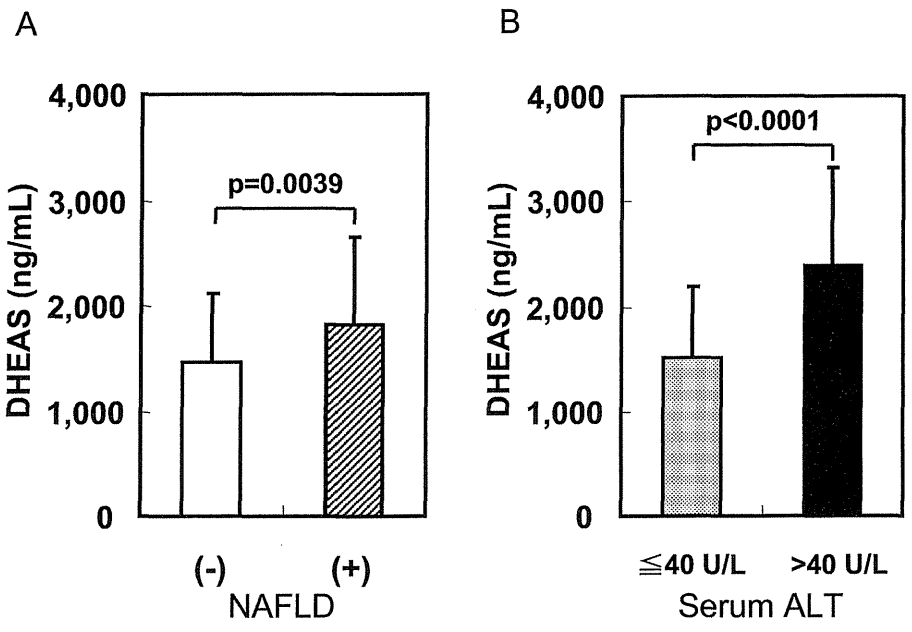

Figure 1. (A) Serum DHEAS levels in 69 patients with NAFLD (hatched column) and in 89 subjects without it (open column). (B) Serum DHEAS levels in 19 patients with NAFLD who had elevated ALT levels (>40 U/L) (closed column) and in 139 men with normal ALT levels (≤40 U/L) (dotted column).

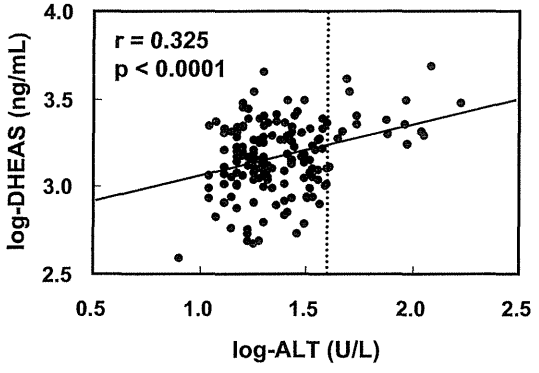

Figure 2. Correlation between serum log ALT levels and serum log DHEAS levels in 158 studied men. Dotted line represents the upper limit of control ranges (40 U/L) of serum ALT level.

ble 2 and Fig. 2). Stepwise multivariate regression analysis showed that serum DHEAS in addition to serum triglycerides and BMI were independently associated with serum ALT (Table 2).

Discussion

In the present investigation, we examined serum DHEAS levels in 158 men without viral hepatic diseases, diabetes mellitus, renal disease, malignant disease, or excess alcohol drinking habits. Since serum DHEAS levels significantly differ between men and women (17), this study was performed in men. The results clearly showed independent association of serum DHEAS levels with serum ALT levels. Serum DHEAS levels were significantly higher in patients with NAFLD than in those without it. In addition, serum DHEAS levels were higher in who had elevated serum ALT levels. Thus, it is suggested that serum DHEAS levels increase in patients with NAFLD with high levels of serum ALT.

Serum DHEAS levels depend on adrenal DHEA production and its hepatic metabolism mediated by DHEA sulfotransferase catalyzing sulfonation of DHEA to form DHEAS. Although the activity and concentration of DHEA sulfotransferase has been shown to be reduced in hepatic tissues derived from primary biliary cirrhosis, primary sclerosing cholangitis, chronic active hepatitis and alcoholic cirrhosis compared with normal liver (18), there is no report on the content and activity of DHEA sulfotransferase in that derived from NAFLD. Thus, it is not known whether or not the increased serum DHEAS levels in NAFLD result from the increased sulfonation of DHEA.

Yoneda et al (19) studied the effects of DHEA treatment on hepatic injury by concanavalin A-induced T lymphocytes in mice. They showed that DHEA reduced hepatic injury by inhibiting several inflammatory mediators such as tumor necrosis factor α and macrophage mitogen inhibitory factor, and prevented the increase of serum ALT levels. Thus DHEA might have a protective effect against hepatotoxicity in this mouse model. Although the roles of DHEA in NAFLD have not yet been proven, the increased serum DHEAS levels in patients with NAFLD may reflect a compensatory increase of DHEA production to protect against hepatic damage. Recently, it has been reported that serum DHEAS levels were rather decreased in patients with histologically diagnosed NASH (12, 13). They have also shown that serum DHEAS levels were lower in NASH patients

Table 2. Univariate and Multivariate Regression Analyses on Serum Log ALT Levels in 160 Studied Men

	Univariate regression analysis		Multivariate regression analysis		
Variable	Correlation coefficient	p value	Partial regression coefficient	F	p value
serum triglyceride	0.394	<0.0001	0.283	14.5	0.0084
BMI	0.418	<0.0001	0.270	13.4	0.0235
serum log-DHEAS	0.322	<0.0001	0.243	12.6	0.0055
HOMA-%S	–0.419	<0.0001	Not entered		
age	–0.224	0.0043	Not entered		
serum HDL cholesterol	–0.186	0.0183	Not entered		
fasting plasma glucose	0.183	0.0204	Not entered		
serum LDL cholesterol	0.138	0.0839	Not entered		

Explanatory variables in multivariate regression analysis included are age, BMI, serum triglyceride, serum high density lipoprotein (HDL) cholesterol, serum low density lipoprotein (LDL) cholesterol, fasting plasma glucose, HOMA-%S and serum DHEAS. R^2=0.307, F=22.4, and p<0.0001.

with incremental fibrosis stage (12, 13). In the present investigations, we failed to perform histological diagnosis of the liver of patients with NAFLD and thus it was not determined how many of these patients represented NASH. We, however, propose that most of them did not suffer from NASH since the frequency of NASH patients among NAFLD patients is suggested to be around 10 % (20). Increased DHEAS in patients with NAFLD may reflect increased adrenal secretion of DHEA for the prevention of the development and progression of hepatic damage (hepato-adrenal axis), while reduced DHEAS levels in patients with advanced stage of NASH may result mainly from the reduced sulphonation of DHEA. Further clinical and experimental studies are necessary to prove these hypotheses.

There is an increasing number of reports demonstrating that insulin resistance and the metabolic syndrome are involved in the development and progression of NAFLD (5, 9). These are also supported by our results in that the insulin sensitivity index HOMA-%S was inversely associated with serum ALT. DHEA is known to have potential to improve insulin sensitivity in vivo (21-24). It also has properties to increase insulin sensitivity in hepatocytes (25). In addition, it has been shown that DHEA can inhibit 11β-hydroxysteroid dehydrogenase 1 expression in liver and adipose tissues (26). It is proposed that the overexpression of 11β-hydroxysteroid dehydrogenase 1 in adipose tissues increases the local cortisol production and thereby leads to the metabolic syndrome-like status (27, 28). In the present study, serum DHEAS was a factor associated with serum ALT, independent of the insulin sensitivity index. Furthermore, serum DHEAS was inversely associated with the insulin sensitivity index HOMA-%S. Therefore, it is not plausible that insulin sensitivity *per se* is involved in the association of serum DHEAS with NAFLD.

Steroid hormones are known to exert their biological effects via specific nuclear receptors (29). Some effects of DHEA are not exactly the same as those of androgens, and thus the presence of DHEA receptors different from classical steroid receptors is postulated. High-affinity binding sites of DHEA have been shown in T lymphocytes, liver, vascular endothelial cells, adrenal medulla cells, which are thought to present in membrane fractions (30, 31). At present, there is no data implying a molecular mechanism as to the relation of DHEAS with NAFLD.

In conclusion, our results indicate that serum DHEAS levels are increased in patients with NAFLD with increased serum ALT levels. Serum DHEAS was found to be associated with serum ALT levels, independent of BMI, HOMA-%S and serum triglycerides. Therefore, DHEAS may be a component of NAFLD, in addition to obesity and its related metabolic disorders.

The authors state that they have no Conflict of Interest (COI).

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ORIGINAL ARTICLES—LIVER, PANCREAS, AND BILIARY TRACT

Characteristics of Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma

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This article has an accompanying continuing medical education activity on page e50. Learning Objectives—At the end of this activity, the learner should identify the clinical features of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma and the role of hepatic fibrosis in the development of hepatocellular carcinoma.

See related article, Villanueva A et al, on page 1501 in *Gastroenterology*.

BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to characterize the clinical features of NASH patients with HCC. **METHODS:** In a cross-sectional multicenter study in Japan, we examined 87 patients (median age, 72 years; 62% male) with histologically proven NASH who developed HCC. The clinical data were collected at the time HCC was diagnosed. **RESULTS:** Obesity (body mass index ≥25 kg/m^2), diabetes, dyslipidemia, and hypertension were present in 54 (62%), 51 (59%), 24 (28%), and 47 (55%) patients, respectively. In nontumor liver tissues, the degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%). The prevalence of cirrhosis was significantly lower among male patients (21 of 54, 39%) compared with female patients (23 of 33, 70%) (P = .008). **CONCLUSIONS: Most patients with NASH who develop HCC are men; the patients have high rates of obesity, diabetes, and hypertension. Male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients.**

Keywords: Liver Cancer; Incidence; Sex; Retrospective Study.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality.[1] HCC mostly occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B and C viruses as well as alcohol consumption, are well-defined, 5%–30% of patients with HCC lack a readily identifiable risk factor for their cancer. It has been suggested that a more severe form of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH), might account for a substantial portion of cryptogenic cirrhosis and HCC cases.[2]

NAFLD is one of the most common causes of chronic liver disease in the world.[3,4] NAFLD is associated with obesity, diabetes, dyslipidemia, and insulin resistance and is recognized as a hepatic manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive NASH associated with fibrosis, necrosis, and inflammation. Despite its common occurrence and potentially serious nature, relatively little is known about the natural history or prognostic significance of NAFLD. Although prospective studies on the natural history of NAFLD and NASH with a larger cohort are awaited, these

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CT, computed tomography; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

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studies might be limited by the long and asymptomatic clinical course of these diseases, by their high prevalence in the general population, and by the lack of serologic markers for NASH. The evidence suggesting that NASH can progress to HCC comes from (1) case reports and case series,[5-8] (2) retrospective studies,[9-12] and (3) prospective studies.[13-17] These studies generally examined limited numbers of cases and follow-ups; therefore, the incidence of HCC and risk factors for HCC in NASH patients remain unclear.

The Japan NASH Study Group (representative, Takeshi Okanoue)[18] was established in 2008 by the Ministry of Health, Labour and Welfare of Japan to address unmet research needs in the area of liver diseases. As a part of this mandate, the study group conducted a cross-sectional multicenter study to characterize the clinical features of histologically proven NASH patients who developed HCC.

Methods

Patients

We retrospectively identified and reviewed 87 Japanese patients with NASH, who developed HCC between 1993 and 2010, at 15 hepatology centers that belong to the Japan NASH Study Group[18] and their affiliated hospitals in Japan. The diagnosis of NASH was based on (1) the histologic features of steatohepatitis, (2) negligible alcohol consumption, and (3) exclusion of liver diseases of other etiology. To determine alcohol consumption as accurately as possible, we reviewed medical records in our institutions, and when patients had been transferred from other institutions, we also reviewed a summary of medical records from those institutions. According to the medical records, alcohol consumption was assessed on the basis of a detailed history that was obtained by physicians and by interviewing family members. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, positivity for anti-hepatitis C virus antibody, the presence of other types of liver diseases (eg, primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. The sections of nontumor liver tissues were reanalyzed by experienced hepatopathologists (T.O., E.H.) who were blinded to the laboratory parameters and clinical data. We excluded patients whose histologic diagnosis of NASH was not confirmed by central review and patients with insufficient or inconclusive information concerning alcohol consumption, body mass index (BMI), and laboratory data including fasting glucose and lipid.

Of the 87 patients, 14 patients had been previously diagnosed as NAFLD or NASH and had been followed at our institutions; 73 patients had been transferred from other institutions to our institutions for investigation and treatment of HCC. Most patients had been identified as having HCC during screening, which included ultrasound and/or computed tomography (CT) of the liver and alpha-fetoprotein (AFP) testing.

The diagnosis of HCC was based on liver histology and, in the absence of histology, on typical features of HCC as assessed by dynamic CT or magnetic resonance imaging (MRI) (ie, hypervascular with washout in the portal/venous phase).[19] Of the 87 patients, 49 patients were diagnosed as HCC after hepatic resection, 21 patients were diagnosed after ultrasound-guided tumor biopsy, and 17 patients were diagnosed by dynamic CT or MRI.

The Ethics Committees of each participating center approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Clinical Assessment and Laboratory Tests

The clinical and laboratory data were collected at the time HCC was diagnosed. BMI was calculated by using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as BMI $\geq$25 kg/m^2 according to the criteria of the Japan Society for the Study of Obesity.[20] Diabetes was defined as fasting plasma glucose concentration of $\geq$126 mg/dL or 2-hour plasma glucose concentration of $\geq$200 mg/dL during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose.[21] Hypertension was defined as systolic blood pressure $\geq$130 mm Hg or diastolic blood pressure $\geq$85 mm Hg or by the use of antihypertensive agents.[22] Dyslipidemia was defined as serum concentrations of triglycerides $\geq$150 mg/dL or high-density lipoprotein (HDL) cholesterol $<$40 mg/dL and $<$50 mg/dL for men and women, respectively, or by the use of specific medication.[22]

Venous blood samples were taken in the morning after 12-hour overnight fast. The laboratory evaluations included blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), fasting plasma glucose, HbA1c, total cholesterol, HDL cholesterol, triglyceride, ferritin, hyaluronic acid, AFP, and des-γ-carboxy prothrombin (DCP). These parameters were measured by using standard clinical chemistry techniques.

Histopathologic Examination

Nontumor liver tissues were obtained from all 87 patients to diagnose the background liver tissue at the time HCC was diagnosed. In 49 patients who underwent hepatic resection for HCC, we examined nontumor liver tissues that were surgically resected. In 21 patients who underwent ultrasound-guided tumor biopsy, nontumor liver tissues far from HCC tumors were biopsied separately. In 17 patients who were diagnosed as HCC by dynamic CT or MRI and did not undergo either hepatic resection or tumor biopsy, only nontumor liver tissues far from HCC tumors were obtained by ultrasound-guided biopsy.

The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin, with Masson trichrome, and by silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory's hyaline (Mallory's body) or fibrosis.[23-25] The necroinflammatory grade and the degree of fibrosis were evaluated and scored according to the criteria proposed by Brunt et al.[26]

Statistical Analysis

Results are presented as numbers with percentages in parentheses for qualitative data or as the medians and ranges (25th-75th percentiles) for quantitative data. Comparisons were made by using a χ^2 test for qualitative factors or a Mann-

Table 1. Patient Characteristics

Characteristic	Total (n = 87)	Male (n = 54)	Female (n = 33)	*P* value[a]
Age (*y*)	72 (69–75)	72 (69–75)	72 (68–75)	.52
BMI (kg/m^2)	26.0 (23.8–28.3)	26.0 (23.8–28.8)	26.2 (23.9–27.7)	.54
Obesity	54 (62%)	35 (65%)	19 (58%)	.50
Diabetes	51 (59%)	31 (57%)	20 (61%)	.77
Dyslipidemia	24 (28%)	13 (24%)	11 (33%)	.35
Hypertension	47 (54%)	22 (41%)	25 (76%)	.001
Platelet count ($\times 10^4/\mu L$)	13.9 (10.1–18.0)	14.5 (11.7–18.0)	10.9 (7.8–18.0)	.05
AST (*IU/L*)	47 (30–59)	46 (27–60)	47 (35–58)	.45
ALT (*IU/L*)	36 (26–55)	43 (26–69)	34 (26–42)	.11
γ-GTP (*IU/L*)	75 (40–115)	68 (36–177)	75 (40–115)	.90
Fasting glucose (*mg/dL*)	114 (99–145)	112 (99–144)	120 (97–152)	.59
HbA1c (*%*)	6.1 (5.4–7.1)	5.9 (5.4–7.0)	6.3 (5.2–7.1)	.78
Total cholesterol (*mg/dL*)	169 (147–202)	169 (147–202)	169 (147–202)	.62
HDL cholesterol (*mg/dL*)	50 (41–60)	45 (41–58)	55 (50–73)	.03
Triglyceride (*mg/dL*)	100 (76–138)	118 (80–147)	96 (74–116)	.06
Ferritin (*ng/dL*)[b]	197 (74–401)	273 (154–703)	98 (23–172)	.005
Hyaluronic acid (*ng/mL*)[c]	166 (67–241)	151 (69–244)	174 (61–332)	.85
AFP (*ng/mL*)	7.1 (5.0–18.0)	6.0 (4.0–14.7)	10.8 (5.9–18.0)	.02
DCP (*mAU/mL*)	66 (22–298)	48 (22–243)	81 (21–942)	.42
HCC tumor size (*cm*)	3.0 (2.0–4.0)	3.1 (2.2–4.5)	2.6 (1.9–4.0)	.18
Number of HCC tumors				.78
1	65 (75%)	39 (72%)	26 (79%)	
2 or 3	16 (18%)	11 (20%)	5 (15%)	
≥4	6 (7%)	4 (8%)	2 (6%)	
Background liver tissue				
Steatosis grade				.64
0: <5%	1 (1%)	1 (2%)	0 (0%)	
1: 5%–33%	60 (69%)	36 (67%)	24 (73%)	
2: 34%–66%	19 (22%)	11 (20%)	8 (24%)	
3: >66%	7 (8%)	6 (11%)	1 (3%)	
Necroinflammatory grade[d]				.22
1: mild	31 (35%)	22 (41%)	9 (27%)	
2: moderate	45 (52%)	26 (48%)	19 (58%)	
3: severe	11 (13%)	6 (11%)	5 (15%)	
Fibrosis stage[d]				.003
1	10 (11%)	10 (18%)	0 (0%)	
2	15 (17%)	10 (18%)	5 (15%)	
3	18 (21%)	13 (25%)	5 (15%)	
4	44 (51%)	21 (39%)	23 (70%)	

NOTE. Values are medians (25th–75th percentiles) or numbers (%). Where no other unit is specified, values refer to number of patients.
[a]χ^2 test or Mann–Whitney *U* test.
[b]Missing data for 27 patients.
[c]Missing data for 29 patients.
[d]According to reference 26.

Whitney *U* test on ranks for quantitative factors with non-equal variance. *P* values less than .05 from two-sided tests were considered to be significant. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc, Chicago, IL).

Results

The characteristics of the 87 NASH patients who developed HCC are summarized in Table 1. The median age was 72 years (25th percentile, 69; 75th percentile, 75); the mean age (standard deviation) was 71.2 (6.7) years. There were 54 male patients (62%) and 33 female patients (38%); the male:female ratio was 1.6:1. The median BMI was 26.0 kg/m^2, and 54 patients (62%) were obese (BMI ≥25 kg/m^2). Diabetes, dyslipidemia, and hypertension were present in 51 (59%), 24 (28%), and 47 (55%) patients, respectively.

The diagnosis of NASH was proved by histologic examination of nontumor liver tissues at the time HCC was diagnosed. The degree of steatosis was grade 1 (5%–33%) in 60 patients (69%), grade 2 (34%–66%) in 19 (22%), and grade 3 (>66%) in 7 (8%). One patient who showed less than 5% steatosis was diagnosed as "burn-out" NASH, because a previous liver biopsy that was performed before development of HCC had demonstrated typical histologic features of NASH. The necroinflammatory grade was mild (grade 1) in 31 patients (35%), moderate (grade 2) in 45 (52%), and severe (grade 3) in 11 (13%). The degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%).

The median diameter of HCC tumors was 3.0 cm (25th percentile, 2.0; 75th percentile, 4.0). A single HCC lesion was present in 65 of 87 patients (75%).

Data were stratified according to sex (Table 1). Compared with female patients, male patients had significantly less hypertension, lower HDL cholesterol and AFP, higher ferritin, and a less advanced stage of fibrosis. The prevalence of cirrhosis was significantly lower in male patients (21 of 54, 39%) than in female patients (23 of 33, 70%) ($P = .008$).

Discussion

In this cross-sectional multicenter study in Japan, we showed the clinical features of a relatively large number (n = 87) of NASH patients with HCC. The male:female ratio was 1.6:1. Men have higher HCC rates than women in almost all populations, with male:female ratios usually averaging between 2:1 and 4:1.[2] In the latest nationwide survey of HCC in Japan,[27] this ratio was 2.5:1. The reasons underlying higher rates of HCC in men might relate to sex-specific differences in exposure to risk factors. Men are more likely to be infected with hepatitis B and C viruses, consume alcohol, smoke cigarettes, and have increased iron stores.[2] Moreover, androgens are considered to influence the development of HCC. With regard to the male:female ratio of HCC associated with NASH, a male:female ratio of 1.3:1 was reported in a summary of 16 published cases of HCC associated with NASH.[28] Ratios of 2.8:1 and 0.67:1 were reported in 2 retrospective studies of HCC arising from cryptogenic cirrhosis in Italy (n = 44)[10] and the United States (n = 30),[9] respectively, and a ratio of 1.6:1 was reported for 36 cases of NASH-associated HCC from a single center in Japan.[15] Overall, NASH patients with HCC are more often men. However, these male:female ratios might be lower than the ratios for HCC of other etiologies, including viral hepatitis and alcohol consumption.

Although it is well-known that male gender is a risk factor for HCC in patients infected with hepatitis B and C viruses,[2] it remains unclear whether male gender is a factor associated with the development of HCC in NASH patients. It is now suspected that there is an even distribution of NASH among men and women.[29] In another study by our group,[30] the male:female ratio was 0.85:1 in 342 NASH patients without cirrhosis and HCC. The male:female ratio (1.6:1) of NASH patients with HCC in the present study is higher than this ratio. In agreement with our observations, a case-control study showed that the male:female ratio was 1.6:1 in 34 NASH patients with HCC, whereas the ratio was 0.69:1 in 348 NASH patients without HCC.[15] A recent prospective study indicated that older age and alcohol consumption were independent risk factors for the development of HCC in patients with NASH-cirrhosis and that male gender tended to be associated with the development of HCC, although this trend did not reach statistical significance.[17]

The median age of our patients was 72 years. There was no significant difference in age between men and women. Although the global age distribution of HCC varies by geographic region, sex, and etiology, in almost all areas the peak female age group in HCC patients is 5 years older than in male HCC patients.[2] In a nationwide survey of HCC in Japan,[27] the mean ages were 65.5 years for men and 69.4 years for women. The male patients in the present study are slightly older than the mean ages reported in these previous studies.

Consistent with the literature,[9-12] more than half of our patients displayed obesity, diabetes, and hypertension. Obesity constitutes a significant risk factor for cancer mortality in general and is an increasingly recognized risk factor for HCC in particular.[31,32] In the present study, body weight was measured at the time HCC was diagnosed. Because advanced HCC might cause weight loss, it is likely that our patients were obese before the development of HCC. Diabetes has also been proposed as a risk factor for HCC.[2] Thus, HCC shares 2 major risk factors, obesity and diabetes, with NASH.

Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. As NASH progresses to cirrhosis, steatosis tends to disappear, so-called burn-out NASH.[5] As expected, the grade of steatosis was mild in most of our cases. It was possible to diagnose 1 case without steatosis as burn-out NASH, because a previous liver biopsy specimen (liver biopsy was performed 25 years prior) was preserved and available. It is likely that many cases of NASH-associated HCC might have been missed because of loss of the telltale sign of steatosis.

Most HCC arises on a background of cirrhosis. It is less clear whether cirrhosis is a necessary predisposition for the development of HCC in patients with NASH. Case reports of HCC arising from NAFLD and NASH patients without fibrosis or cirrhosis have been accumulating.[33-36] Cirrhosis (fibrosis stage 4) was present in 51% of cases, and advanced stages of fibrosis (stage 3 or 4) were found in 72% of cases in the present study. Indeed, cirrhosis or advanced fibrosis appeared to be the predominant risk factors for HCC development. However, in the remaining 28% of cases, HCC developed in patients with less fibrosis (stage 1 or 2). Interestingly, male patients developed HCC at a less advanced stage of fibrosis than female patients, and the prevalence of cirrhosis was significantly lower in men (39%) than in women (70%). Although the reason for the sex differences is unclear, these findings indicate that screening for HCC is needed not only in NASH patients with advanced fibrosis but also in those with less fibrosis, particularly if they are men. Further studies are needed to confirm this potentially important observation. Paradis et al[37] reported that in patients whose only risk factors for chronic liver disease are features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis. In addition, they found that some of these HCCs developed on preexisting liver cell adenomas. However, no preexisting adenomas were observed in the present cases.

Compared with female patients, male patients had significantly higher serum ferritin value. The normal value for ferritin varies according to the age and gender of the individual. Adult men have serum ferritin values averaging approximately 100 ng/mL (range, 75–250), whereas adult women have levels averaging approximately 30 ng/mL (range, 20–75).[38] Thus, normal men have higher ferritin levels than women. Elevation of ferritin levels is associated with NASH.[39] Because we excluded patients with alcohol consumption as rigorously as possible, we believe that alcohol consumption did not contribute to the elevation of ferritin levels in our patients.

The median diameter of the HCCs in the present study was 3.0 cm, which is equal to or smaller than the size of previously reported HCCs.[9,10,12,28,37] This is probably because most of our patients had been identified as having HCC during screening. A single HCC lesion was present in 75% of patients. For early detection of NASH-associated HCC, vigilant screening is important,[9] and the development of serologic markers for NASH is necessary.

The mechanisms of carcinogenesis in NASH remain to be elucidated. Possible mechanisms include hyperinsulinemia

caused by insulin resistance in NASH, increased levels of insulin-like growth factor that promotes tumor growth, increased susceptibility of the steatotic liver to lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, disordered energy and hormonal regulation in obesity, and aberrations in regenerative processes occurring in cirrhosis.[25]

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding the causality of NASH in HCC. Second, the study did not include a control group of HCC patients with other liver diseases. Third, there might be a bias in patient selection, because patients were retrospectively identified as having NASH-associated HCC. Finally, although our patients were negative for hepatitis B virus surface antigen, it is still possible that occult hepatitis B virus infection might be associated with the development of HCC in some of our cases.

In summary, we showed the clinical features of NASH patients with HCC. NASH patients with HCC were more often men and frequently displayed obesity, diabetes, and hypertension. Our results suggest that male patients might develop HCC at a less advanced stage of fibrosis than female patients. Further prospective studies with a longer follow-up time and larger cohorts are needed to determine the causal association of NASH with HCC and to identify risk factors for the development of HCC in NASH patients.

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Conflicts of interest

The authors disclose no conflicts.

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BASIC STUDIES

A novel and comprehensive mouse model of human non-alcoholic steatohepatitis with the full range of dysmetabolic and histological abnormalities induced by gold thioglucose and a high-fat diet

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Keywords
animal models – gold thioglucose – hyperphagia – NASH – obesity

Abbreviations:
AdipoR1/R2, adiponectin receptor1/receptor2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FAS, fatty acid synthase; GTG, gold thioglucose; GTT, glucose tolerance test; HF, high-fat diet; IGT, impaired glucose tolerance; IR, insulin resistance; ITT, insulin tolerance test; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; QUICKI, quantitative insulin sensitivity check index; SC, standard chow; TG, Triglyceride; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases.

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Abstract

Background: The search for effective treatments of non-alcoholic steatohepatitis (NASH), now the most common chronic liver disease in affluent countries, is hindered by a lack of animal models having the range of anthropometric and pathophysiological features as human NASH. *Aims:* To examine if mice treated with gold thioglucose (GTG) – known to induce lesions in the ventromedial hypothalamus, leading to hyperphagia and obesity – and then fed a high-fat diet (HF) had a comprehensive histological and dysmetabolic phenotype resembling human NASH. *Methods:* C57BL/6 mice were injected intraperitoneally with GTG and then fed HF for 12 weeks (GTG+HF). The extent of abdominal adiposity was assayed by CT scanning. A glucose tolerance test and an insulin tolerance test were performed to evaluate insulin resistance (IR). Histological, molecular and biochemical analyses were also performed. *Results:* Gold thioglucose+HF induced dysmetabolism, with hyperphagia, obesity with increased abdominal adiposity, IR and consequent steatohepatitis, with hepatocyte ballooning, Mallory–Denk bodies, perivenular and pericellular fibrosis as seen in adult NASH, paralleled by an increased expression of the profibrogenic factors, transforming growth factor-β1 and TIMP-1. Plasma adiponectin and the expression of adiponectin receptor 1 and receptor 2 were decreased, while PPAR-γ and FAS were increased in the livers of GTG+HF mice. In addition, GTG+HF mice showed glucose intolerance and severe IR. *Conclusions:* Treatment with GTG and HF diet induce, in mice, a comprehensive model of human NASH, with the full range of dysmetabolic and histological abnormalities.

Obesity-induced liver disease, non-alcoholic steatohepatitis (NASH), now the most important cause of chronic liver disease in industrialized nations (1, 2), defines the spectrum from steatohepatitis to cirrhosis (3). The search for treatments of NASH is hampered by the lack of animal models having the same range of dysmetabolic and pathophysiological features as the human disease, namely obesity and insulin resistance (IR), with consequent liver disease (4). A comprehensive animal model of NASH should have a liver pathology that features steatosis, inflammation, liver cell injury (hepatocyte ballooning and Mallory–Denk bodies) and fibrosis, particularly the perivenular and pericellular fibrosis usually seen in adult NASH. Additionally, the animal model must exhibit metabolic abnormalities such as obesity, IR, hyperglycaemia, dyslipidaemia and altered adipokine profiles.

It was reported recently that chronic administration of high-fat diet (HF, 60% fat) led to the development of steatohepatitis in male C57BL/6J mice (5). After 10 weeks, HF-fed mice were obese with hyperinsulinaemia, impaired glucose tolerance and hepatic steatosi. To engender comprehensive features of human NASH, however, required HF feeding for 50 weeks, an impractical time scale for most study protocols.

Now, gold thioglucose (GTG) is known to induce lesions in the ventromedial hypothalamus, resulting in hyperphagia and obesity, because GTG treatment down-regulates GPR7, the endogenous G protein-coupled receptor for identified ligands, neuropeptide B and neuropeptide W (6). GTG-treated mice are hyperphagic and obese with IR and mild hepatic steatosis.

The aim of the present study therefore was to observe if GTG treatment with HF feeding provided a comprehensive mouse model of NASH, with the full range of dysmetabolic and histological abnormalities paralleling human NASH.

Materials and methods

Animal preparation

All procedures conformed to our Institutions guidelines for the care and use of animals. Four-week-old male C57BL/6 mice were purchased from CLEA Japan Inc. (Tokyo, Japan). All animals were housed for 12 weeks on 12 h light/12 h dark cycling, with food and water freely available. Mice were fed standard chow (SC) or HF as supplied by Oriental Yeast (Tokyo, Japan). SC (346.7 kcal/100 g) compromised 16.9% calories from fat, 60% from carbohydrate and 23.1% from protein. HF (F2HFD2, 640 kcal/100 g) compromised 58% lard (wt/wt), 30% fish powder, 10% skimmed milk and 2% vitamin and mineral mixture equivalent to 7.5 g% carbohydrate (3 cal%), 24.5 g% protein (15 cal%) and 60 g% fat (82 cal%) as described previously (7). Three experimental groups were studied: (i) SC alone for 12 weeks (SC group); (ii) SC for 1 week, then HF alone for 11 weeks (HF group); (iii) an intraperitoneal administration of GTG (2 mg/g of body weight, Sigma-Aldrich, St Louis, MO, USA), followed by SC for 1 week then HF for 11 weeks (GTG+HF group). At the end of the treatment period, all animals were fasted overnight, anaesthetized with pentobarbital sodium intraperitoneally (25–50 mg/kg of body weight, Nembutal; Abbott Laboratories, Abbott Park, IL, USA), and 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.

Computed tomography scan analysis for body fat composition and evaluation of hepatic steatosis

The extent of adiposity and hepatic steatosis in each experimental group was assayed by CT scanning (La Theta, Aloka, Tokyo, Japan) after isoflurane (2% v/v) anaesthesia, according to the manufacture's protocol. Animals were scanned at 2 mm intervals from the diaphragm to the pelvis. Visceral fat and subcutaneous fat volumes were quantified with a LATHETA software (V1.00) (8, 9). For evaluation of hepatic steatosis, regions of interest was set on the dorsal right lobe of the livers.

Histopathological examinations

Five-micrometre sections of formalin-fixed/paraffin-embedded livers (right dorsal lobe) were processed for haematoxylin and eosin (H&E) and Azan staining. Oil red-O staining of intracellular neutral lipids was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, sliced frozen liver samples were washed three times with cold PBS and fixed in 10% neutral formalin. The samples were again washed with PBS and stained with a filtered oil red-O stock solution for 15 min at room. The dishes were then rinsed in distilled water and counterstained with haematoxylin. For estimation of the extent of hepatic steatosis, the areas of digital photomicrographs were quantified with a computerized image analysis system (macintosh MacSCOPE ver. 2.591) as described previously (10, 11).

The level of oxidative stress was determined by staining with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody as described previously (10, 12).

Glucose tolerance test, insulin tolerance test and quantitative insulin sensitivity check index

At 12 weeks, mice ($n = 6$) were fasted for 18 h for glucose tolerance test (GTT), and then intraperitoneally loaded with 20% glucose at a dose of 1 g/kg body weight. Mice for insulin tolerance test (ITT) ($n = 6$) were fasted for 6 h, and then intraperitoneally challenged with human insulin at 1 U/kg body weight (11, 13). With both GTT and ITT, blood samples from the orbital sinus were taken at times 0, 30, 45 and 60 min, and plasma glucose concentrations were measured using an automatic blood glucose test meter (Glutest; Sanwa Kagaku Kenkyusho Co. Ltd, Nagoya, Japan). Plasma insulin level was measured using an ultrasensitive mouse insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacture's instructions. Quantitative insulin sensitivity check index (QUICKI) was calculated as a measure of IR from the fasting insulin and glucose levels.

Measurement of plasma adiponectin

Plasma adiponectin level was measured by a mouse adiponectin/Acrp30 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacture's instructions.

Chemical parameters

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

Real-time reverse transcription polymerase chain reaction for quantitative assessment of mRNA expression

Total RNA was extracted with trizol reagent (Life Technologies, Grand Island, NY, USA) and reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (Takara, Kyoto, Japan). Real-time reverse transcription polymerase chain reaction was performed on an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Probes and primers for tumour necrosis factor (TNF)-α, PPAR-α, PPAR-γ, transforming growth factor (TGF)-β, TIMP-1, FAS and adiponectin receptor 1/receptor 2 (AdipoR1/R2) were all purchased from Applied Biosystems. The relative expression of target gene mRNA was normalized to the amount of GAPDH mRNA.

Statistics

Data are shown as means ± 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 (SAS Institute, Cary, NC, USA).

Results

Gold thioglucose and high-fat diet feeding induces obesity

The body weight of mice administered GTG and then fed a HF diet (GTG+HF) was higher and rose more rapidly than that observed in HF diet alone or SC-fed groups. At 4 weeks, GTG+HF mice had a significantly increased body weight (39.5 ± 4.5 g) compared with HF-fed (28.3 ± 1.4 g) or SC-fed mice (22.1 ± 1.2 g) (Fig. 1). The increased body weight of GTG+HF mice was 5-fold faster than SC mice. At 12 weeks, GTG+HF-fed mice had body weights of almost 60 g.

We next evaluated induced adiposity by CT scanning at 12 weeks (Fig. 2). As shown in Figure 2A, GTG+HF mice were extremely obese compared with HF- or SC-fed mice. Induced obesity was reflected in an increased volume of visceral and subcutaneous adiposity on CT scanning (Fig. 2A). The time course of adiposity showed that adiposity increased faster and more severely in GTG+HF mice compared with HF- or SC-fed mice (Fig. 2B). At 4 weeks, total abdominal adiposity volume in GTG+HF mice was more than 3-fold than in HF mice (16.1 ± 1.1 vs. 5.1 ± 1.9 cm^3). The pattern at 12 weeks was similar to that at 4 weeks (Fig. 2B).

Gold thioglucose+high-fat diet-fed mice had severe hepatic steatosis and hepatomegaly

We next evaluated the degree of hepatic steatosis and hepatomegaly in the three mice groups by CT scanning (Fig. 3). At 4 weeks, the liver 'CT number', an indicator of steatosis, was markedly reduced in the GTG+HF group compared with the SC-fed group (18.7 ± 15.8 vs. 100 ± 6.4 HU). At 12 weeks, the difference was even more marked (− 47.6 ± 8.1 vs. 100 ± 6.4 HU) for GTG+HF mice and SC-fed groups, respectively, indicating that GTG+HF mice developed more robust and rapid hepatic steatosis compared with SC-fed mice. The pattern for HF-fed mice was similar to that for GTG+HF mice but the latter induced more profound steatosis (68.3 ± 5.1 vs. 18.7 ± 15.8 HU) at 4 weeks and (60.5 ± 23.0 vs. − 47.6 ± 8.1 HU, Fig. 3B) at 12 weeks. CT scanning at 12 weeks demonstrated more marked hepatomegaly in GTG+HF mice compared with HF- or SC-fed mice (Fig. 3A) as confirmed by the liver weights and the liver/body weight ratios (4.5 ± 0.5 g and 8.3 ± 0.7% in GTG+HF mice, 3.1 ± 0.5 g and 6.2 ± 0.7% in HF mice, ***$P < 0.001$ vs. SC and HF, Fig. 3C).

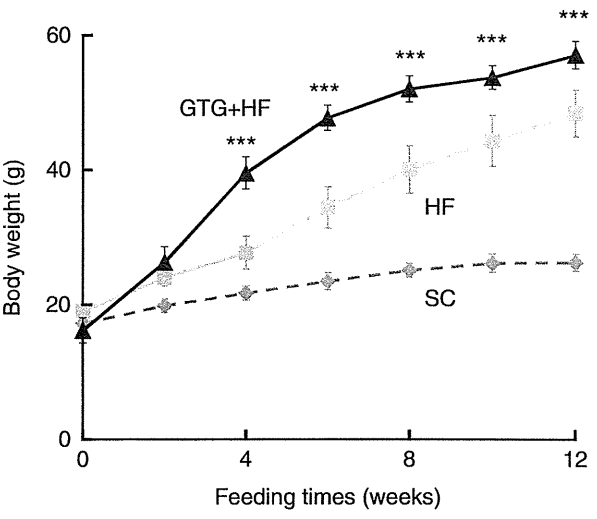

Fig. 1. Time courses of body weight gain in GTG and HF induce obesity, GTG+HF mice gained more and faster weight compared with HF or SC groups. ***$P < 0.001$ vs. HF or SC, $n = 6$. GTG, gold thioglucose; GTG+HF: GTG treated/HF fed; HF, high-fat diet; SC, standard chow.

Biochemical and histological characterizations in non-alcoholic steatohepatitis model mice induced by gold thioglucose+high-fat diet

We now examined biochemical and histological characteristics of the three groups of mice at 12 weeks. GTG+HF mice had markedly elevated plasma AST and ALT compared with HF- or SC-fed mice. For example, GTG+HF feeding induced a significantly raised ALT as compared with HF or SC feeding (514 ± 170 vs. 142 ± 80 vs. 31 ± 9, Table 1). GTG+HF mice had significantly lowered plasma adiponectin compared with HF- or SC-fed mice.

Oil red-O staining and quantitative image analysis of liver sections at 12 weeks showed that GTG+HF feeding induced marked hepatic steatosis compared with HF or

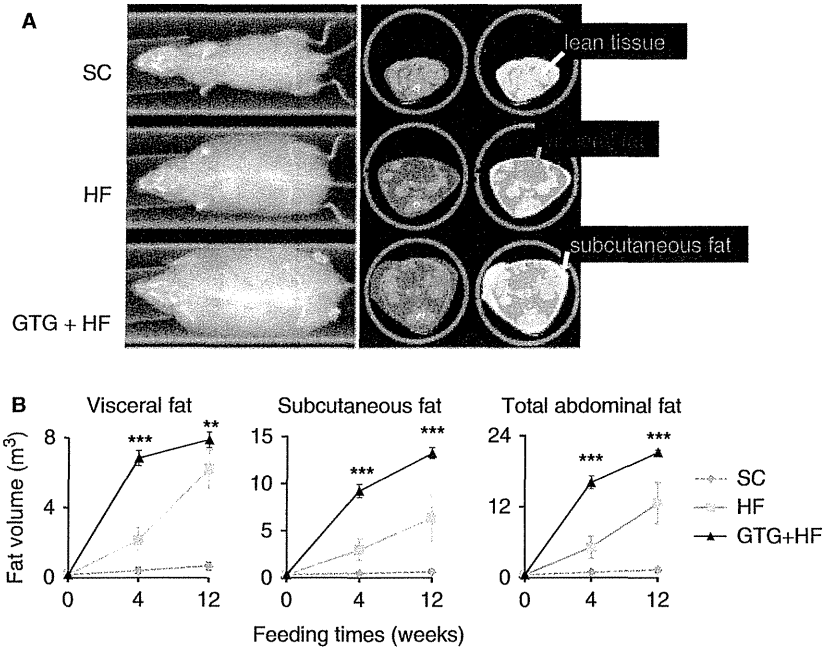

Fig. 2. Body shape and adiposity in GTG+HF mice: (A) mice body shapes and abdominal adiposity by CT scanning. GTG+HF mice at 12 weeks were demonstrably obese and had larger volumes of visceral and subcutaneous fat than HF or SC mice; purple, visceral fat; yellow, subcutaneous fat; sky blue, lean tissue. (B) The time course of increased volume of visceral, subcutaneous and total abdominal fat evaluated by CT scanning. GTG+HF mice had larger volume of subcutaneous and visceral fat than HF or SC mice. The rate of increase of the fat was in tandem much faster and severe in GTG+HF mice than in other groups. $^{***}P < 0.001$ vs. HF or SC, $^{**}P < 0.01$ vs. HF, $n = 6$. CT, computed tomography; GTG, gold thioglucose; GTG+HF, GTG treated/HF fed; HF, high-fat diet; SC, standard chow.

SC feeding (Fig. 4A). To now determine the effects of GTG+HF feeding on the hepatic expression of genes involved in lipid synthesis, we assayed the hepatic expression of FAS, PPAR-γ, PPAR-α and AdipoR1/R2 (14–16). In the livers of GTG+HF-fed mice, the relative mRNA expression of FAS, a key regulator of hepatic fatty acid synthesis (17, 18) ($^{***}P < 0.001$ vs. SC, $n = 6$) and PPAR-γ mRNA ($^{***}P < 0.001$ vs. SC, $n = 6$) increased (Fig. 4B). The hepatic expression of PPAR-α mRNA was not different between any of the groups. Because the plasma concentration of adiponectin was significantly reduced in this NASH model (Table 1), we also examined hepatic mRNA expression of adiponectin receptors, AdipoR1 and AdipoR2. The relative mRNA expressions of AdipoR1 ($^{**}P < 0.005$ vs. HF, $^{***}P < 0.001$ vs. SC, $n = 6$) and AdipoR2 ($^{*}P < 0.05$ vs. SC, $^{***}P < 0.001$ vs. HF, $n = 6$) in the livers of GTG+HF mice were suppressed compared with other groups (Fig. 4C).

Haematoxylin and eosin staining of the liver sections of GTG+HF mice showed marked steatohepatitis with lobular inflammation (arrowhead, Fig. 5A). In addition, hepatocyte ballooning and Mallory–Denk bodies, features of steatohepatitis, were also recognized only in the livers of GTG+HF mice (arrows, Fig. 5A). Furthermore, Azan staining of the liver sections showed significant histological evidence of fibrosis only in the livers of GTG+HF mice (Fig. 5B). The pattern of fibrosis was as commonly observed in NASH – namely hepatic zone 3 and pericellular fibrosis (Fig. 5B). In parallel, the hepatic mRNA expression of profibrogenic cytokines, TIMP-1 and TGF-β, was elevated by 11- and 1.6-fold, respectively, in the livers of GTG+HF mice compared with SC mice. As compared with HF mice, the hepatic TIMP-1 expression was 2-fold higher in GTG+HF mice ($^{**}P < 0.01$), while TGF-β level was unchanged. Additionally, the hepatic expression of TNF-α in GTG+HF mice compared with SC mice was increased 3-fold ($P < 0.0001$, Fig. 5C), while hepatic oxidative stress, assessed by anti-8-OHdG immunostaining, was marked in the GTG+HF group compared with the SC-fed group (57.6 ± 4.8 vs. $25.8 \pm 3.7\%$, $P < 0.0001$, Fig. 5C).

Glucose intolerance and insulin resistance of non-alcoholic steatohepatitis model mice induced by gold thioglucose+high-fat diet

Given the central role of IR to the pathogenesis of NASH (19), we now measured basal fasting plasma glucose and insulin levels, and calculated QUICKI, as an index of IR. Basal fasting plasma glucose in GTG+HF mice was markedly increased compared with that in SC mice, and GTG+HF mice exhibited fasting hyperinsulinaemia compared with HF-fed ($^{*}P < 0.05$) and SC-fed mice ($^{***}P < 0.001$). QUICKI indicated the presence of IR in GTG+HF mice compared with SC-fed mice ($^{***}P < 0.001$ vs. SC, Fig. 6A).

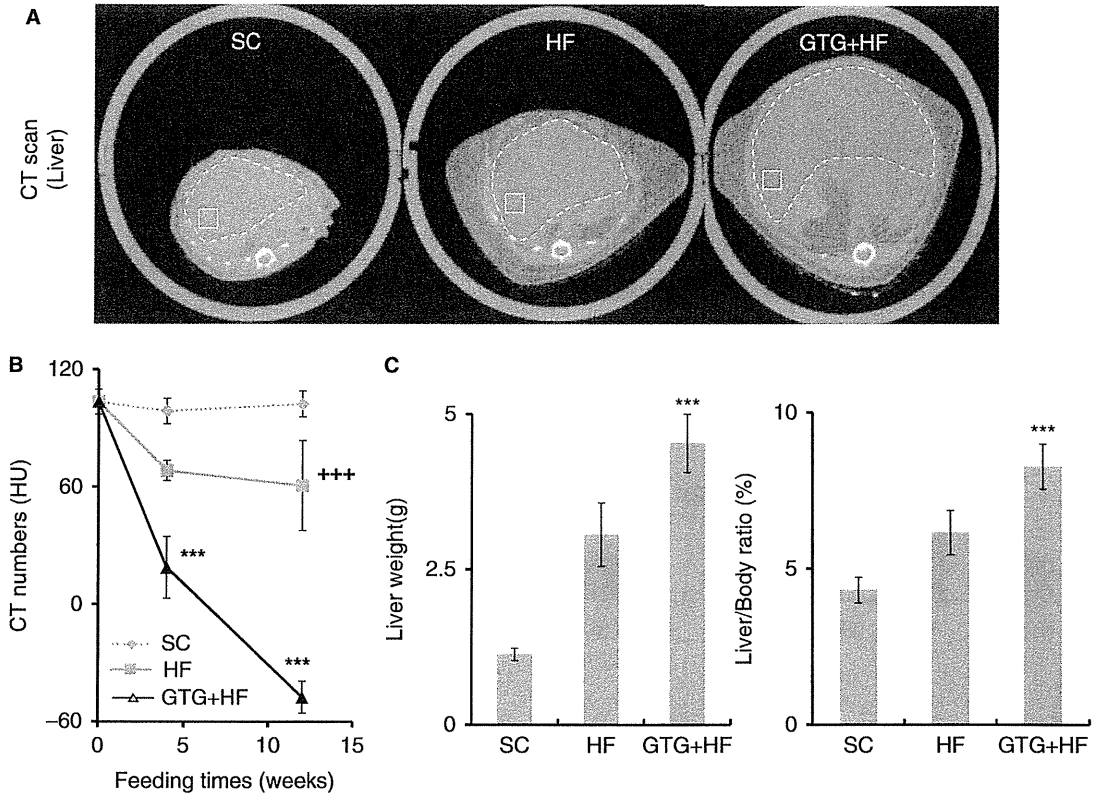

Fig. 3. GTG+HF induced hepatomegaly and hepatic steatosis: (A) evaluation of hepatomegaly by CT scanning. GTG+HF mice had demonstrably more severe hepatomegaly compared with other groups. (B) Time course of hepatic steatosis as assessed by CT scan. GTG+HF mice had more severe and much faster hepatic steatosis in the dorsal right lobe (ROIs were shown as in Fig. 3A) of the liver compared with HF mice at 4 and 12 weeks. $^{***}P < 0.001$ vs. HF or SC, $^{+++}P < 0.001$ vs. SC, $n = 6$. (C) Liver weight and liver/body weight ratio (liver/body). Both liver weight and liver/body ratio were significantly increased in GTG+HF group compared with other groups. $^{***}P < 0.001$ vs. HF and SC, $n = 6$. CT, computed tomography; GTG, gold thioglucose; GTG+HF, GTG treated/HF fed; HF, high-fat diet; ROI, region of interest; SC, standard chow.

Table 1. Concentration of biochemical markers in plasma

	SC ($n = 6$)	HF ($n = 6$)	GTG ± HF ($n = 6$)
AST (U/l)	86 ± 11	140 ± 32***	310 ± 114*,**
ALT (U/l)	31 ± 9	214 ± 80***	514 ± 170*,**
TG (mg/dl)	36 ± 7	50 ± 9	37 ± 13^{NS}
Adiponectin (ng/ml)	7.74 ± 0.36	7.77 ± 0.33	6.72 ± 0.48^{++}

The levels of plasma AST and ALT in GTG+HF mice were significantly higher compared with SC ($^{**}P < 0.01$) and HF mice ($^{*}P < 0.05$). There were no differences in TG level between the groups. Plasma adiponectin in GTG+HF mice was remarkably decreased compared with other groups ($^{++}P < 0.01$ vs. HF and SC), $^{***}P < 0.001$ vs. SC, $n = 6$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTG, gold thioglucose; HF, high-fat diet; SC, standard chow; TG, triglyceride.

We next confirmed the presence of glucose intolerance and IR in the various groups, using the GTT and the ITT (Fig. 6B). GTT revealed that GTG+HF mice had more severe glucose intolerance compared with HF- and SC-fed mice ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. HF, $^{***}P < 0.001$ vs. SC, $n = 6$). ITT showed extremely severe IR in GTG+HF mice compared with others ($^{***}P < 0.001$ vs. HF and SC, $n = 6$). In GTG+HF mice, plasma glucose levels were not decreased in ITT even at 60 min.

These results, taken together, indicate that GTG+HF mice are a novel comprehensive model of human NASH, with the full range of dysmetabolic and histological abnormalities.

For completeness, we also studied GTG+SC mice. These developed obesity and hepatic steatosis as reported previously (6). However, their body weights and degree of hepatic steatosis were much lower than seen with GTG+HF or HF feeding alone (supporting information, Figs S1 and S2). Additionally, ALT and AST in GTG+SC-fed mice were lower than in GTG+HF- or HF alone-fed mice (supporting information, Table S1). Finally, the degree of IR in GTG+SC-fed mice was lower than that in GTG+HF mice or HF alone-fed mice (supporting information, Fig. S3). Therefore, GTG+HF induced a more robust NASH phenotype compared with GTG+SC or HF feeding alone.

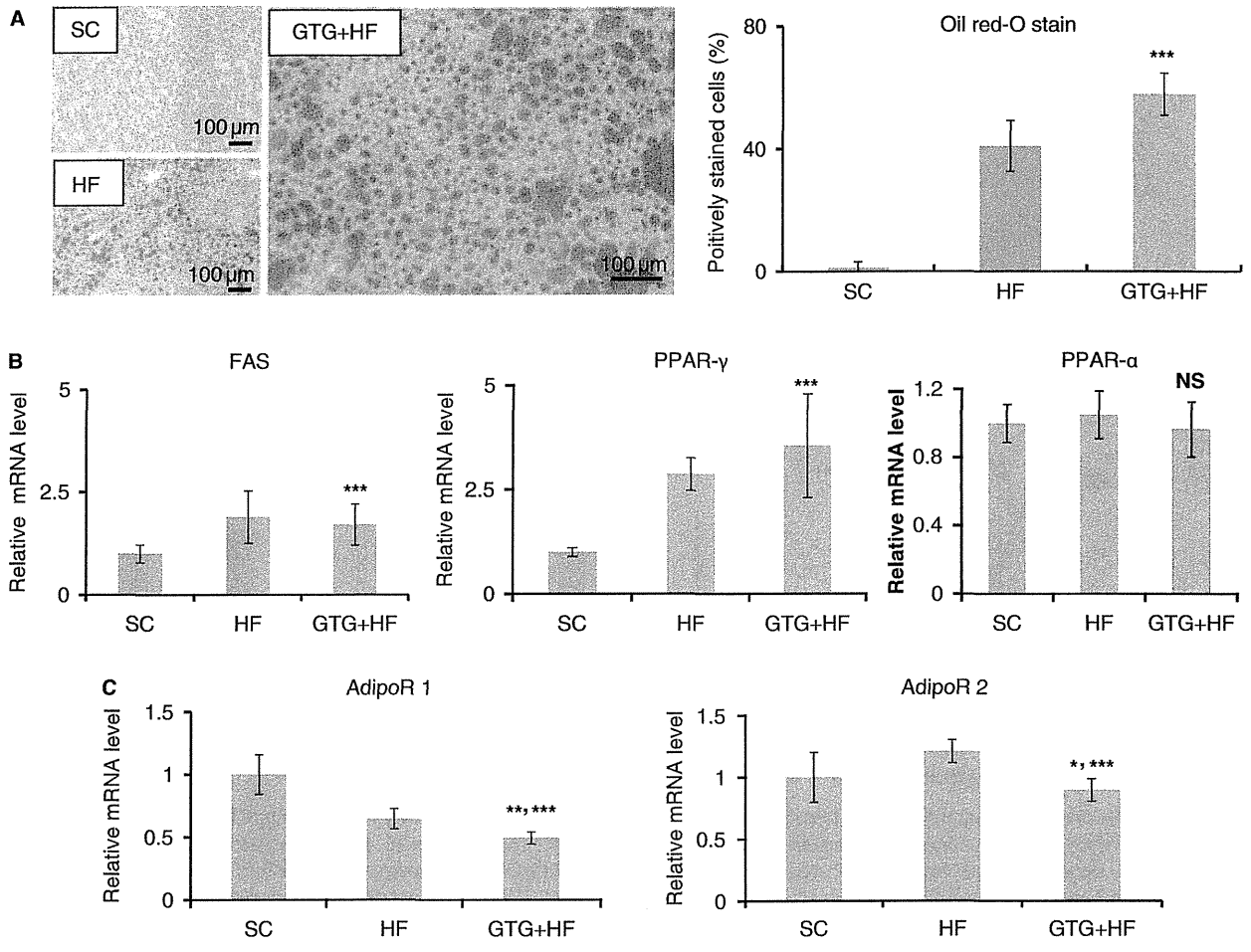

Fig. 4. Hepatic steatosis and expression of genes involved in lipid synthesis: (A) oil red-O staining of livers and image analysis. GTG+HF mice at 12 weeks had more severe hepatic steatosis compared with that of HF and SC mice. ***$P < 0.001$ vs. HF and SC, $n = 6$. (B) Gene expressions of FAS, PPAR-γ and PPAR-α. The relative mRNA expression of FAS and PPAR-γ in the livers of GTG+HF mice were increased (***$P < 0.001$ vs. SC, $n = 6$). The hepatic expression of PPAR-α mRNA was not different between any of the groups. (C) Hepatic gene expression of Adipo R1 and Adipo R2. Both gene expressions of Adipo R1 (**$P < 0.005$ vs. HF, ***$P < 0.001$ vs. SC, $n = 6$) and Adipo R2 (*$P < 0.05$ vs. SC, ***$P < 0.001$ vs. HF, $n = 6$) was reduced in the livers of GTG+HF mice. GTG, gold thioglucose; GTG+HF, GTG treated/HF fed; HF, high-fat diet; SC, standard chow.

Discussion

The public health importance of NASH (1, 2) and the unavailability of proven and effective therapies drive the search for greater understanding of its pathophysiology and novel therapeutic pathways. This search is hampered, however, by the unavailability of experimental models having the full range of pathophysiological features of the human NASH.

Gold thioglucose, which we used here, known to downregulate GPR7, the endogenous G protein-coupled receptor for the identified ligands neuropeptide B and neuropeptide W, in the hypothalamus has been reported to induce hyperphagia, obesity, IR and mild hepatic steatosis in mice (6). In addition, mice treated with HF diet alone have been shown previously to develop obesity and some features of human NASH, after a 50-week period (5), an impractical time line for most experimental protocols. Therefore, we combined here GTG treatment with HF diet feeding, and found that this combination induces a rapid onset and more severely obese phenotype with the full range of dysmetabolic and histological features paralleling human NASH, unlike previous models of NASH (20–22). Our novel murine model has all the hallmarks of the human disease with dysmetabolism as shown by obesity, IR, fasting hyperglycaemia, altered adipokine profile with hepatic steatosis, hepatic inflammation, liver cell injury (hepatocyte ballooning and Mallory–Denk bodies) and progression to fibrosis, particularly perivenular and pericellular fibrosis as seen in adult NASH.

Insulin resistance is the essential requirement for development of NASH (23). Similar to patients with NASH, GTG+HF mice here also had severe IR and glucose intolerance as confirmed by ITT and GTT (Fig. 6). The development of IR has been reported to be

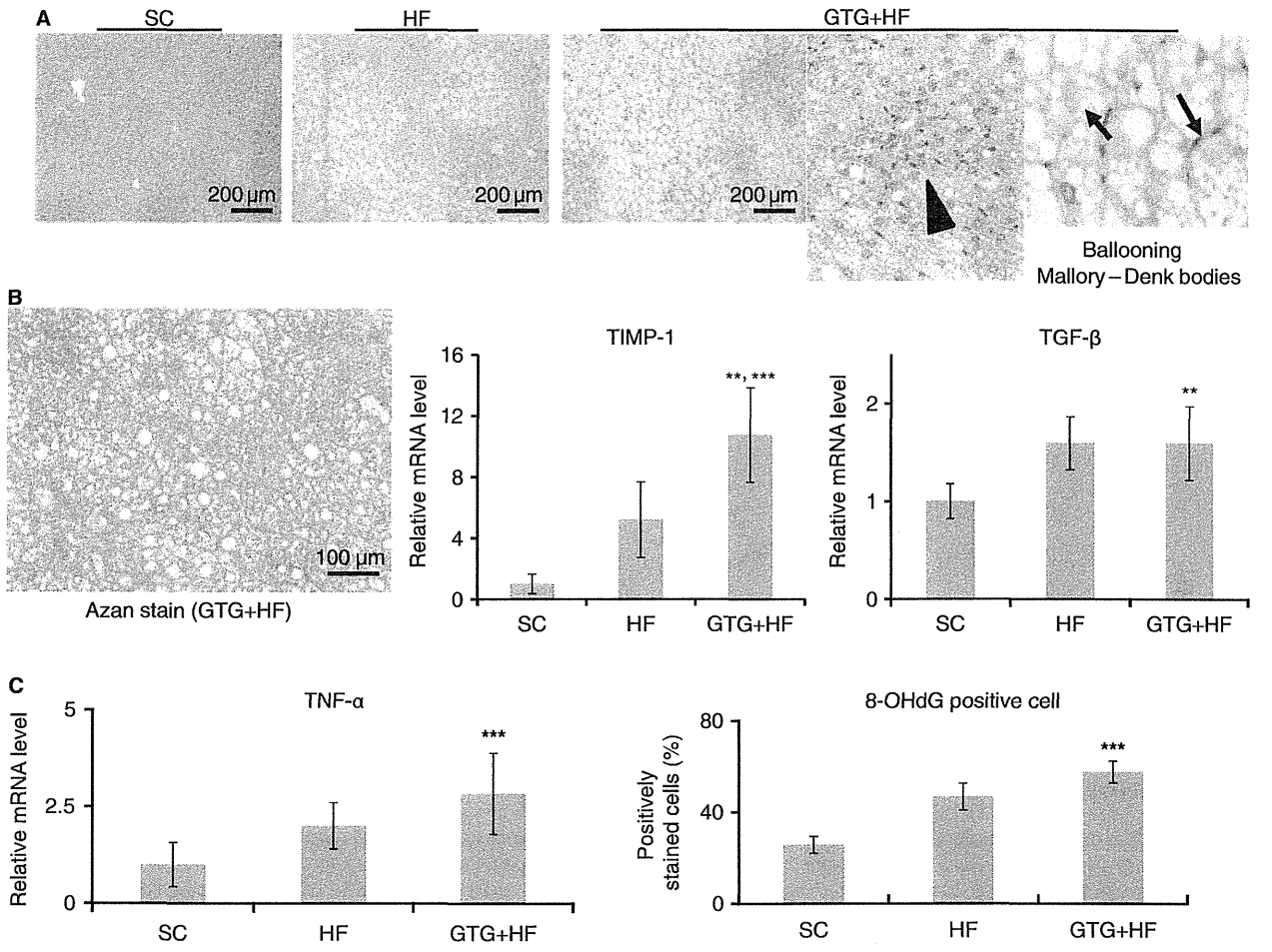

Fig. 5. Pathological and biological features of NASH: (A) H&E staining of liver sections. Liver sections of GTG+HF mice at 12 weeks showed marked steatohepatitis with lobular inflammation (arrowhead), hepatocyte ballooning and Mallory–Denk bodies (arrows). (B) Hepatic fibrosis and expression of profibrogenic markers. With Azan staining, liver sections from GTG+HF mice at 12 weeks showed marked hepatic fibrosis: zone 3 and pericellular. TIMP-1 (**$P < 0.01$ vs. HF, ***$P < 0.001$ vs. SC, $n = 6$) and TGF-β (**$P < 0.01$ vs. SC, $n = 6$) mRNA expression were elevated in tandem in the livers of GTG+HF mice. (C) Gene expression of TNF-α and oxidative stress in the livers of GTG+HF mice. Hepatic expression of TNF-α mRNA in GTG+HF mice was increased compared with SC (***$P < 0.001$ vs. SC, $n = 6$). The numbers of nuclei stained positive for 8-OHdG were also evaluated in the liver of GTG+HF mice compared with other groups. GTG+HF mice had significantly more positive-staining cells than either HF or SC groups (***$P < 0.001$ vs. HF and SC, $n = 6$). 8-OHdG, 8-hydroxy-2′-deoxyguanosine; GTG, gold thioglucose; GTG+HF, GTG treated/HF fed; H&E, haematoxylin and eosin; HF, high-fat diet; NASH, non-alcoholic steatohepatitis; SC, standard chow; TGF, transforming growth factor; TIMP, tissue inhibitors of matrix metalloproteinases; TNF, tumour necrosis factor.

involved in central obesity, decreased adiponectin action, elevation of TNF-α level and oxidative stress (18, 23–26). In GTG+HF mice, the combination of these factors would contribute to the development of IR.

Obesity, particularly, visceral adiposity is almost universal in NASH (24, 27) and is thought to contribute to the pathogenesis of fatty liver disease (28). GTG+HF mice showed severe obesity with increased volume of visceral and subcutaneous fat as evaluated by CT scan. The development of increased body weights and abdominal adiposity, and consequent hepatic steatosis was faster and more severe in GTG+HF mice. At 4 weeks, GTG+HF mice already had reached around 40 g body weight, and hepatic steatosis also had developed as demonstrated by CT scanning. At 12 weeks, liver sections of GTG+HF mice showed the typical histological features of NASH (Fig. 5).

Adiponectin is an important adipokine in the pathogenesis of NASH (14) with serum adiponectin decreased in NASH patients compared with controls or those with simple steatosis. In addition, several reports inversely correlate serum adiponectin levels with NASH severity (29, 30). In our study, not only plasma adiponectin but also hepatic expression of Adipo R1/R2 was decreased in GTG+HF mice (Table 1 and Fig. 4). These results indicated that decreased function of adiponectin action

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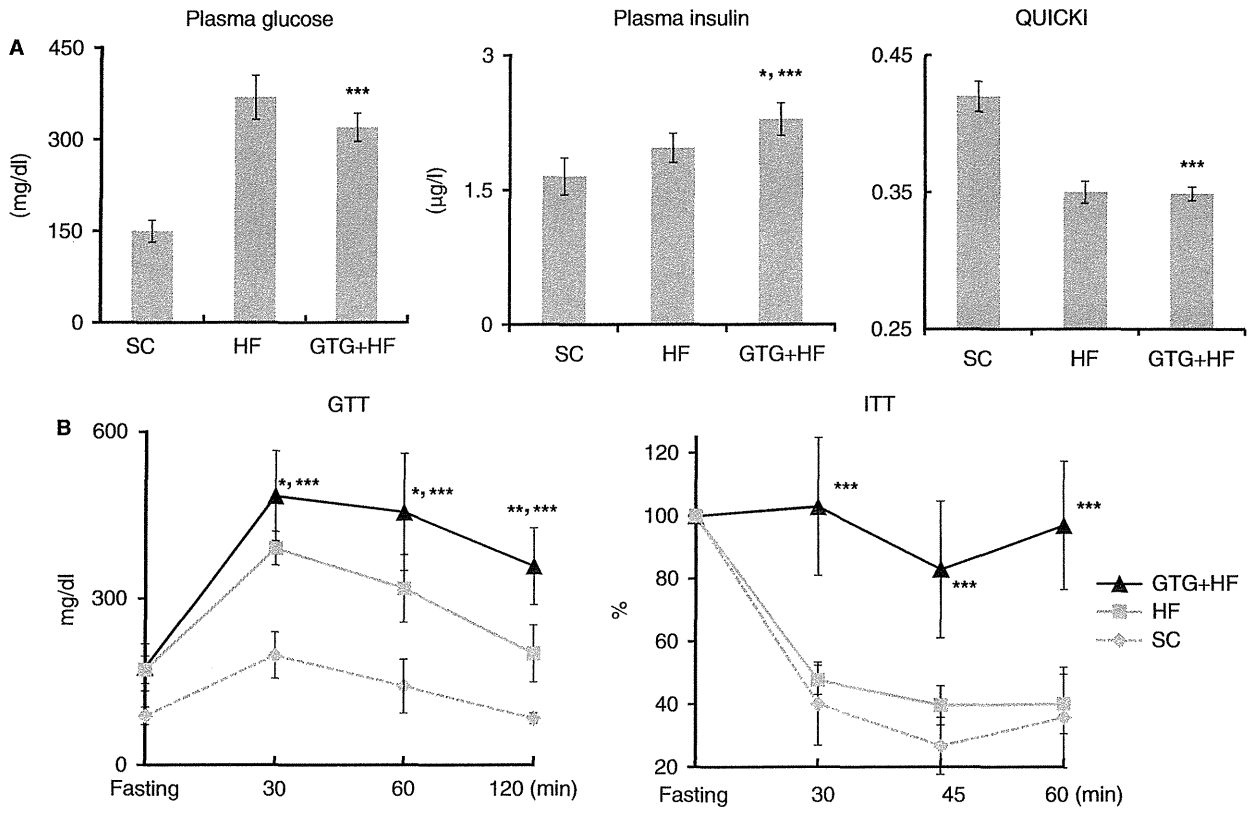

Fig. 6. Analysis of glucose intolerance and insulin resistance. (A) Fasting plasma glucose, insulin levels and QUICKI. In GTG+HF mice, plasma fasting glucose and insulin levels at 12 weeks were increased compared with controls (SC). QUICKI also showed that GTG+HF mice had severe IR compared with SC. $^{***}P < 0.001$ vs. SC, $^{*}P < 0.05$ vs. HF, $n = 6$, (B) GTT and ITT. At 12 weeks in GTG+HF mice, GTT revealed severe glucose intolerance ($^{*}P < 0.05$, $^{**}P < 0.01$, vs. HF, $^{***}P < 0.001$ vs. SC, $n = 6$), and ITT confirmed severe IR ($^{***}P < 0.001$ vs. HF and SC, $n = 6$). GTG, gold thioglucose; GTG+HF, GTG treated/HF fed; GTT, glucose tolerance test; HF, high-fat diet; ITT, insulin tolerance test; QUICKI, quantitative insulin sensitivity check index; ROI, region of interest; SC, standard chow.

would also contribute more to the progression of NASH in GTG+HF mice. It has also been reported that the expression of PPAR-γ is increased in a HF diet-induced hepatic steatosis in mice (31). Hepatic over expression of PPAR-γ provokes hepatic steatosis, whereas liver-specific PPAR-γ disruption improved hepatic steatosis in ob/ob mice (32). We have also shown here an increased hepatic expression of PPAR-γ in GTG+HF mice. In addition, TNF-α has been known to have important role in the pathogenesis of NASH with elevated serum TNF-α levels in human NASH (14), while anti-TNF antibodies have been shown to ameliorate steatohepatitis in ob/ob mice (26). Moreover, increased hepatic oxidative stress has also been implicated in the pathogenesis of NASH (25). In keeping with the above findings, we have shown here that GTG+HF mice have an increased hepatic expression of TNF-α plus evidence of enhanced oxidative stress. Taken together with our data, the increased hepatic expression of TNF-α and enhanced oxidative stress might contribute the development of NASH in GTG+HF mice.

A variety of animal models of NASH have been reported. For example, the methionine–choline-deficient diet (MCD) model shows that steatohepatitis with perivenular and pericellular fibrosis with multiple foci of necro-inflammation typically seen in human NASH (10). MCD-fed animals, however, are not obese, do not have IR and serum adiponectin levels are unchanged or increased (10, 33, 34). Genetic animal models of NASH have also been reported but none of these have the necessary obesity (35, 36), IR (20, 21) or hepatic fibrosis (37, 38). Therefore, there has been an ongoing search for an overnutritional model of NASH. The gastric overnutrition model of NASH with an implanted gastrostomy tube offered some promise and was reported previously as an excellent murine model of NASH (22). However, the induction of this model required a high degree of technical expertise that prevented this model from becoming popular. On the other hands, treatment with GTG and HF feeding easily induced hyperphagia, obesity, dysmetabolism and steatohepatitis with fibrosis, paralleling human NASH. Furthermore, this method easily provides a comprehensive mice model of NASH not only in wild-type mice, C57BL/6 mice but also may of course be used on any

genetic background of interest to enhance the search for effective treatment of NASH.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Time course of body weight gain in GTG+HF, HF, GTG+SC and SC fed mice.
Fig. S2. Oil red-O staining of livers and image analysis.
Fig. S3. Glucose tolerance test (GTT) and insulin tolerance test (ITT).
Table S1. Biochemical evidence of liver injury.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease

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Abstract
Background Liver histology is the gold standard for the diagnosis of nonalcoholic steatohepatitis (NASH). Noninvasive, simple, reproducible, and reliable biomarkers are greatly needed to differentiate NASH from nonalcoholic fatty liver disease (NAFLD).
Methods To construct a scoring system for predicting NASH, 177 Japanese patients with biopsy-proven NAFLD were enrolled. To validate the scoring system, 442 biopsy-proven NAFLD patients from eight hepatology centers in Japan were also enrolled.
Results In the estimation group, 98 (55%) patients had NASH. Serum ferritin [≥200 ng/ml (female) or ≥300 ng/ml (male)], fasting insulin (≥10 μU/ml), and type IV collagen 7S (≥5.0 ng/ml) were selected as independent variables associated with NASH, by multilogistic regression analysis. These three variables were combined in a weighted sum [serum ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male) = 1 point, fasting insulin ≥10 μU/ml = 1 point, and type IV collagen 7S ≥5.0 ng/ml = 2 points] to form an easily calculated composite score for predicting NASH, called the NAFIC score. The area under the receiver operating characteristic (AUROC) curve for predicting NASH was 0.851 in the estimation group and 0.782 in the validation group. The NAFIC AUROC was the greatest among several previously established scoring systems for detecting NASH, but also for predicting severe fibrosis.
Conclusions NAFIC score can predict NASH in Japanese NAFLD patients with sufficient accuracy and simplicity to be considered for clinical use.

All authors are members of the Japan Study Group of NAFLD (JSG-NAFLD).

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