lower than those in HFHC diet-fed rats (data not shown), suggesting that rosuvastatin treatment significantly decreased total triglyceride, free cholesterol and T-CHO contents per liver in HFHC diet-fed rats. Furthermore, despite the fact that rosuvastatin had no effect on serum triglyceride levels, it significantly decreased HFHC diet-induced elevation of serum T-CHO levels, which is caused by the decrease in serum free and very LDL (VLDL)/LDL cholesterol levels (Fig. 1D). These findings indicated that rosuvastatin may ameliorate hypercholesterolaemia and liver steatosis.

The effects of rosuvastatin on the hepatic mRNA levels of lipid-related gene expression

The major mechanism driving hepatic triglyceride accumulation is increased delivery of FFAs from peripheral adipose depots to the liver. Hepatic lipid disposal *via* mitochondrial/peroxisomal β-oxidation and lipoprotein export are central mechanisms for eliminating potentially toxic FFAs. To investigate why liver steatosis was ameliorated by rosuvastatin treatment, we assessed the mRNA expression of hepatic sterol regulatory element-binding protein (SREBP)-1, -2, FA synthase (FAS), Acetyl-CoA carboxylase (ACC)-1, LDL-C receptor (LDL-R) and microsomal transport protein (MTP)-1. While HFHC diets significantly increased hepatic SREBP-1c, FAS and ACC1, and decreased MTP-1 and

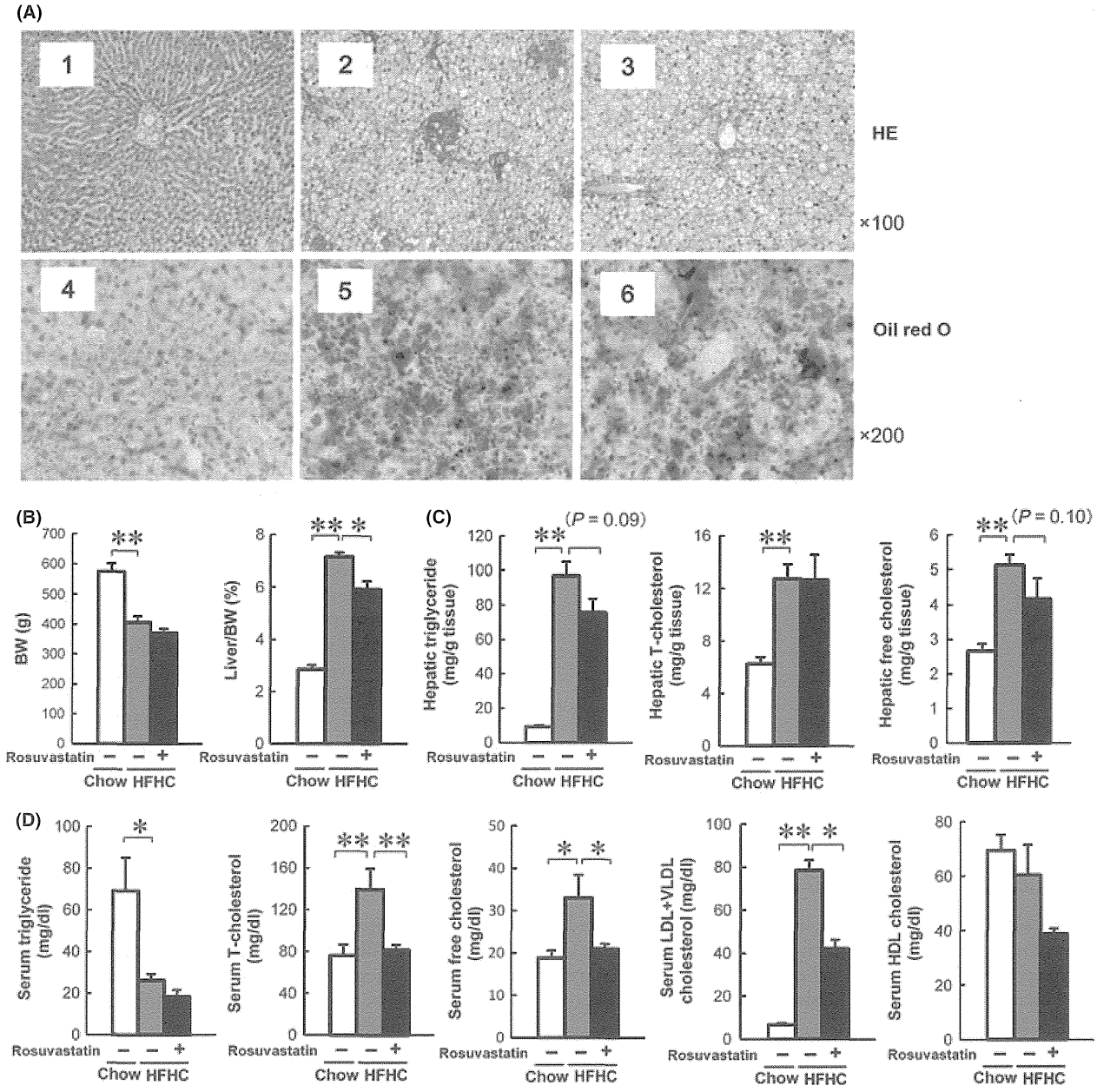


Fig. 1. Liver histology, liver/BW ratio, liver triglyceride and total cholesterol contents and serum triglyceride and total-cholesterol levels. (A) Liver sections from representative rats from each treatment group. HE staining of control rats (A-1), HFHC diet-fed rats (A-2), HFHC diet-fed rats treated with rosuvastatin (A-3). Oil Red O staining of control mice (A-4), HFHC diet-fed rats (A-5), HFHC diet-fed rats treated with rosuvastatin (A-6). (B) Body weight and Liver/body weight (BW) ratio were assessed at the end of 12 weeks treatment. Mean ± SE data from each group are plotted at 8 weeks (*$P < 0.05$, **$P < 0.01$). (C) Hepatic triglycerides, total-cholesterol (T-CHO) and free cholesterol contents also were measured (n = 5/group). Results are expressed per g tissue, respectively. Mean ± SE data from each group are plotted at 12 weeks (**$P < 0.01$). (D) Serum triglyceride, T-CHO, free cholesterol, LDL + VLDL cholesterol and HDL cholesterol levels were determined in each group at the end of the 12-week treatment period (n = 5/group). Data are presented as mean ± SE (*$P < 0.05$, **$P < 0.01$).

acyl-CoA oxidase (ACO) gene expression, it exerted no effect on SREBP-2 and, rather, decreased LDL-R gene expression (Fig. 2A, B, 5E). These observations were consistent with recent studies (30, 33) indicating that HFHC diet-induced hepatic lipid accumulation was caused by up-regulated lipogenic gene and down-regulated VLDL export-related gene expression, without increased uptake of LDL-C into the liver. Furthermore, as previously reported (34), rosuvastatin treatment significantly decreased the expression of SREBP-1c in the

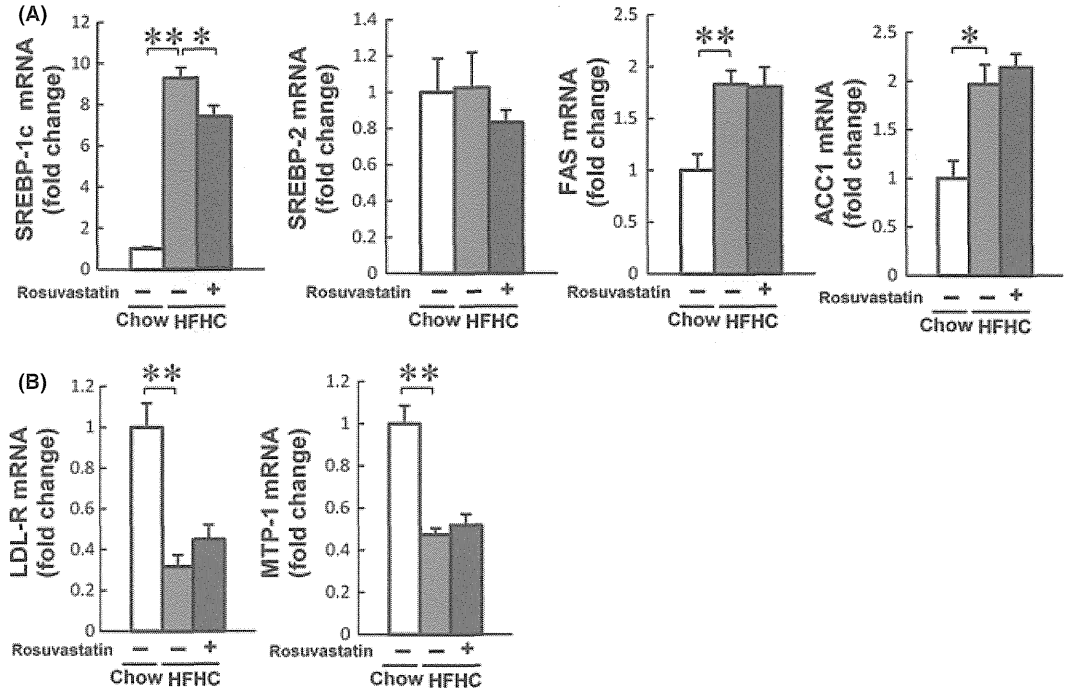


Fig. 2. Lipid metabolism related gene expression in rat livers. (A) The mRNA levels of hepatic SREBP-1c, SREBP-2, FAS and ACC1 were determined by quantitative real time PCR analysis after 12 weeks of treatment. Results were normalized to GAPDH expression and then expressed as fold changes relative to gene expression in chow-fed control rats (n = 5/group). Data are presented as mean ± SE data from each group (*P < 0.05, **P < 0.01). (B) The mRNA levels of hepatic LDL-R and MTP-1 were determined by quantitative real time PCR analysis after 12 weeks of treatment. Results were normalized to GAPDH expression. Mean ± SE data are displayed as fold changes relative to chow-fed control rats (**P < 0.01).

liver (Fig. 2A), suggesting that improvement in hepatic steatosis with rosuvastatin treatment was, at least in part, owing to the suppression of hepatic lipogenesis (Fig. 2A, B). Rosuvastatin treatment had no effect on the expression of the other hepatic genes.

Rosuvastatin ameliorated high-fat and high-cholesterol diet-induced liver injury

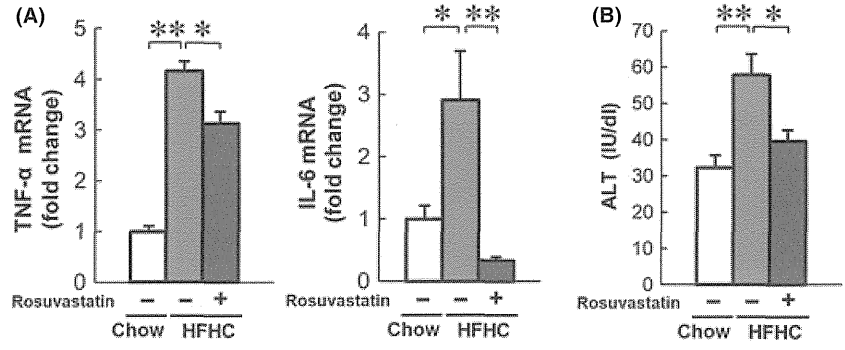
We compared hepatic injury-related parameters among the three groups. While serum levels of TNF-α and IL-6 were below the limit of detection (data not shown), 12-week treatment with rosuvastatin considerably suppressed HFHC diet-induced mRNA expression of hepatic TNF-α and IL-6 (Fig. 3A). Consistent with the evidence that HFHC diets increase hepatic TNF-α and IL-6 expression, the HFHC diet-fed group exhibited two-fold higher serum ALT levels than the chow-fed group. Rosuvastatin treatment significantly decreased serum ALT levels (Fig. 3B). These findings suggested that rosuvastatin treatment ameliorated liver injury. To confirm this finding, liver sections were evaluated for lobular inflammation. Lobular inflammatory grades in the HFHC diet-fed group were higher than those in the chow-fed group (Fig. 3C). As expected, rosuvastatin treatment for 12 weeks significantly decreased HFHC diet-induced lobular inflammation (Fig. 3C).

Rosuvastatin ameliorated high-fat and high-cholesterol diet-induced liver fibrosis

To evaluate the effect of rosuvastatin treatment on liver fibrosis, mRNA levels of various fibrosis markers were compared among the three groups. Feeding rats with an HFHC diet significantly increased hepatic mRNA expression of TGFβ-1, CTGF and collagen, and rosuvastatin treatment significantly suppressed them (Fig. 4A). To further assess the effects of rosuvastatin treatment on HFHC diet-induced hepatic fibrosis, Sirius red staining and α-SMA immunohistochemical analysis were performed (Fig. 4B). As predicted from the results of analysis of hepatic fibrosis markers, although the HFHC diet-fed group had the largest Sirius red stained and α-SMA stained-positive areas, as demonstrated by liver morphometry among three groups, rosuvastatin treatment decreased the proportion of these areas (Fig. 4C).

Rosuvastatin decreased lipid peroxidation/oxidant stress

To elucidate the mechanism of ameliorated liver damage and fibrosis by rosuvastatin treatment in this model, we examined liver FFA content and the markers of lipotoxicity at the end of 12 weeks of treatment. Rosuvastatin treatment suppressed HFHC diet-induced



(C)	Inflammatory score	
Control	0.11 (±0.07)	**
HFHC	1.35 (±0.13)	**
HFHC+statin	0.50 (±0.10)	

0: No inflammatory foci per 200x field
1: 1-2 Inflammatory foci per 200x field
2: 3-4 Inflammatory foci per 200x field
3: >4 Inflammatory foci per 200x field

Fig. 3. Hepatic injury-related parameters in rats at the end of 12 weeks. (A) The mRNA levels of hepatic TNF-α and IL-6 were evaluated by quantitative real time PCR analysis from each group at 12 weeks (n = 5/group). Results were normalized to GAPDH expression. Mean ± SE data are displayed as fold changes relative to chow-fed control rats (*P < 0.05, **P < 0.01). (B) Serum ALT levels were measured after 12 weeks of treatment. Mean ± SE data from each group (n = 5/group) (*P < 0.05, **P < 0.01). (C) The number of inflammatory foci per 200x field was counted in sections from each group. Mean ± SE data from each group (n = 5/group) (**P < 0.01).

elevation of liver FFA levels (Fig. 5A). Furthermore, rosuvastatin normalized hepatic 8-OHdG to control chow levels and slightly suppressed hepatic accumulation of 4-HNE, indicating that ROS-induced DNA damage was suppressed (Fig. 5B, C). Thus, decreased lipotoxicity as a result of rosuvastatin treatment may have ameliorated liver damage in this model. To investigate why rosuvastatin treatment decreased hepatic FA levels and suppressed lipid peroxidation/oxidant stress, we assessed hepatic mitochondrial and peroxisomal β-oxidation-related gene expression of carnitine palmitoyltransferase (CPT)-1, long-chain acyl-CoA dehydrogenase (LCAD), ACO and catalase by real-time PCR. We found that rosuvastatin treatment had no effect on hepatic mRNA expression of CPT-1 and LCAD, but increased ACO and catalase expression (Fig. 5D, E). To confirm this finding, we measured hepatic catalase activity by ELISA, and found that catalase activity returned to near control levels with rosuvastatin treatment (Fig 5F). Taken together, we suggest that rosuvastatin may activate peroxisomal β-oxidation via induction of catalase expression.

Discussion

Statins are commonly used in the treatment of hypercholesterolaemia and coronary artery disease (6–12). Several studies have already shown that statins can decrease serum lipid and aminotransferase levels, but whether statins improve histological activity of NAFLD or not has not been clarified (35–38). In this study, we assessed the beneficial effect of rosuvastatin on the progression of NAFLD in three major manifestations of liver disease, that is, steatosis, inflammation and fibrosis. In the results, we were able to demonstrate that rosuvastatin treatment significantly ameliorated liver steatosis, inflammation and fibrosis in a HFHC diet-fed rat model of NASH with hypercholesterolaemia.

Hepatocytes can sense decreased serum cholesterol levels and enhance hepatic SREBP-2 expression, leading to compensatory synthesis of LDL-R to take cholesterol out of circulation (6, 7, 9, 12, 31). LDL and VLDL are removed from circulation and transported into the liver where cholesterol is reprocessed into bile salts (6, 7, 34). In contrast, despite hypercholesterolaemia, hepatic SREBP-1c expression remains up-regulated by LXR, resulting in hepatic lipid accumulation, exportation of VLDL to the liver and hepatic ROS production in patients with NAFLD (39). In general, while statins inhibit endogenous cholesterol synthesis and hepatic SREBP-1c expression, the compensatory induction of hepatic SREBP-2 expression may enhance LDL-R synthesis and LDL-C uptake (34). This may lead to temporary accumulation of lipids within the liver,

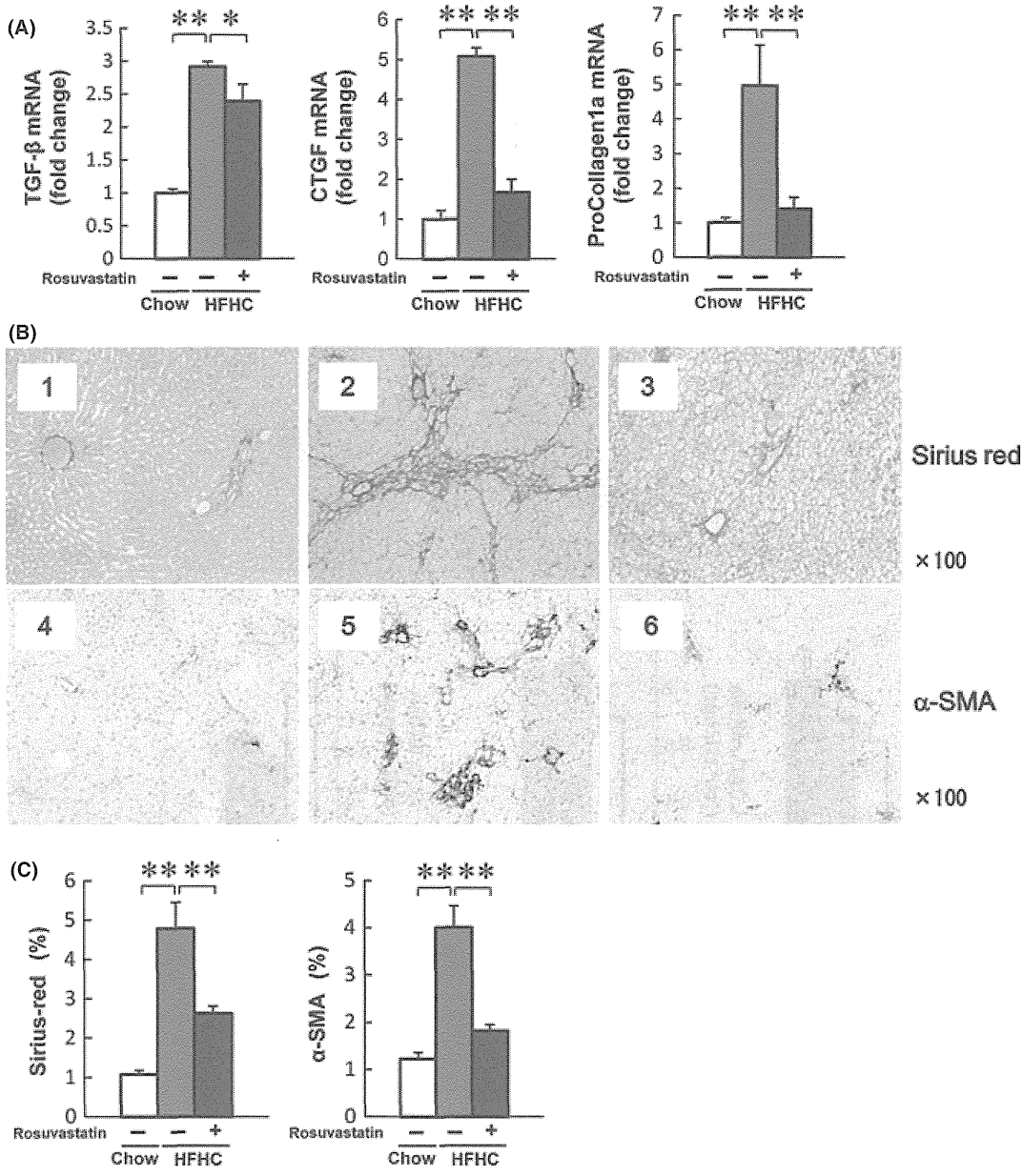


Fig. 4. Hepatic fibrosis markers in rat livers. (A) The mRNA levels of hepatic TGFβ-1, CTGF and collagen were determined by quantitative real time PCR analysis after 12 weeks of treatment. Results were normalized to GAPDH expression. Mean ± SE data from each sample are displayed as fold changes relative to chow-fed control rats (*$P < 0.05$, **$P < 0.01$). (B) Liver sections from all rats were stained with Sirius Red and α-SMA antibody after 12 weeks of treatment. Photomicrographs from representative rats are shown. Sirius Red staining of control rats (B-1), HFHC diet-fed rats (B-2), HFHC diet-fed rats treated with rosuvastatin (B-3). α-SMA staining of control rats (B-4), HFHC diet-fed rats (B-5), HFHC diet-fed rats treated with rosuvastatin (B-6). (C) Morphometric analysis of Sirius Red- and α-SMA-stained sections from each group at 12 weeks (n = 5/group). Results are expressed as percentage of section staining (+) for Sirius Red and α-SMA (**$P < 0.01$).

which may be the source of ROS production. A recent study focused on 'dysregulation of cholesterol metabolism in human and animal models of NAFLD'. In patients with NAFLD and in more advanced stage 'NASH', hepatic LDL receptor expression was significantly decreased, although the expression of SREBP-2 was not depressed (40). Histological severity of NAFLD was closely correlated with up-regulated HMG-CoA reductase activity and hepatic free cholesterol levels, demonstrating that dysregulated cholesterol metabolism in NAFLD might lead to more severe disease (33, 41, 42). Hence, inhibition of HMG-CoA reductase by statins could improve the dysregulated cholesterol metabolism and protect the liver from potentially toxic

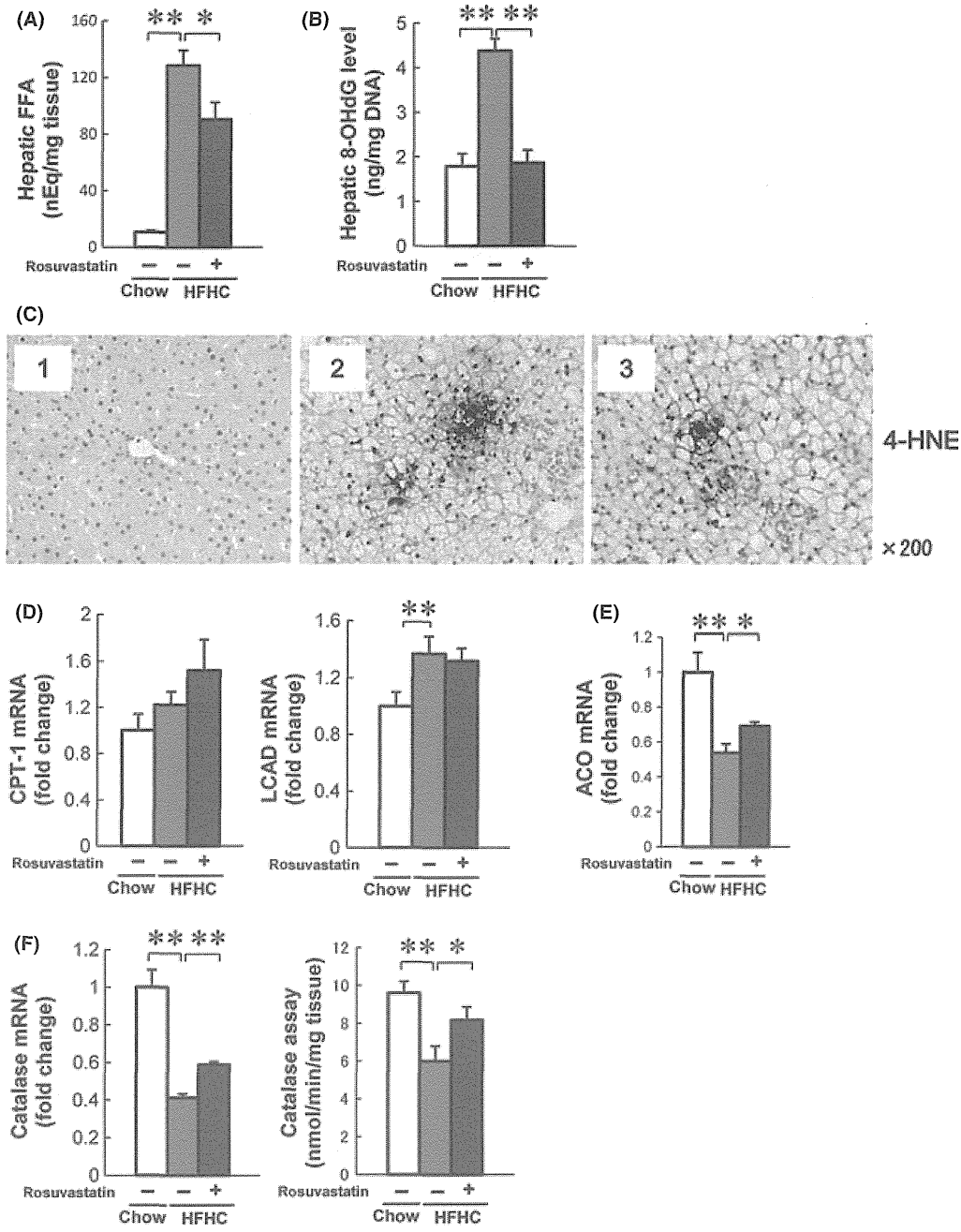


Fig. 5. Parameters of lipotoxicity, oxidant stress in rat livers. (A) Hepatic FFA contents were measured. Results are expressed per mg tissue, respectively. Mean ± SE data from each group are plotted at 12 weeks (*$P < 0.05$, **$P < 0.01$). (B) Hepatic 8-OHdG levels were determined in each group at the end of the 12-week treatment period (n = 5/group). Results are expressed per mg genomic DNA. Mean ± SE data from each group are plotted at 12 weeks (**$P < 0.01$). (C) 4-HNE was evaluated by immunohistochemistry in liver samples from chow-fed control rats (c-1), HFHC diet-fed rats (c-2), and HFHC diet-fed rosuvastatin-treated rats (c-3) at the end of the 12-week treatment period. Photomicrographs from representative rats are shown. (D) The mRNA levels of hepatic CPT-1 and LCAD were evaluated by quantitative real time PCR analysis from each group at 12 weeks (n = 5/group). Results were normalized to GAPDH expression. Mean ± SE data are displayed as fold changes relative to chow-fed control rats (**$P < 0.01$). (E) The mRNA levels of hepatic ACO were evaluated by quantitative real time PCR analysis from each group at 12 weeks (n = 5/group). Results were normalized to GAPDH expression. Mean ± SE data are displayed as fold changes relative to chow-fed control rats (*$P < 0.05$, **$P < 0.01$). (F) The mRNA levels of hepatic catalase were evaluated by quantitative real time PCR analysis from each group at 12 weeks (n = 5/group). Results were normalized to GAPDH expression. Mean ± SE data are displayed as fold changes relative to chow-fed control rats (*$P < 0.05$, **$P < 0.01$). Hepatic catalase activities were also valuated at 12 weeks. Results were normalized per mg tissue. Mean ± SE data from controls and each HFHC diet-fed group (n = 5/group) (*$P < 0.05$, **$P < 0.01$).

Liver International (2013)

hepatic free cholesterol. Recently, however, the more potent statins have been reported to be capable of restoring impaired peroxisomal FA β-oxidation *via* the peroxisome proliferator-activated receptor-alpha-mediated signalling pathway, and they are sufficiently potent to ameliorate severe hepatic steatosis (43–45). This finding is consistent with increased ACO expression and the enhancement of catalase activity (one of the potential disposal systems of toxic hepatic FAS) by rosuvastatin treatment in the HFHC diet-fed group in this study. We speculate that despite impaired VLDL export from livers, dietary cholesterol itself and decreased LDL uptake into livers may cause elevated serum VLDL/LDL/t-cholesterol levels in HFHC diet-treated rats. Rosuvastatin may decrease serum LDL levels and ameliorate liver steatosis in HFHC diet-fed rats without up-regulation of hepatic SREBP-2 and LDL-R expression, which may be caused by the inhibition of cholesterol synthesis and enhancement of peroxisomal FA oxidation. Furthermore, although 2mg/kg of rosuvastatin is sufficient to inhibit cholesterol synthesis in rodents fed with chow or high-fat diets, it may be insufficient to completely remove the lipotoxicity of HFHC diets, which contain 1.25% dietary cholesterol (46).

Statins have also been reported to exhibit systemic anti-inflammatory and anti-oxidant effects (8–16). Statins reduce mevalonate-derived molecules such as farnesyl pyrophosphate and GGPP, which activate the Ras, Rho, Rab and Ran superfamily (12). These are small GTP-binding proteins. They are directly modulated by the mevalonate pathway, and act as regulators of growth and apoptosis in endothelial cells, smooth muscle cells, fibroblasts and infiltrating monocytes/macrophages, of which important sources of ROS are NADPH oxidases (12). Recently, the anti-oxidant effects of statins were suggested to contribute to (i) inhibition of oxidant formation by affecting NADPH oxidase, (ii) blocking of ROS effects by up-regulation of anti-oxidant enzymes or (iii) increases in nitric oxide production in these cells. Statins were shown not only to block Rho-dependent profibrogenic cytokine production but also to increase the expression of endothelial nitric oxide synthase by blocking Rho geranylgeranylation, suggesting a statin-mediated reduction in oxidative stress (47).

The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) inhibitor, rosuvastatin, exhibits more potent affinity for the active sites of HMG-CoA reductase than other statins (6, 7, 31). Furthermore, rosuvastatin treatment has been shown to attenuate the transcription of monocyte chemoattractant protein-1, TGF-β, IL-1 and TNF-α in the kidney (22) and directly inhibit IL-6-induced CRP expression in liver cells (48), indicating the anti-inflammatory effects of rosuvastatin. These observations are consistent with the decrease in hepatic TNF-α and IL-6 expression in the HFHC diet-fed rosuvastatin-treated group in this study.

Statins also exhibit antifibrotic efficacy in various organs, and can inhibit the angiotensin II/Smad pathway and related fibrosis by a TGF-β-dependent/independent process (18–21). Recent reports have demonstrated that statins could be effective antifibrotic agents in hepatic fibrosis by inducing the apoptosis of fibrogenic cells such as HSCs. In this study, we showed that hepatic markers of liver fibrosis were considerably decreased in the HFHC diet-fed rosuvastatin-treated group with a reduction in hepatic profibrotic cytokine expression such as TGF-β and CTGF. Activation of HSCs and infiltration of T-cells and macrophages appeared to be lowered in the livers of rosuvastatin-treated rats.

Upon consideration of the results of this study and other reports, we conclude that rosuvastatin can prevent oxidative stress, liver steatosis, inflammation and fibrosis, at least in part, via improved peroxisomal β-oxidation. Hopefully, further studies using other animal models of NAFLD and NASH, including the HFHC diet model without cholic acid (33), will clarify the mechanisms.

Acknowledgements

Financial support: This study was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Program of Science (Kanji Yamaguchi and Yoshito Itoh).

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J Gastroenterol (2013) 48:1051–1060
DOI 10.1007/s00535-012-0704-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Noninvasive scoring systems in patients with nonalcoholic fatty liver disease with normal alanine aminotransferase levels

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Received: 16 August 2012 / Accepted: 14 October 2012 / Published online: 27 November 2012

Abstract
Background The severity of liver fibrosis must be estimated to determine the prognosis, for surveillance, and for optimal treatment of nonalcoholic fatty liver disease (NAFLD). However, the severity of hepatic fibrosis tends to be underestimated in patients with normal ALT.
Methods We investigated histological data and scoring systems (FIB-4 index, NAFLD fibrosis score, BARD score, and AST/ALT ratio) of 1,102 liver-biopsy-confirmed NAFLD patients.
Results A total of 235 NAFLD patients with normal ALT were estimated to exist. The ratio of advanced fibrosis (stage 3–4) was seen in 16.1 % of subjects with normal ALT. Scoring systems, especially the FIB-4 index and NAFLD fibrosis score, were clinically very useful (AUROC >0.8), even in patients with normal ALT. Furthermore, with resetting of the cutoff values, the FIB-4 index (>1.659) and NAFLD fibrosis score (>0.735) were found to have a higher sensitivity and higher specificity for the prediction of advanced fibrosis, and all of these scoring systems (FIB-4 index, NAFLD fibrosis score, BARD score, and AST/ALT ratio) had higher negative predictive values (>90.3 %). By using the resetting cutoff value, liver biopsy could have been avoided in 60.4 % (FIB-4), 66.4 % (NAFLD fibrosis score), 51.9 % (BARD score), and 62.1 % (AST/ALT ratio).

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Conclusions We reset the cutoff values of numerous non-invasive scoring systems to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal ALT, and these non-invasive scoring systems with the reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

Keywords NAFLD · NASH · Normal ALT · Scoring systems

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUROC	Area under the receiver-operating characteristic curve
BMI	Body mass index
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
NPV	Negative predictive value
PPV	Positive predictive value
AAR	AST/ALT ratio

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an important clinical subtype of chronic liver disease in many countries around the world [1]. The histological changes range over a wide spectrum, extending from simple steatosis, which is generally non-progressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, liver failure, and sometimes even hepatocellular carcinoma [2–5]. The severity of liver fibrosis must be estimated to determine the prognosis, for surveillance, and for optimal treatment of NAFLD, similar to the case for other liver diseases [6]. Liver biopsy is recommended as the gold standard for the diagnosis and staging of fibrosis in patients with NASH [1, 2, 7]. This procedure, however, is invasive and is associated with a high risk of complications [8]. Approximately 24.6 % of all patients complain of pain during/after the biopsy procedure [9], and the estimated risk of severe complications is 3.1 per 1,000 procedures [10]. Furthermore, it is impossible to enforce liver biopsy in all NAFLD patients, because the estimated number of NAFLD patients has reached 80–100 million in the US and over 20 million in Japan [11]. These considerations underscore the need for the development of simple non-invasive methods for assessing the severity of fibrosis.

Numerous non-invasive panels of tests have been developed for the staging of liver disease consisting of combinations of clinical and routine laboratory parameters, as well as specialized tests, such as direct markers of fibrosis and elastography [12–15]. Especially serum alanine aminotransferase (ALT) has long been used as a surrogate marker of liver injury [16, 17] and has been used in many scoring systems for various liver diseases, including NAFLD, such as the aspartate aminotransferase (AST)-to-ALT ratio (AAR) [18], NAFLD fibrosis score [19], BARD score [20], and FIB-4 index [21]. It is, however, well known that both fatty liver and NASH may exist without elevation of the serum ALT value [22, 23]. It is also well known that the serum ALT values may not always be well correlated with the severity of liver disease [17].

The purpose of this study was to compare the distribution of histological fibrosis stage and scoring systems in various serum ALT levels and to investigate the clinical usefulness of established clinical scoring systems for detecting the presence of advanced liver fibrosis (bridging fibrosis or cirrhosis) and resetting the reported cutoff values, as appropriate, in a large retrospective cohort of Japanese patients with NAFLD patients with normal ALT levels.

Patients and methods

Patients

A total of 1,102 patients with liver-biopsy-confirmed NAFLD between 2002 and 2011 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following ten hepatology centers in Japan: Nara City Hospital, Yokohama City University, Hiroshima University, Kochi Medical School, Saga Medical School, Osaka City University, Kyoto Prefectural University of Medicine, Asahikawa Medical College, Kurume University, and Saiseikai Suita Hospital. We performed liver biopsy for the purpose of diagnosis and staging of NASH. The principal indications for liver biopsy were a persistent decrease of the platelet count and increase in the serum levels of the direct markers of fibrosis (type IV collagen 7s and hyaluronic acid) according to the consensus of the Japan Society of Hepatology (JSH). In addition, older age, presence of diabetes, obesity, a prolonged history of steatosis, and the results of elastography were also considered on an individualized basis. The histological criterion used for the diagnosis of NAFLD was the presence of macrovesicular fatty changes in the hepatocytes, with displacement of the nuclei to the edges of the cells [24]. The criteria for exclusion from this study included a history of hepatic disease, such as chronic hepatitis C or concurrent active hepatitis B (seropositive for hepatitis B

surface antigen), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, as well as a current or past history of consumption of more than 20 g of alcohol daily. Informed consent was obtained from each patient included in the study, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Anthropometric and laboratory evaluation

The weight and height of the patients were measured using a calibrated scale after requesting the patients to remove their shoes and any heavy clothing. Venous blood samples were obtained in the morning after the patients had fasted overnight for 12 h. Laboratory evaluations in all patients included determination of the blood cell counts, and measurement of the serum levels of AST, ALT, γ-glutamyl transpeptidase (GGT), cholinesterase (ChE), albumin, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, fasting immunoreactive insulin (IRI), hyaluronic acid and type IV collagen 7s domain, and fasting plasma glucose. All of the parameters were measured using standard techniques.

Based on the previous study, the upper normal limit of the serum ALT was set at 40 IU/l [25, 26]. The FIB-4 index was calculated as age × AST (IU/l)/platelet count ($\times 10^9$/l)/$\sqrt{}$ALT (IU/l) [21]. The NAFLD fibrosis score was calculated according to the following formula: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+$ $1.13 \times$ impaired fasting glycemia or diabetes (yes = 1, no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/l) $- 0.66 \times$ albumin (g/dl) [19]. The BARD score was estimated as the weighted sum of three variables (BMI >28 = 1 point, AST/ALT ratio >0.8 = 2 points, diabetes = 1 point) [20]. AAR was calculated as AST/ALT [18].

Histologic evaluation

All patients enrolled in this study had undergone a percutaneous liver biopsy under ultrasound guidance. Fatty liver was defined as the presence of >5 % steatosis, while steatohepatitis was defined as the presence of steatosis, inflammation, and hepatocyte ballooning [27–29]. The degree of steatosis was assessed based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, 5–33 % hepatocytes containing macrovesicular fat droplets; grade 2, 33–66 % hepatocytes containing macrovesicular fat droplets; grade 3, >66 % hepatocytes containing macrovesicular fat droplets. The individual parameters of fibrosis were scored independently according to the NASH Clinical Research Network (CRN) scoring system developed by the NASH CRN [30]. Advanced fibrosis was classified as stage 3 or 4 (bridging fibrosis or cirrhosis).

Statistical analysis

Statistical analysis was conducted using SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data were represented as numbers, with the percentages indicated within parentheses. The statistical significances of differences in the quantitative data were determined using the t test or Mann-Whitney's U test. Because the variables were often not normally distributed, group comparisons of more than two independent groups were performed using the Kruskal-Wallis test. The percentage of cases with advanced fibrosis was compared between the ALT ≤40 and ALT >40 groups using Fisher's exact test. The diagnostic performances of the scoring systems were assessed by analyzing the receiver-operating characteristic (ROC) curves. The probabilities of a true-positive (sensitivity) and true-negative (specificity) assessment were determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cutoff points. Differences were considered to be statistically significant at $p < 0.05$.

Results

Patient characteristics

Using a multicenter database, 1,102 biopsy-proven cases of NAFLD were investigated. Of these, the serum ALT levels were more than 40 IU/l in 867 (78.7 %) patients and less than or equal to 40 IU/l in 235 (17.4 %) patients. In NAFLD patients with serum ALT levels ≤40 IU/l, steatosis grade, inflammatory activity, and fibrosis stage were not correlated with the serum ALT levels ($p = 0.4536$, 0.6238, and 0.1158 respectively by Kruskal-Wallis analysis). The distribution of histological fibrosis stage in various serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) is shown in Table 1. The distribution of the fibrosis stage in ALT level ≤40 was as follows: stage 0, $n = 91$ (38.7 %); stage 1, $n = 65$ (27.7 %); stage 2, $n = 41$ (17.4 %); stage 3, $n = 21$ (8.9 %); stage 4, $n = 17$ (7.2 %). The ratio of advanced fibrosis was 16.1 % (ALT ≤40 IU/l), 24.5 % (ALT 41–60 IU/l), 16.2 % (ALT 61–80 IU/l), 27.9 % (ALT 81–100 IU/l), and 25.0 % (≥101 IU/l) (Table 1). The percentage of cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l was