than in females. Five of 67 HCC patients consumed >60 g alcohol daily, and two of these five patients were anti-HCV Ab positive. HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence in the HCC patients was 8.6, 50.0, and 41.4 %, respectively. In a Japanese nationwide survey of 19,499 HCC patients [3], HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence was 15.0, 67.7, and 17.3 %, respectively. Non-B non-C prevalence was higher in our DM patients with HCC than in the nationwide HCC survey participants ($p < 0.001$). Mean PLT count in DM patients with HCC was as follows: HBsAg-positive patients, 12.4 ± 6.8; anti-HCV Ab-positive patients, 12.4 ± 5.6; and non-B non-C patients, 16.0 ± 7.0 ($\times 10^4/\mu L$); PLT count was significantly higher in the non-B non-C patients than in the anti-HCV-positive patients ($p < 0.05$). Mean BMI in these three patient groups was as follows: HBsAg-positive patients, 23.2 ± 5.1; anti-HCV Ab-positive patients, 22.8 ± 3.3; and non-B non-C patients, 27.2 ± 4.4 (kg/m^2); BMI was significantly higher in the non-B non-C patients than in the anti-HCV Ab-positive patients ($p < 0.001$).

Discussion

This is the first multicenter study, as per our knowledge, that clarifies the cause of liver injury in DM patients in Japan. Most Japanese HBV carriers are genotype C, acquired via perinatal vertical transmission or early childhood infection [12]. The HBV carrier rate in Japan is higher than that in western countries and significantly lower than that in other Asian countries [13]. In 1986, the Japanese government initiated a nationwide hepatitis B immunization program for infants born to HBV carrier mothers to prevent perinatal transmission. Consequently, the number of young serum HBsAg-positive individuals is extremely low. In our study, although the HBV carrier rate in DM patients was significantly higher than that in blood donors, 72 % of HBsAg-positive patients were serum HBV-DNA negative. Only 10 % of HBsAg-positive patients exhibited high serum HBV-DNA levels (≥4.0 log copies/ml), which is likely to induce hepatitis. These results indicate that a majority of DM patients who are HBV carriers may be asymptomatic.

Chronic hepatitis C may result in life-threatening complications, including cirrhosis and HCC. Worldwide, cirrhosis can be attributed to HBV (30 %) and HCV infection (27 %) [14]. The leading cause of cirrhosis among HBV and HCV sufferers and alcohol consumers varies with individual countries. A recent nationwide Japanese survey reported the etiology of cirrhosis in Japan as follows: HCV 60.9 %, HBV 13.9 %, alcoholism 13.6 %, primary biliary cirrhosis 2.4 %, NASH-related 2.1 %, and autoimmune hepatitis 1.9 % [15]. However, we must consider that hepatic triglycerides diminish with liver fibrosis progression in NASH patients (so-called "burned-out" NASH), resulting in difficulty in diagnosing NASH. Sixty-two percent of anti-HCV Ab-positive DM patients were HCV-RNA positive; these patients showed significantly higher serum ALT levels compared with HCV-RNA-negative patients. These results indicate that HCV infection is involved in the etiology of liver disease in DM patients.

There is no doubt that the positive rates of serum HBsAg and anti-HCV Ab in the general population are higher than in blood donors. Unfortunately, there were no data in the distribution of the rate of hepatitis virus carriers in each age group in Japan. In the present study, the positive rates of HBsAg and anti-HCV Ab in DM patients were significantly higher than that in blood donors. However, the present study demonstrated that most of HBsAg positive patients were negative for serum HBV DNA or had low serum HBV DNA levels and around one-third of anti-HCV Ab positive patients were negative for serum HCV RNA.

These results indicate the possibility that the frequency of hepatitis virus carriers in DM patients is higher than that in general population but no significant differences might be noted between DM patients and the general population.

Alcohol consumption is reportedly a significant factor associated with the risk of HCC development in patients with NASH-associated cirrhosis [16]. In our study, serum AST and ALT levels were comparable between drinkers consuming 20–59 g alcohol daily and nondrinkers. The ratio of heavy drinkers consuming >60 g alcohol daily was low (4.3 %) in our study. Moreover, drinking was not chosen as a variable related to elevated serum ALT levels. These results suggest that alcohol intake is not an important factor in the pathogenesis of liver disease in DM patients.

In our study, the frequency of anti-HCV Ab-positive DM patients was 5 %, whereas the serum HCV-RNA positivity rate in anti-HCV Ab-positive patients was 62 %. Therefore, the HCV carrier rate was calculated as 3 %. Since the proportion of HCV carriers and patients with elevated ALT levels were 3 % and up to 29 %, respectively, the influence of HCV infection is estimated to be no more than 10 % (3 % divided by 29 %) among DM patients with elevated ALT levels. There was no significant change in the number of DM patients with elevated ALT levels before and after elimination of HBV and/or HCV carriers and heavy drinkers. These results suggested that the major cause (up to 90 %) of liver injury in DM patients may be NAFLD.

In the present study, the frequency of advanced stage NASH was significantly higher in male DM patients than

in male non-DM patients. Neuschwander-Tetri et al. [17] reported that patients with advanced stage NASH were more likely to have DM. Mayaaki et al. [18] also examined the relationship between hepatic fibrosis stage and DM prevalence. In the mild fibrosis group, only 42 % were complicated with DM, whereas in the severe fibrosis group, the prevalence was as high as 71 % ($p = 0.020$). Lo et al. [19] reported that DM exacerbated diet-induced NASH fibrosis in mice. Therefore, DM may be an important factor in hepatic fibrosis development in NAFLD patients.

HCC frequency is significantly higher in obese and DM patients than in non-obese and non-DM patients [20, 21]. Recently, Tokushige et al. [22] reported on the backgrounds of Japanese HCC patients, and non-B non-C HCC accounted for 16 % of cases. A recent report has shown that NASH patients are likely to develop HCC in an earlier stage of fibrosis compared with chronic hepatitis C patients [23]. Our previous study analyzed 87 histologically proven NASH-HCC patients [24]; 37 % (20/54) of male HCC patients had a mild to moderate stage of liver fibrosis (F1 or F2); however, no female HCC patients were F1 stage, and only 15 % (5/33) were F2 stage. In the present study, DM patients with non-B non-C HCC exhibited a tendency to have higher PLT counts than those in DM patients with HCV-HCC, indicating that non-B non-C HCC is more likely to occur in DM patients with less advanced liver disease than in those with viral hepatitis.

In conclusion, HBsAg and anti-HCV Ab positivity rates were high; however, most of these patients were HBV-DNA negative or had low serum HBV-DNA levels. One-third of anti-HCV Ab-positive patients were HCV-RNA negative, and 4.3 % patients were drinkers whose ALT levels were comparable with those of nondrinkers. From these results, we conclude that up to 90 % of Japanese DM patients with liver injury may have NAFLD/NASH.

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Conflict of interest The authors declare that they have no conflicts of interest to disclose.

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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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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

Table 1 The distribution of histological fibrosis stage in serum ALT levels

ALT range	Patients no.	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Advanced fibrosis ratio (%)
All patients	1,102	240 (21.8 %)	339 (30.8 %)	290 (26.3 %)	196 (17.8 %)	46 (4.2 %)	22.0
≤40	235	91 (38.7 %)	65 (27.7 %)	41 (17.4 %)	21 (8.9 %)	17 (7.2 %)	16.1
41–60	229	48 (21.0 %)	76 (33.2 %)	49 (21.4 %)	41 (17.9 %)	15 (6.6 %)	24.5
61–80	172	35 (20.3 %)	53 (30.8 %)	56 (32.6 %)	23 (13.4 %)	5 (2.9 %)	16.2
81–100	122	18 (14.8 %)	41 (33.6 %)	38 (31.1 %)	31 (25.4 %)	3 (2.5 %)	27.9
≥101	344	48 (14.0 %)	104 (30.2 %)	106 (30.8 %)	80 (23.3 %)	6 (1.7 %)	25.0

significantly lower than that among the NAFLD patients with serum ALT levels >40 IU/l, as evaluated by Fisher's exact test ($p = 0.0163$).

The demographic and laboratory characteristics of NAFLD patients with serum ALT levels ≤40 IU/l and the clinical and laboratory features of the subjects with no or mild fibrosis (stage 0–2) compared with those of the patients with advanced fibrosis (stage 3–4) are shown in Table 2. In the patient group with serum ALT levels ≤40 IU/l, comparison of the characteristics of the subjects with no or mild fibrosis with those of subjects with advanced fibrosis revealed significantly higher values of age, serum AST, serum HDL cholesterol, fasting plasma glucose, serum fasting IRI, HOMA-IR, serum hyaluronic acid and serum type IV collagen 7s domain, and lower values of serum cholinesterase, serum albumin, hemoglobin, and platelet count in subjects with advanced fibrosis. In addition, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR were all significantly higher in patients with advanced fibrosis as compared with the values in the patients with no or mild fibrosis in the patient group with serum ALT levels ≤40 IU/l (Table 2).

The AUROC of the platelet count and serum level of the type IV collagen 7s domain for detecting cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l

Because significant differences in the platelet count and type IV collagen 7s domain were observed between patients with advanced fibrosis and those with no or mild fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l, the AUROCs of the platelet count and serum level of type IV collagen 7s domain for detecting fibrosis stages ≥ stage 3 were calculated. The AUROC for estimating the diagnostic performance of the platelet count for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.786 (optimal cutoff value 19.3 × 10^4/μl, sensitivity 81.6 %, specificity 65.5 %) (Fig. 1a). The AUROC for estimating the diagnostic performance of the serum level of type IV collagen 7s for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.794 (optimal cutoff value 5.0 ng/ml, sensitivity 63.2 %, specificity 84.3 %) (Fig. 1b).

The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

In order to investigate the diagnostic accuracy of the scoring systems in NALFD with various serum ALT levels, the AUROC for detecting fibrosis stages ≥ stage 3 was calculated in various distributions of serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) (Table 3). The AUROCs were calculated for the FIB-4 index (0.706–0.878), NAFLD fibrosis score (0.657–0.843), BARD score (0.517–0.684), and AAR (0.684–0.804). The diagnostic accuracy of each scoring system was more than equivalent also in case of ALT ≤40 IU/l. Furthermore, concerning the AUROC of the FIB-4 index, the NAFLD fibrosis score had the highest value in case of ALT ≤40 IU/l.

In NAFLD patients in the ALT >40 group, the optimal cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were 1.499, 0.502, 2, and 0.723, which were close to the cutoff values reported before [19, 20, 31, 32].

Prediction of the presence of advanced liver fibrosis and resetting of the cutoff value in NAFLD with normal ALT levels

In order to investigate the diagnostic accuracy of the scoring systems, ROC curves were constructed (Fig. 2). Then, the AUROC, optimal cutoff value, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined for each of the scoring systems. The FIB-4 index ranged from 0.305 to 12.482 in the NAFLD group with serum ALT values ≤40 IU/l. The FIB-4 index values stratified by the fibrosis stage were as follows: stage 0, 1.233 ± 0.711; stage 1, 1.570 ± 1.167; stage 2, 1.870 ± 1.496; stage 3, 3.071 ± 1.703; stage 4, 6.010 ± 3.704. Thus, in the NAFLD group with serum ALT values ≤40 IU/l, the FIB-4 index increased with increasing histological severity of the

Table 2 Characteristics of the NAFLD patients with serum ALT values ≤40 IU/l

Variable	Total ($n = 235$)	No or mild fibrosis (stage 0–2)	Advanced fibrosis (stage 3, 4)	p value
Age (years)	59.9 ± 12.1	58.6 ± 11.3	66.7 ± 8.4	0.0112
Body mass index (kg/m^2)	26.9 ± 4.0	26.5 ± 4.0	28.7 ± 4.7	0.0564
AST (IU/l)	24.7 ± 10.2	23.3 ± 6.93	31.9 ± 8.60	<0.001
ALT (IU/l)	23.7 ± 7.0	23.8 ± 4.56	23.0 ± 5.38	0.5293
Alkaline phosphatase (IU/l)	271.9 ± 124.4	264.3 ± 123.3	311.3 ± 205.3	0.2206
GGT (IU/l)	52.7 ± 74.1	51.9 ± 43.3	56.9 ± 54.1	0.6927
Cholinesterase (IU/l)	345.6 ± 90.6	366.3 ± 91.0	238.1 ± 104.4	<0.001
Albumin (g/dl)	4.28 ± 0.79	4.39 ± 1.06	3.71 ± 0.45	0.0177
Total cholesterol (mg/dl)	202.6 ± 37.9	203.5 ± 34.9	198.1 ± 43.7	0.6152
LDL cholesterol (mg/dl)	124.7 ± 31.3	127.1 ± 29.2	112.5 ± 31.6	0.1426
HDL cholesterol (mg/dl)	53.9 ± 13.5	52.4 ± 13.3	61.9 ± 16.2	0.0300
Triglyceride (mg/dl)	140.4 ± 72.2	146.2 ± 74.8	110.1 ± 60.8	0.0934
FPG (mg/dl)	123.3 ± 48.2	117.7 ± 40.1	152.1 ± 93.0	0.0176
Fasting insulin (μU/ml)	11.4 ± 8.31	10.6 ± 9.00	15.7 ± 9.01	0.0901
HOMA-IR	3.96 ± 3.56	3.39 ± 3.63	6.96 ± 7.77	0.0127
HbA1c (%)	5.96 ± 0.94	6.07 ± 0.80	5.42 ± 0.78	0.0885
Hemoglobin (g/dl)	13.5 ± 1.61	13.7 ± 1.38	12.2 ± 2.51	0.0010
Platelet count (×10^4/μl)	21.1 ± 6.90	22.7 ± 6.56	12.6 ± 6.18	<0.001
Hyaluronic acid (ng/ml)	87.3 ± 119.3	76.9 ± 128.9	141.0 ± 86.3	0.1302
Type IV collagen 7s (ng/ml)	4.74 ± 1.65	4.29 ± 1.38	7.05 ± 2.26	<0.001
Dyslipidemia	150 (63.8 %)	132 (67.0 %)	18 (47.3 %)	–
Diabetes mellitus	108 (46.0 %)	88 (44.7 %)	20 (52.6 %)	–
Steatosis grade (1/2/3)	124/84/27	94/79/24	30/5/3	–
Inflammatory grade (0/1/2/3)	59/138/38/0	57/117/23/0	2/21/15/0	
Fibrosis stage (0/1/2/3/4)	91/65/41/21/17	–	–	–
FIB 4 index	2.03 ± 1.93	1.47 ± 1.09	4.92 ± 3.42	<0.001
AST/ALT	1.07 ± 0.34	1.01 ± 0.32	1.41 ± 0.30	<0.001
NAFLD fibrosis score	−0.69 ± 1.81	−1.18 ± 1.63	1.82 ± 1.41	<0.001
BARD score	2.46 ± 1.23	2.28 ± 1.09	3.39 ± 0.65	0.0006

Values are mean ± SD. p values from Student's t test, Mann-Whitney test or χ^2 test, as appropriate

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *FPG* fasting plasma glucose, *HOMA-IR* homeostasis model assessment-insulin resistance

hepatic fibrosis ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the FIB-4 index for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT ≤40 IU/l was 0.878 (Fig. 2a) (optimal cutoff value 1.659, sensitivity 89.5 %, specificity 71.1 %). Using the previously published cutoff value proposed by Shah et al. [31] (>2.67), the sensitivity of this index for the detection of advanced fibrosis was calculated as 63.2 % and the specificity as 88.3 % (Table 4).

The NAFLD fibrosis score ranged from −6.304 to 4.639 in the NAFLD patients with serum ALT values ≤40 IU/l. The NAFLD fibrosis scores stratified by the fibrosis stage were as follows: stage 0, −1.439 ± 1.538; stage 1, −1.290 ± 1.592; stage 2, −0.762 ± 1.591; stage 3, 0.256 ± 1.400; stage 4, 2.110 ± 1.332; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the NAFLD fibrosis score for hepatic fibrosis stages ≥ stage 3 in the NAFLD patients with serum ALT values ≤40 IU/l was 0.843 (Fig. 2b) (optimal cutoff value 0.735, sensitivity 68.4 %, specificity 88.3 %). Using the previously published cutoff point proposed by Angulo et al. [19] (>0.676), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 68.4 % and the specificity as 87.8 % (Table 4).

The BARD score ranged from 0 to 4 in the NAFLD patients with serum ALT values ≤40 IU/l. The BARD scores stratified by the fibrosis stage were as follows: stage 0, 2.000 ± 1.200; stage 1, 1.967 ± 1.303; stage 2, 2.100 ± 1.215; stage 3, 2.316 ± 1.057; stage 4, 3.333 ± 0.724. The AUROC calculated to estimate the

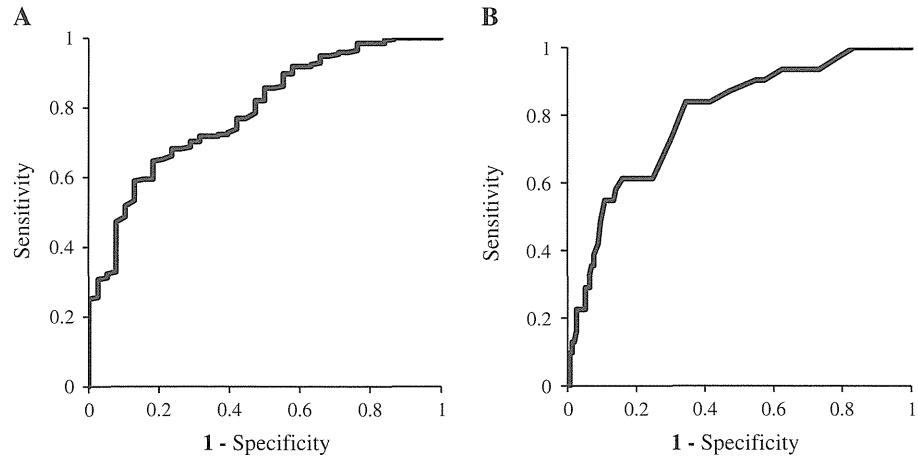

Fig. 1 Receiver-operating characteristic (ROC) curves for detecting advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** The platelet count, **b** type IV collagen 7s

Table 3 The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

ALT levels (IU/l)	FIB4 index	NAFLD fibrosis score	BARD score	AST/ ALT
≤40	0.878	0.843	0.671	0.794
41–60	0.818	0.726	0.684	0.804
61–80	0.706	0.654	0.663	0.737
81–100	0.752	0.657	0.517	0.684
≥101	0.773	0.670	0.578	0.728

diagnostic performance of the BARD score for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.671 (Fig. 2c) (optimal cutoff value 3, sensitivity 65.8 %, specificity 59.9 %). Using the previously published cutoff point proposed by Harrison et al. [20] (>2), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 86.8 % and the specificity as 32.5 % (Table 4).

AAR ranged from 0.448 to 2.630 in the NAFLD patients with serum ALT values ≤40 IU/l. The AAR values stratified by the fibrosis stage were as follows: stage 0, 0.869 ± 0.221; stage 1, 0.942 ± 0.269; stage 2, 0.998 ± 0.424; stage 3, 1.232 ± 0.423; stage 4, 1.359 ± 0.361; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the AAR for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.794 (Fig. 2d) (optimal cutoff value 0.975, sensitivity 78.9 %, specificity 70.1 %). Using the previously published cutoff point proposed by McPherson et al. [32] (>0.8), the sensitivity of this ratio for the detection of advanced fibrosis was calculated as 89.5 % and the specificity as 37.1 % (Table 4).

Discussion

The incidence of NAFLD is rising rapidly in both adults and children because of the currently ongoing epidemics of obesity and type 2 diabetes [33]. Thus, development of a rapid and non-invasive method for the detection of fibrosis in NAFLD patients is of major clinical interest. In recent years, Shah et al. [31] reported, from a multicenter trial, the usefulness of scoring systems for NAFLD patients. In their study, they evaluated 541 NAFLD patients and concluded that the AUROC values calculated to estimate the diagnostic performances of FIB4, the NAFLD fibrosis score, and AAR in which the serum ALT is included for hepatic fibrosis stages ≥ stage 3 were 0.802, 0.768, and 0.720, respectively. We also previously validated these scoring systems in 576 biopsy-proven Japanese NAFLD patients [34]. Furthermore, in this study, the cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were close to the cutoff values reported before [19, 20, 31, 32].

NAFLD often presents as abnormal liver enzyme levels in the absence of markers of other common liver diseases, e.g., hepatitis C. The severity of hepatic fibrosis tends to be underestimated in patients with serum ALT values within normal limits, even though normal serum ALT values do not guarantee the absence of advanced fibrosis in patients with NAFLD [23]. It is not uncommon for patients to present with complications of previously unrecognized cirrhosis despite being under long-standing medical care, because these patients often do not manifest the classical physical changes associated with cirrhosis. At present,

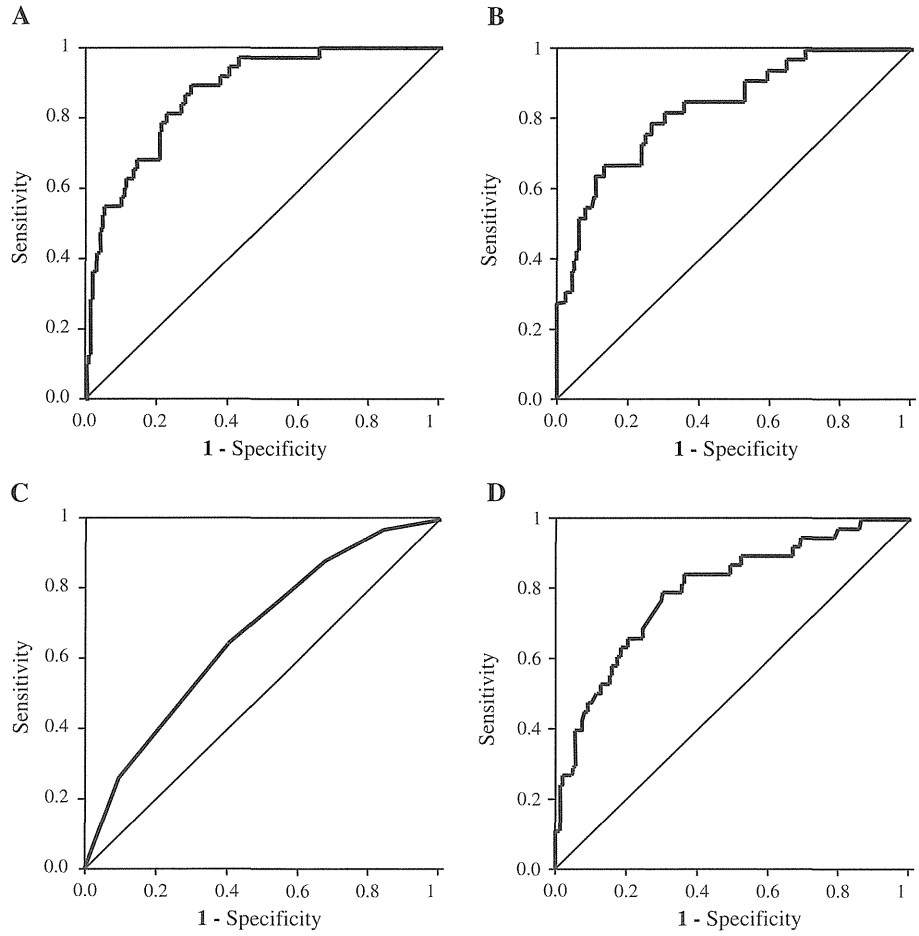

Fig. 2 Receiver-operating characteristic (ROC) curves for the noninvasive scores for a diagnosis of advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** FIB-4 index, **b** NAFLD fibrosis score, **c** BARD score, **d** AST/ALT ratio (AAR)

Table 4 Comparison of the performance of each of the scoring systems for the diagnosis of advanced fibrosis in 235 NAFLD patients with serum ALT values under 40 IU/l using reported cutoff and reset cutoff values

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	References
Fib-4						
Reported by Shah et al.	2.67	63.2	88.3	51.1	92.6	[31]
Re-setup	1.659	89.5	71.1	37.4	97.2	
NAFLD fibrosis score						
Reported by Angluo et al.	0.676	68.4	87.8	52.0	93.5	[19]
Re-setup	0.735	68.4	88.3	53.0	93.5	
BARD score						
Reported by Harrison et al.	2	86.8	32.5	19.9	92.8	[20]
Re-setup	3	65.8	59.9	24.0	90.1	
AST/ALT (AAR)						
Reported by McPherson et al.	0.8	89.5	37.1	21.5	94.8	[32]
Re-setup	0.975	78.9	70.1	30.7	94.5	

PPV positive predictive value, *NPV* negative predictive value

NAFLD patients with normal serum ALT values are very rarely investigated or subjected to liver biopsy. Mofrad et al. [23] and Fracanzani et al. [25] found that the histological features of NAFLD sometimes progress even in persons with normal serum ALT values and that the liver histology in these persons is not very different from that in

patients with high serum ALT levels; in addition, a low or normal serum ALT level does not serve as a reliable criterion to exclude the need for liver biopsy in NAFLD patients [23, 25]. Fracanzani et al. [25] reported that a persistent increase of the serum ferritin level, persistent evidence of severe steatosis on ultrasonography, and a persistent increase of the serum GGT levels were the main reasons for liver biopsy in patients with normal serum ALT levels. Mofrad et al. [23] reported that the principal indications for liver biopsy in patients with normal ALT levels were persistent hepatomegaly, donor evaluation for living donor liver transplantation, elevated serum ferritin levels, abnormal imaging characteristics of the liver suggestive of parenchymal liver disease, baseline biopsy to initiation of methotrexate therapy, and clinical features of portal hypertension without other evidence of liver disease.

A first finding in our study is the ratio of advanced fibrosis (stage 3–4) in various distributions of ALT. Advanced fibrosis was seen in 16.1 % of subjects with serum ALT levels ≤40 IU/l. Thus, caution must be exercised in evaluating the disease severity in NAFLD patients with normal serum ALT values. While the platelet count and serum level of the collagen 7s domain were reported to be useful for predicting the presence of advanced fibrosis in NAFLD patients [35, 36], it appears that they may also be useful for predicting severe fibrosis in cases of NAFLD with normal serum ALT levels. However, the specificity of the platelet count and sensitivity of type IV collagen 7s were slightly low. So far, no previous studies have investigated the usefulness of the available tests for the prediction of liver fibrosis in NAFLD patients with normal serum ALT values, because the small sample size of NAFLD subjects with normal serum ALT levels hampers any attempt to construct scoring systems for predicting NASH or fibrosis [25]. Thus, the previous scoring systems, especially their cutoff values, seem to be insufficient for the diagnosis of fibrosis in the NAFLD patients with normal ALT.

A second finding of this study was that the scoring systems investigated in NALFD with normal ALT. Of these, especially the FIB-4 index and NAFLD fibrosis score were clinically very useful (AUROC >0.8) even in patients with normal serum ALT values. Furthermore, with resetting of the cutoff values, they were found to have a higher sensitivity and higher specificity for the prediction of advanced fibrosis in a retrospective cohort of NAFLD patients with normal serum ALT values. The BARD score failed to detect the outstanding sensitivity and specificity in all the ALT groups. Consistent with the present study, Fujii and colleagues [37] reported significantly poorer applicability of the BARD score in Japanese patients with NAFLD compared to Caucasian subjects. It has been suggested that the BARD score is less predictive of advanced fibrosis in Japanese NAFLD patients because they are less obese that those in western countries.

As a third finding of this study, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR all had high NPVs (>90.1 %) for advanced fibrosis in the cohort of patients with NAFLD. This suggests that these scoring systems could be used clinically to exclude advanced fibrosis in subjects with NAFLD. For example, using the FIB-4 index (<1.659) to exclude advanced fibrosis, liver biopsy could have been avoided in 60.4 % of the patients in our cohort of patients with serum ALT values ≤40 IU/l. Similarly, prediction of the presence/absence of fibrosis based on the NAFLD fibrosis score (<0.735), BARD score (<3), and AAR allowed avoidance of liver biopsy in 66.4, 51.9, and 62.1 % of patients, respectively. Given the large numbers of NAFLD patients with normal serum ALT values, use of these non-invasive tests with reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

As a fourth finding of this study, in contrast to the NPVs, the PPVs of the tests did not have sufficient accuracy for the diagnosis of advanced fibrosis. It would, therefore, seem appropriate to consider liver biopsy in all patients with values above the cutoff of the selected index. We previously reported, for the first time in the world, that transient elastography and acoustic radiation force impulse (ARFI) elastography can be used to measure the severity of fibrosis in patients with NAFLD [15, 38]. It is possible that a combination of transient elastography and one of the aforementioned scoring systems may provide better performance than each of them used alone, although this needs to be verified in future studies.

This study had several limitations. First, the proportion of subjects with advanced fibrosis was small. Second, the patients were recruited from hepatology centers in Japan with a particular interest in the study of NAFLD; therefore, the possibility of some referral bias cannot be ruled out. Patient selection bias could also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. The findings may thus not represent those of the NAFLD patients in the community at large. The question remains as to whether the revised cutoff values of the various scoring systems might be useful in real clinical practice. Another limitation is that the supposedly normal range of ALT values is incorrect. The public health implications and clinical usefulness of reducing the upper limits of the normal value for the serum ALT continue to be under debate, and the currently proposed cutoff values for the upper limits of the serum ALT levels are 30 IU/l for men and 19 IU/l for women [39]. Recently, the upper limit of the normal range of serum ALT levels in the Asian population was reported as 35 IU/l for men and 26 IU/l for patients with a normal liver

histology [40]. According to our preliminary data, the AUROC calculated for detecting advanced fibrosis was 0.907 (FIB4 index), 0.916 (NAFLD fibrosis score), 0.793 (BARD), and 0.859 (AAR) in 127 biopsy-proven NAFLD patients with ALT ≤30 (data not shown). We also acknowledge that the pathologic diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling errors and/or inter-observer variability [41, 42].

In conclusion, the issue of development of a non-invasive method for the assessment of disease severity remains crucial in patients with NAFLD given the high number of subjects with steatosis and normal serum ALT values in the general population. We reset the cutoff values of numerous non-invasively determined indices to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal serum ALT values. In the absence of biopsy or of an adequate score capable of identifying subjects at risk, these patients could miss being included in the list for careful follow-up and might be scarcely motivated to adopt lifestyle modifications that could potentially cure their liver disease. Clinicians should be aware of the importance of complete clinical evaluation for early diagnosis and treatment of liver diseases. Non-invasive scoring systems, especially the FIB-4 index and the NAFLD fibrosis score showed high sensitivity and specificity, and they can be reliably used to exclude advanced fibrosis in NAFLD subjects with normal serum ALT levels.

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Conflict of interest The authors have no conflicts of interest to disclose.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Bofutsushosan, a Japanese herbal (Kampo) medicine, attenuates progression of nonalcoholic steatohepatitis in mice

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Abstract

Background Obesity-induced liver disease (nonalcoholic fatty liver disease, NAFLD) is now the commonest cause of chronic liver disease in affluent nations. There are presently no proven treatments for NAFLD or its more severe stage, nonalcoholic steatohepatitis (NASH). Bofutsushosan (BTS), a Japanese herbal (Kampo) medicine, long used as an anti-obesity medicine in Japan and other Asian countries, has been shown to reduce body weight and improve insulin resistance (IR) and hepatic steatosis. The precise mechanism of action of BTS, however, remains unclear. To evaluate the ability of BTS to prevent the development of NASH, and determine the mediators and pathways involved.

Methods C57BL/6 mice were injected intra-peritoneally with gold-thioglucose and fed a high-fat diet (HF) or HF diet admixed with either 2 or 5 % BTS for 12 weeks. The effectiveness of BTS in attenuating features of NASH and the mechanisms through which BTS attenuated NASH were then assayed through an assessment of the anthropometric, radiological, biochemical and histological parameters.

Results BTS attenuated the progression of NASH through induction of adiponectin and its receptors along with an induction of PPAR-α and PPAR-γ, decreased expression of SREBP-1c, increased hepatic fatty acid oxidation and increased hepatic export of triglycerides. BTS moreover, reduced IR through phosphorylation of the protein kinase, Akt.

Conclusions BTS through induction of adiponectin signaling and Akt attenuated development of NASH. Identification of the active entity in BTS should allow development of novel treatments for NASH.

Keywords NAFLD · Adiponectin · Bofutsushosan · Kampo medicine

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
GTG	Gold-thioglucose
HF	High-fat-diet
BTS	Bofutsushosan
IR	Insulin resistance
IGT	Impaired glucose tolerance
GTT	Glucose tolerance test

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ITT	Insulin tolerance test
QUICKI	Quantitative insulin sensitivity check index
MCAD	Medium-chain acyl-CoA dehydrogenase
TIMP	Tissue inhibitor of metalloproteinases
TGF	Transforming growth factor
MTP	Microsomal triglyceride transfer protein
AMPK	AMP-activated protein kinase

Introduction

Obesity-induced liver disease (nonalcoholic fatty liver disease, NAFLD), is now the commonest cause of chronic liver disease in affluent nations. The disease comprises obesity and insulin resistance (IR), with a consequent histopathological spectrum of hepatosteatosis, steatohepatitis (nonalcoholic steatohepatitis, NASH), cirrhosis and possible hepatocellular cancer [1–3]. There are presently no proven treatments for NAFLD or its more severe stage NASH. Bofutsushosan (BTS), a Japanese herbal (Kampo) medicine, long used as an anti-obesity medicine in Japan and other Asian countries [4] has recently been shown in obese Japanese women, to reduce body weight and improve IR [4]. In addition, BTS in experimental animals prevented adipogenesis [5], reduced weight, suppressed visceral and subcutaneous fat accumulation and in parallel decreased plasma glucose, triglycerides (TG), insulin, tumor necrosis factor-alpha [6], and hepatic steatosis induced by high-fat diet feeding [7, 8]. The mechanism of action of BTS however, is not known. Our aim here was to evaluate the ability of BTS to prevent the development of NASH in a recently described murine model involving administration of gold-thioglucose (GTG) and high-fat feeding to induce NASH [9], and to determine the mediators and pathways involved.

Materials and methods

Animal preparation

All procedures conformed to our institutions' guidelines for the care and use of animals in Kochi Medical School. Four-week-old male C57BL/6 mice were purchased from CLEA Japan Inc. All animals were housed for 12 weeks on a 12 h light/12 h dark cycle, with food and water freely available. Mice were fed high-fat-diet (HF, 640 kcal/100 g, F2HFD2, Oriental Yeast, Tokyo, Japan) or HF admixed with 2 or 5 % BTS (TJ-62, Tsumura & Co., Tokyo, Japan). All groups were fed standard chow (SC) for the first week and then continued on their respective group diets for remainder of the protocol.

Three experimental groups were studied: (1) intra-peritoneal administered GTG (2 mg/g of body weight, Sigma-Aldrich, St. Louis, MO, USA) and then SC for 1 week followed by HF diet for 11 weeks (GTG + HF) [9]; (2 and 3) intra-peritoneal GTG, SC for 1 week followed by HF diet admixed with either 2 %BTS or 5 % BTS for further 11 weeks (2 %BTS or 5 % BTS). At the end of the treatment period, all animals were fasted overnight, anesthetized with pentobarbital sodium intraperitoneally (25–50 mg/kg of body weight, Nembutal; Abbott Laboratories, Abbott Park, IL, USA). Blood and liver samples were harvested. Livers were fixed in 10 % formalin, or snap frozen in liquid nitrogen and stored at −80 °C, for later analyses.

CT scan analysis for body fat composition

The extent of adiposity in each experimental group was assayed by CT scanning (La Theta, ALOKA, Tokyo, Japan) under isoflurane (2 % v/v) anesthesia as described previously [9]. Animal were scanned at 2-mm intervals from the diaphragm to the pelvis, and visceral fat and subcutaneous fat volumes quantified with La Theta software (version 1.00) [9–11].

Histopathological examination

Five-micrometer sections of formalin-fixed/paraffin-embedded livers were processed for haematoxylin and eosin (H&E). Oil Red-O staining of intracellular neutral lipids was performed according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). For estimation of extent of hepatic steatosis, the areas of digital photomicrographs were quantified with a computerized image analysis system (macintosh MacSCOPE version 2.591) as described previously [9, 12]. Degree of oxidative stress was determined by staining and quantification with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) and anti-4-hydroxy-2-nonenal (4-HNE) as previously described [9, 12, 13].

Glucose tolerance test (GTT), insulin tolerance test (ITT) and QUICKI

At 12 weeks, a glucose tolerance test (GTT) ($n = 6$) and an insulin tolerance test (ITT) ($n = 6$) were performed. For GTT, mice were fasted for 18 h, and then intra-peritoneally loaded with 20 % glucose at a dose of 1.0 g/kg body weight. For ITT, mice were fasted for 6 h, and then intra-peritoneally challenged with human insulin at 1.0 U/kg body weight [14, 15]. With both GTT and ITT blood

samples from the orbital sinus were taken at times 0, 30, 45, 60 and 120 min and plasma glucose concentrations measured using an automatic blood glucose meter (Glutest; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Plasma insulin level was measured by Ultrasensitive Mouse Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacture's protocol. The quantitative insulin sensitivity check index (QUICKI), as a measure of IR, was calculated from the fasting insulin and glucose levels.

Measurement of plasma adiponectin

Plasma adiponectin levels were measured by Mouse Adiponectin/Acrp30 (R&D Systems, Minneapolis, MN, USA) according to the manufacture's instructions.

Laboratory evaluation

Asparate aminotransferase (AST), alanine aminotransferase (ALT) and TG were measured by an autoanalyzer (BM6010; JEOL Ltd., Tokyo, Japan).

Real-time RT-PCR for quantitative assessment of mRNA expression

Total RNA was extracted using trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacture's protocol. RNA extracts were reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (Takara, Kyoto, Japan). Real time RT-PCR were performed for quantitative assessment of mRNA expression on an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Probes and primers for TNF-α, PPAR-α, PPAR-γ, MTP, DGAT2, Cyp2E1, adiponectin receptor 1/receptor 2 (AdipoR1/R2) were all purchased from Applied Biosystems. Relative expression of target gene mRNA was normalized to the amount of GAPDH mRNA.

Western blot analysis

For in vivo analysis of phosphorylated Akt and total Akt, mice were fasted for 18 h, injected intra-peritoneally with human insulin (10 U/kg) or control, and sacrificed 4 min later. Livers were snap frozen in liquid nitrogen. Liver total protein was analyzed by western blot with a polyclonal antibody to phosphorylated Akt/Akt and phosphorylated AMPK/AMPK (Cell Signaling Technology, Inc., Danvers, MA, USA) as described [16]. For analysis of SREBP-1c, mice were fasted for 18 h, sacrificed, and livers dissected and homogenized to prepare cell nuclear extracts which were then analyzed by western blotting with anti-SREBP-1c antibody (Santa Cruz Biotechnology, Inc., California, CA, USA) as described [17]. For analysis of 4-HNE, liver total protein was analyzed by western blot with a monoclonal anti-4-HNE antibody (Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistics

Data are shown as mean ± SD. A univariate analysis was conducted with the Mann–Whitney U test to determine significance between groups. Qualitative data were compared using Fisher's exact test. Statistical significance was accepted at $p < 0.05$. All analyses were performed using Stat View software (SAS Institute, Cary, NC, USA).

Results

BTS treatment reduces GTG + HF induced obesity and steatohepatitis

Mice administered GTG and then fed a HF diet (GTG + HF), had a comprehensive histological and dysmetabolic phenotype resembling human NASH as reported recently [9]. To then evaluate the effectiveness of BTS in the GTG + HF model, we studied the anthropometric, radiological, biochemical and histological parameters as detailed above in the presence or absence of BTS 2 or 5 % admixed with the HF component of the GTG + HF. Administration of BTS attenuated weight gain in a dose dependent manner (Fig. 1a). Unexpectedly, GTG + HF induced increase in the volume of the visceral and subcutaneous fat were not markedly attenuated by treatment with BTS (Fig. 1b). However, hepatic steatosis on H&E or Oil Red-O staining in 12 weeks was attenuated in a dose dependent manner by BTS treatment (Figs. 2a, 3a). Additionally, BTS treatment also attenuated GTG + HF induced hepatomegaly in a dose-dependent manner (Fig. 2b).

GTG + HF mice livers showed steatohepatitis with marked steatosis and inflammation, hepatocyte ballooning and Mallory–Denk bodies as described previously (Fig. 3a) [9]. BTS treatment attenuated hepatic steatosis and hepatic inflammation (Fig. 3a), and inhibited hepatocyte ballooning and Mallory–Denk bodies. In parallel, oxidative stress makers, 8-OHdG (Fig. 3b) and 4-HNE (supplemental figure), were remarkably reduced by BTS, as was the expression of TNF-α (Fig. 3b). BTS treatment moreover, attenuated GTG + HF induced elevation of transaminases (Table 1).

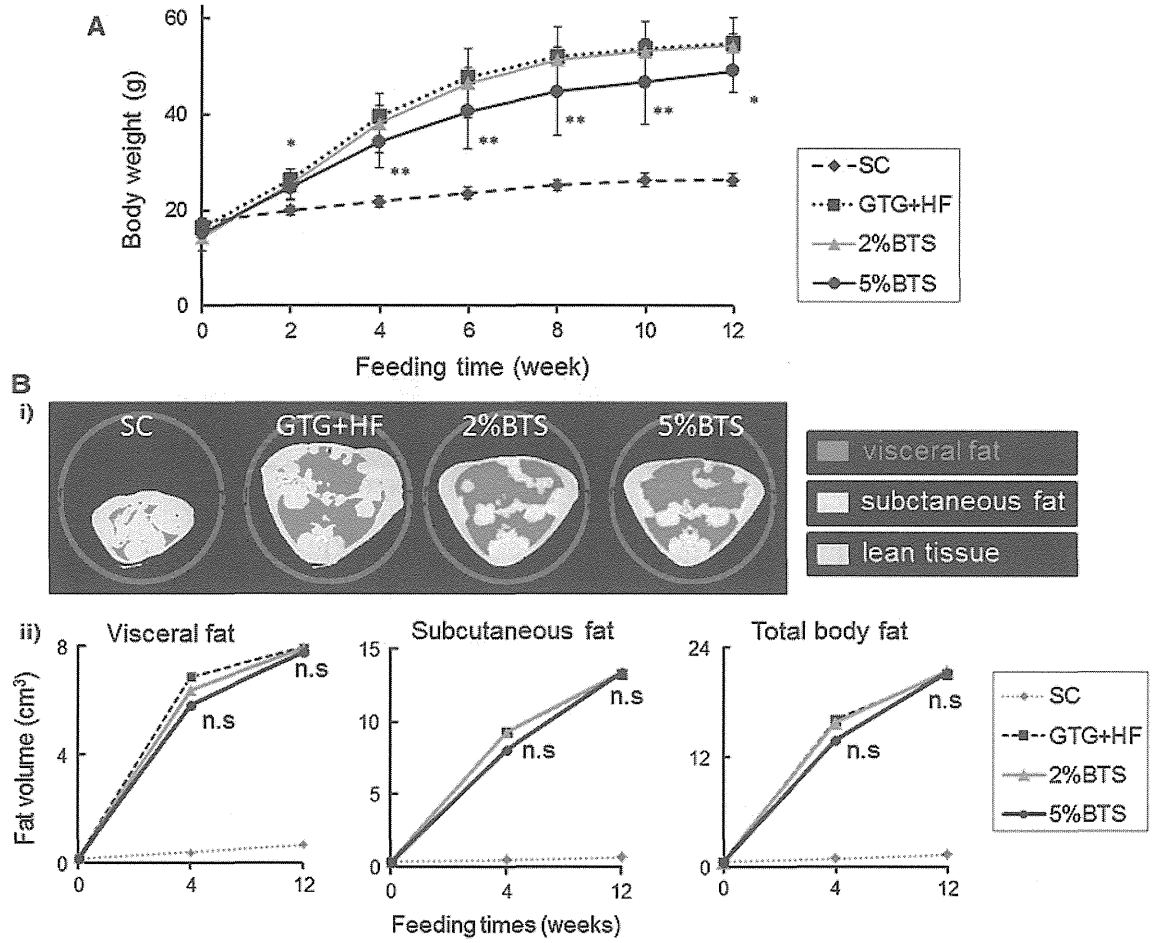

Fig. 1 Effectiveness of BTS for obesity and adiposity. **a** BTS dose-dependently attenuated weight gain in GTG + HF mice. $^{**}p < 0.01$, $^{*}p < 0.05$ vs GTG + HF, $n = 6$. *SC* standard chow fed mice, *GTG + HF* GTG treated and HF fed mice, *2 %BTS* 2 %BTS + GTG + HF fed mice, *5 %BTS* 5 %BTS + GTG + HF fed mice. **b** Anthropometry and evaluation of subcutaneous fat and visceral fat volumes by abdominal CT *i* representative photomicrographs of abdominal CT scan of SC, GTG + HF, 2 %BTS and 5 %BTS mice shown at 12 weeks. *Yellow* subcutaneous fat, *purple* visceral fat, *blue* lean tissue. *ii* Time course of increase of volume of subcutaneous and visceral fat. Neither subcutaneous fat nor visceral fat was significantly attenuated by treatment with BTS; 2 %BTS, 5 %BTS vs GTG + HF, p = ns, $n = 6$

BTS inhibition of hepatic lipid metabolism occurs through induction of adiponectin signaling

We examined plasma adiponectin levels and liver expression of the adiponectin receptors (AdipoR1 and AdipoR2), an important anti-inflammatory cytokine and receptors [18–20], because both plasma adiponectin levels and liver expression of the adiponectin receptors were decreased in GTG + HF mice as described previously [9]. Plasma adiponectin level and hepatic expression of AdipoR1, R2 were significantly enhanced by BTS (Fig. 4a). We next investigated the pathways of suppression of hepatic lipid metabolism by adiponectin in the presence of BTS. The expression of SREBP-1c was decreased dose dependently by BTS (Fig. 4b). The phosphorylation of AMPK (P-AMPK/AMPK) was here increased by BTS treatment (Fig. 4c) in parallel with activation of AdipoR1 signaling (Fig. 4a). In addition, expression of PPAR-γ, an activator of AMPK, was increased by BTS treatment (Fig. 4d).

Attenuation of hepatic steatosis with BTS treatment involves activation of fatty acid oxidation

Additionally, the expression of PPAR-α and its target genes, MCAD, involved in mitochondrial β-oxidation and CYP2E-1 involved in microsomal ω-oxidation [21, 22] were increased by treatment with BTS (Fig. 4e–g).

BTS promotes hepatic lipid export

We next determined if enhanced secretion of TG from the liver could contribute to the attenuation of hepatic steatosis by BTS treatment. The expression of microsomal triglyceride transfer protein (MTP) known to play a central role in

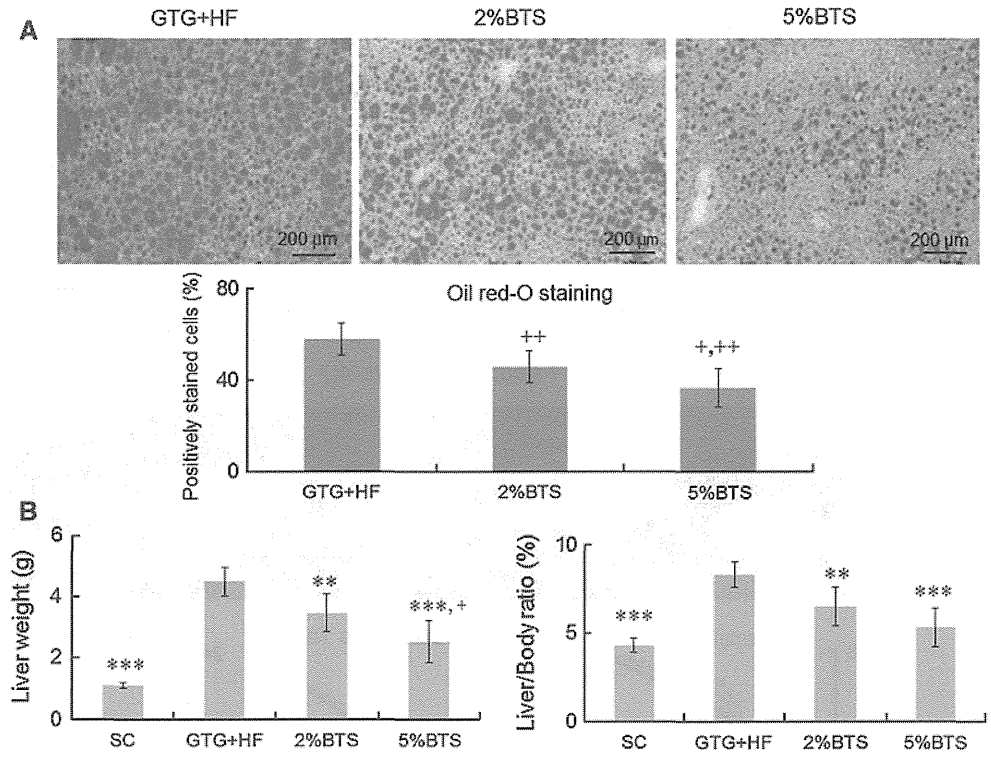

Fig. 2 BTS reduces hepatomegaly and hepatic steatosis. **a** Oil red-O staining and image analysis of livers: Oil red-O staining showed that BTS attenuated hepatic steatosis in GTG + HF fed mice. In addition, image analysis for Oil red-O staining of liver sections confirmed that BTS treatment attenuated hepatic steatosis. *Plus symbol* $p < 0.0001$ vs 2 %BTS, *double plus symbol* $p < 0.00001$ vs GTG + HF, $n = 6$. **b** Liver weight and liver/body weight ratio (liver/body): the increase of both liver weight and liver/body ratio was significantly attenuated by BTS treatment in a dose dependent manner. *Triple asterisk* $p < 0.001$, *double asterisk* $p < 0.01$ vs GTG + HF, *plus symbol* $p<0.05$ vs 2 %BTS, $n = 6$

lipoprotein assembly [23] was increased with BTS treatment (Fig. 4h). Interestingly, expression of DGAT2 reported to be involved in the conversion of free fatty acids into TG in the liver [24] was also increased in the BTS treated groups (Fig. 4i).

BTS reduces glucose intolerance and insulin resistance through induction of Akt

Since IR is regarded as a central pathogenic feature of NAFLD [25], we now investigated the effect of BTS treatment on IR. Fasting plasma glucose and insulin levels were markedly reduced in a dose dependent manner by BTS treatment, and QUICKI as an index of IR was also increased by BTS (Fig. 5a). We next evaluated the attenuation of glucose intolerance and IR using the GTT and the ITT in the presence of BTS. GTT revealed that treatment with BTS remarkably attenuated severe glucose intolerance induced by GTG + HF, and ITT showed that treatment with BTS attenuated IR induced by GTG + HF (Fig. 5b). The reduction of IR by BTS involved its promotion of the phosphorylation of Akt (Fig. 5c) an important factor in glucose metabolism [26].

Discussion

The public health importance of NAFLD [1, 2] and the unavailability of proven and effective therapies drive the search for a greater understanding of its pathophysiology and novel therapeutic pathways. In this study, we have clarified the mechanisms through which BTS attenuates NASH based on a novel animal model of NASH [9]. BTS attenuated the GTG + HF induced increases in body and liver weight, serum transaminases, hepatic steatosis, degree of oxidative stress and TNF-α expression (Figs. 1, 2, 3; Table 1) without reducing intake volume of diets (data not shown). However, in contrast to previous reports [6], we did not demonstrate statistically remarkable reduction by BTS, in GTG + HF mice, of induced increases in visceral or subcutaneous fat (Fig. 1b), perhaps because the volume of these fats in our mice were much larger than previously reported [6]. Additionally, the reduction of body weight might mainly be through the reduction of fat accumulation in muscles, since it has been reported that the degree of hepatosteatosis is well correlated with the degree of fat accumulation in muscles [27].

To examine the mechanisms though which BTS attenuated hepatic steatosis, we firstly evaluated the effect of BTS on adiponectin, thought to be a central adipokine in the pathogenesis for NASH [18, 19]. The expression of AdipoR1 and AdipoR2 in the livers was increased in a dose dependent manner by BTS (Fig. 4a), indicating that BTS could have PPAR-α agonist like effect, since PPAR-α agonists are known to increase expression of AdipoR1 and AdipoR2 [28]. Interestingly, plasma adiponectin levels were also remarkably induced by BTS treatment (Fig. 4a) even though neither visceral nor subcutaneous fat were decreased. These data indicated that BTS through putative

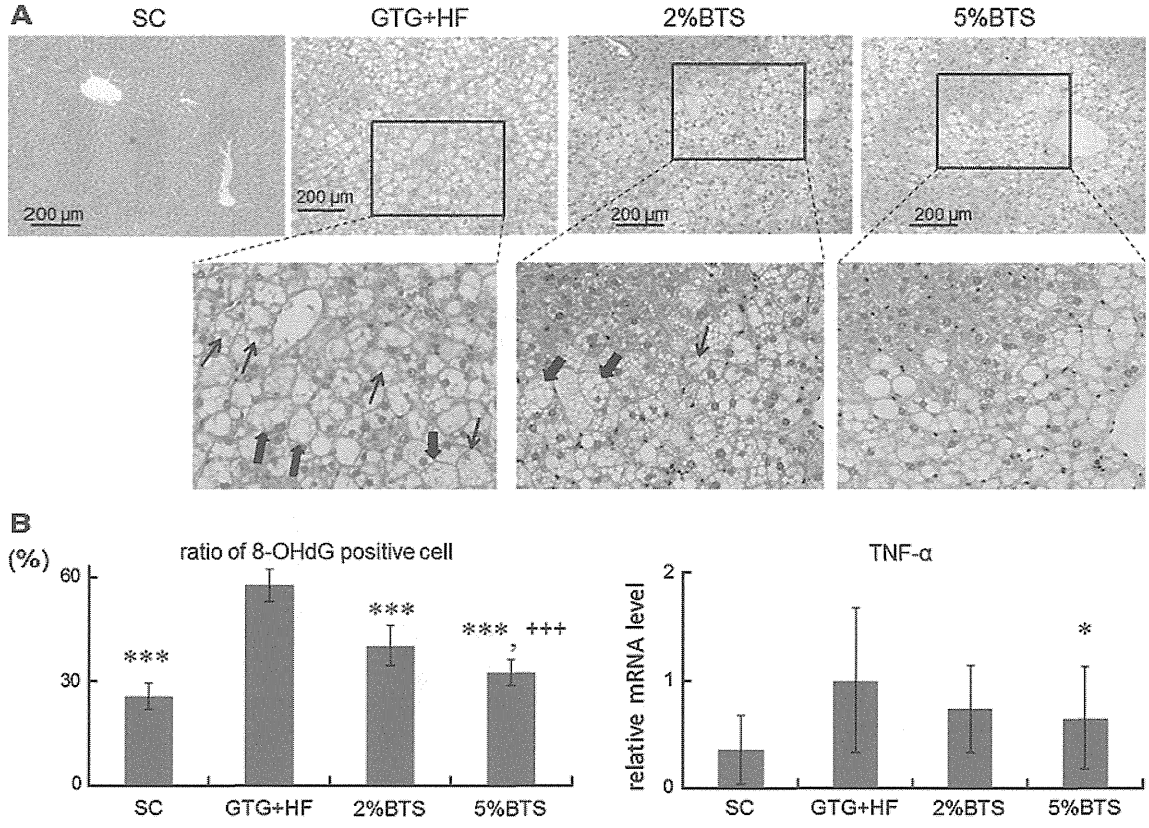

Fig. 3 BTS treatment reduces hepatic inflammation and oxidative stress. **a** The extent of hepatic inflammation was markedly attenuated with BTS: hepatocyte ballooning and Mallory–Denk bodies in the livers of GTG + HF fed mice were absent in BTS treatment livers. *Filled arrow* ballooning hepatocyte, *arrow* Mallory–Denk body. **b** Oxidative stress and TNF-α expression: BTS reduced hepatic oxidative stress as shown by reduction of numbers of nuclei stained positive for 8-OHdG in GTG + HF mice. BTS similarly reduced hepatic TNF-α mRNA expression. *Triple asterisk* $p < 0.001$, *asterisk* $p < 0.01$ vs GTG + HF, *Triple plus symbol* $p < 0.001$ vs 2 %BTS, $n = 6$

Table 1 Physiological and biochemical analyses in mice treated with BTS

	SC ($n = 6$)	GTG + HF ($n = 6$)	2 %BTS ($n = 6$)	5 %BTS ($n = 6$)
AST (U/L)	86 ± 11	310 ± 114	271 ± 27	170 ± 46*
ALT (U/L)	31 ± 9	514 ± 170	433 ± 37	299 ± 133*
TG (mg/dL)	36 ± 7	37 ± 13	52 ± 11*	49 ± 8

Serum ALT and AST levels were significantly reduced in mice treated with GTG + HF + 5 % BTS compared to the control GTG + HF group, *$p < 0.05$. There was no change in serum TG level between the GTG + HF + 5 % BTS and GTG + HF groups although TG was elevated by 2 %BTS compared to GTG + HF (*$p < 0.05$)

* $p < 0.05$ vs GTG + HF, $n = 6$

PPAR-γ effects may also function to increase serum adiponectin, since PPAR-γ agonists have been shown to positively regulate serum adiponectin independently of adipose tissue volume regulation [28, 29].

To now study the mechanisms of inhibition of liver lipid metabolism by BTS in the presence of the activated adiponectin signaling pathway, AdipoR1 signaling pathways and their target genes were examined. It is known that activated AdipoR1 signaling decreases expression of SREBP-1c [30], a key regulator of hepatic fatty acid synthesis [31, 32], through AMPK activation [33]. Here, we showed increased phosphorylation of AMPK by BTS in parallel with activated AdipoR1 signaling (Fig. 4c). Additionally, the phosphorylation of AMPK could also have been induced by activated PPAR-γ, in BTS treated mice, since as above BTS could also have PPAR-γ agonist like actions. Moreover, in the livers of mice treated with BTS, there was a reduced expression of SREBP-1c (Fig. 4b) in parallel with activated adiponectin signaling and phosphorylation of AMPK.

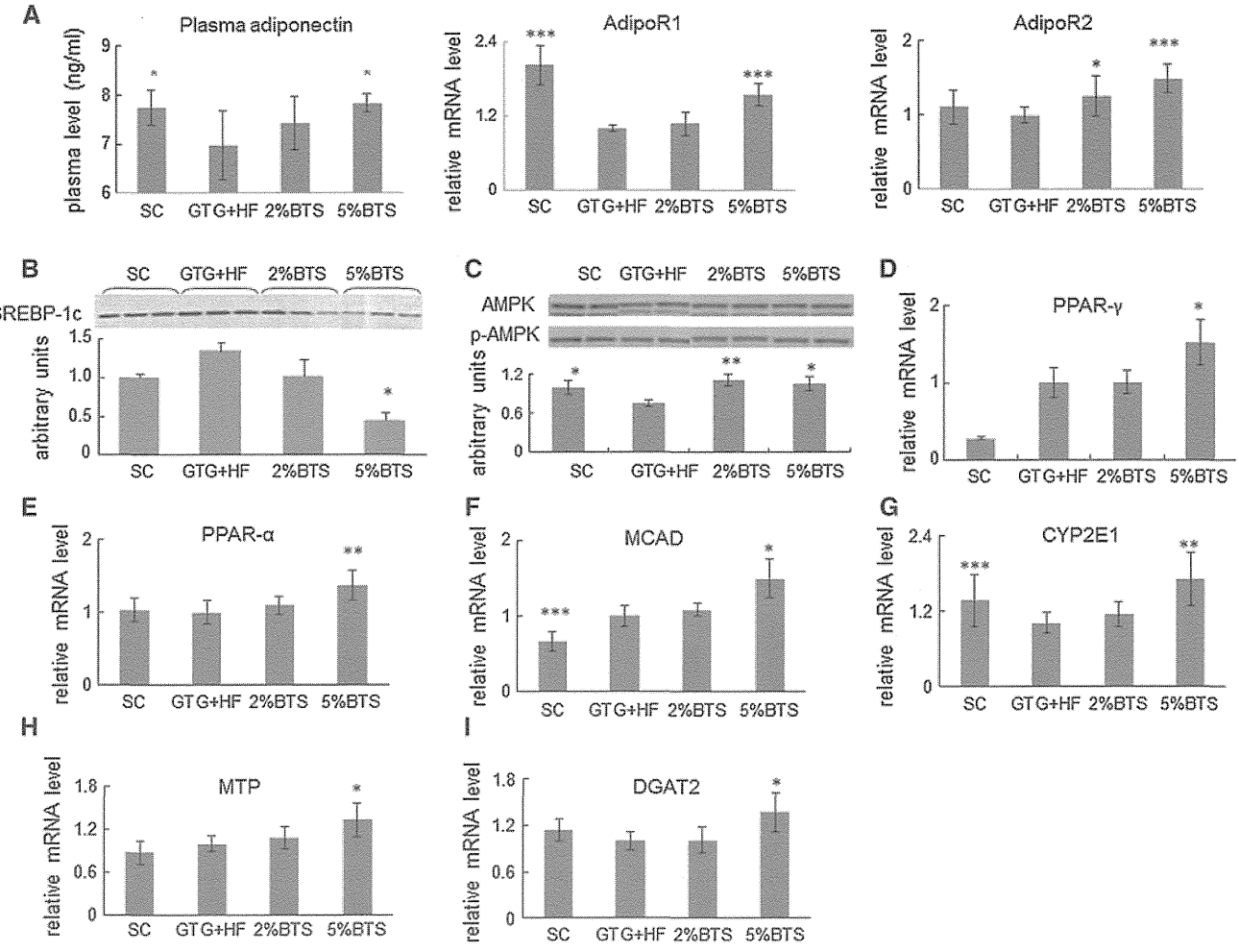

Fig. 4 BTS attenuates hepatosteatosis through activation of adiponectin. **a** Plasma adiponectin and expression of adiponectin receptors 1 (AdipoR1) and 2 (AdipoR2): plasma adiponectin levels and hepatic mRNA expression of AdipoR1 and AdipoR2 were dose dependently increased by BTS. *Asterisk* $p < 0.05$, *triple asterisk* $p < 0.001$ vs GTG + HF, $n = 6$. **b–d** AdioR1 signaling and target genes expression: BTS induced AdipoR1 signaling suppressed nuclear expression of SREBP-1c ($n = 4$) through phosphorylated AMPK ($n = 4$). Expression of PPAR-γ mRNA was also increased by BTS treatment ($n = 6$). *Asterisk* $p < 0.05$, *double asterisk* $p < 0.01$ vs GTG + HF. **e–g** Expression of PPAR-α and its target genes: Expression of PPAR-α mRNA was increase by BTS ($n = 6$). Expression of MCAD ($n = 6$) and CYP2E1 ($n = 6$), PPAR-α target genes, was similarly increased by BTS. *Asterisk* $p < 0.05$, *double asterisk* $p < 0.01$, *triple asterisk* $p < 0.001$ vs GTG + HF. **h, i** BTS promotes hepatic lipid export: MRNA expression of MTP ($n = 6$) and DGAT2 ($n = 6$) was increased by treatment with BTS. *Asterisk* $p < 0.05$ vs GTG + HF

TNF-α expression is also known to regulate expression of SREBP-1c through activation of AMPK [31, 34]. Therefore, the suppression of TNF-α expression by BTS, as observed here, could also have contributed to the decreased SREBP-1c expression. Taken together, therefore, the data here suggest that activation of AdipoR1 signaling and consequent suppression of SREBP-1c may be central mechanisms through which BTS attenuates hepatic steatosis.

BTS may also have enhanced hepatic fatty acid oxidation via AdipoR2 signaling, known to increase the expression of PPAR-α [24] and its fatty acid oxidation related target genes. Here, the expression of PPAR-α (Fig. 4e) and its target gene MCAD (Fig. 4f) and CYP2E1 (Fig. 4g), were increased by treatment with BTS. Therefore, activated fatty acids oxidation could also contribute to the attenuation of hepatic steatosis by BTS. Activation of fatty acid oxidation would be expected to increase production of reactive oxygen species (ROS) in the liver [35, 36]. BTS treatment here reduced ROS levels, probably contributing to the improved hepatic inflammation observed with BTS.

Additionally, MTP expression was confirmed here to be increased by treatment with BTS, possibly contributing to attenuation of steatosis by increasing hepatic export of TG (Fig. 4h). Interestingly, expression of DGAT2 was also increased by BTS treatment (Fig. 4i). Reduced hepatic accumulation of TG in BTS-treated livers may therefore have been due to increased MTP expression and increased expression of DGAT2 to reduce the content of FFA.