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我が国における飲酒の実態把握およびアルコールに関連する生活習慣病と
その対策に関する総合的研究
（研究代表者　樋口　進）

平成23年度分担研究報告書
人間ドック受診者における飲酒習慣と生活習慣病との関連の研究

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研究要旨：適度の飲酒は、high density lipoprotein-cholesterol (HDL-C) を増加させ、low density lipoprotein cholesterol (LDL-C) を減少させることで、動脈硬化性疾患のリスクを減らす可能性が報告されているが、飲酒は中性脂肪を増加させることで動脈硬化性疾患のリスクを高める可能性もある。本研究では、LDLの亜型で動脈硬化惹起性が強いsmall dense LDL-C (sd-LDL-C) に着目して、飲酒と脂質代謝異常の関連を解析した。対象は、人間ドッグを受診し、sd-LDL-Cを測定した男性のうち、脂質異常症・糖尿病治療中、もしくは中性脂肪値が400mg/dl以上の受診者を除いた467名である。平均飲酒量（エタノール換算g/日）で4群（飲まない、20g/日未満、20g以上60g未満、60g以上）に分けて検討すると、飲酒量の増加に伴い、中性脂肪は増加傾向を示し、HDL-Cは有意に増加した。一方、飲酒量の増加に伴い、LDL-Cは減少するものの、sd-LDL-Cは4群間で有意差はなかった。以上のことから、飲酒は脂質代謝において、動脈硬化促進と抑制に働くような二面性を有していると考えられるが、sd-LDL-C値には影響しないと考えられた。飲酒が動脈硬化性疾患のリスクを減らすかは、新たなマーカーやsd-LDL-Cの経時的な変化なども含めたさらなる検討が必要と考えられた。

研究協力者
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A．研究目的

今までに、7年間の縦断研究から、飲酒は脂肪肝発症に抑制的に作用する傾向であることを報告した。さらに、適度の飲酒は、high density lipoprotein-cholesterol (HDL-C) を増加させ、low density lipoprotein cholesterol (LDL-C) を減少させることで、動脈硬化性疾患のリスクを減らす可能性が報告されている。しかし、飲酒は中性脂肪を増加させることで動脈硬化性疾患のリスクを高める可能性もあり、動脈硬化に着目した飲酒と脂質代謝異常との関連は十分明らかにされていない。

LDLの亜型であるsmall dense LDL (sd-LDL) が、動脈硬化惹起性の強いリポ蛋白粒子として最近注目されていることから、本研究では、sd-LDL-Cに着目して、飲酒と脂質代謝異常の関連を解析した。

B．研究方法

対象は、人間ドッグを受診し、sd-LDL-Cを測定した男性のうち、脂質異常症・糖尿病治療中、もしくは中性脂肪値が400mg/dl以上の受診者を除いた467名である。血清sd-LDL-Cは平野らの方法に準じて測定した。アルコールの飲酒状況はアンケート調査に基づいた。

（倫理面への配慮）

a．個人の人権の擁護：参加者のデータは、連結可能匿名化を行い、厳重な秘密保持のもとに

管理され、本研究のデータが参加者に不利益を及ぼすことはないと考えられる。

b. 個人情報の管理：ID 番号、氏名、住所、電話番号などの個人を特定できる情報を除いたものを作製し、新たな番号を付与し、本研究にはこの番号のみを用い、個人が特定できる名前などを用いない。

c. 研究等によって生じる個人への不利益：超音波検査や静脈穿刺は人間ドッグで行われるものであり、被験者に不当な危険が生じることはない。個人のプライバシーに関わる点については十分な配慮を行い、対象者の不利益が生じないようにする。

C．研究結果

平均飲酒量(エタノール換算 g/日)で 4 群(飲まない、20g/日未満、20g 以上 60g 未満、60g 以上) に分けて検討すると、sd-LDL-C は 60g/日未満の飲酒で低値を呈する傾向であったが、有意ではなかった。また、平均飲酒量が増加すると中性脂肪は増加傾向を示し、LDL-C は有意に低下、HDL-C は有意に増加した。

D．考察

飲酒は脂肪肝発症に抑制的に作用する傾向であることを以前報告しているが、脂肪肝は動脈硬化性疾患とも関連することから、飲酒は脂肪肝のみならず、動脈硬化性疾患のリスクを減らす可能性がある。また、適度の飲酒は、HDL-C を増加させ、LDL-C を減少させることで、動脈硬化性疾患のリスクを減らす可能性も報告されている。一方、飲酒は中性脂肪を増加させることから、飲酒は動脈硬化を促進する可能性もあり、飲酒と動脈硬化の関連は十分明らかになっていない。本研究では、LDL の亜型である sd-LDL が動脈硬化促進に重要な役割を担うことに着目し、飲酒と sd-LDL-C との関連を解析し、飲酒が動脈硬化の重要な要因である脂質異常とどのように関連するかを検討した。

本年度の研究では、今までの報告と同様に、平均飲酒量が増加すると LDL-C は低下し、HDL-C は増加、中性脂肪は増加傾向を示すことを示した。一方、本研究では、sd-LDL-C は飲酒による影響を受けない可能性を示した。sd-LDL-C は LDL-C と中性脂肪濃度に影響を受けることから、飲酒による LDL-C の低下と中性脂肪の増加が、sd-LDL-C 濃度が飲酒量とは関連しない結果に影響したと考えられた。

今までに、飲酒習慣と脂肪肝の発生頻度との関連を横断研究で検討し、飲酒量の増加に伴い脂肪肝の頻度は増えるが、飲酒頻度の増加に伴い脂肪肝の頻度は減少することを報告している。飲酒と脂質の動脈硬化惹起性の関係では、sd-LDL-C の挙動も考慮し、飲酒パターンや肥満の有無などを踏まえた上で、さらなる検討が必要である。

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F．知的財産権の出願・登録状況

1．特許取得

なし。

2．実用新案登録

なし。

3．その他

なし。

研究成果の刊行に関する一覧

雑誌

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Hamabe A, Uto H, Imamura Y, et al.	Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period	J Gastroenterol	46	769-778	2011
Hiramine Y, Imamura Y, Uto H, et al.	Alcohol drinking patterns and the risk of fatty liver in Japanese men	J Gastroenterol	46	519-528	2011

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Liver regeneration after partial hepatectomy in rat is more impaired in a steatotic liver induced by dietary fructose compared to dietary fat

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ABSTRACT

Hepatic steatosis (HS) has a negative effect on liver regeneration, but different pathophysiologies of HS may lead to different outcomes. Male Sprague-Dawley rats were fed a high fructose (66% fructose; H-fruc), high fat (54% fat; H-fat), or control chow diet for 4 weeks. Based on hepatic triglyceride content and oil red O staining, HS developed in the H-fruc group, but was less severe compared to the H-fat group. Hepatic mRNA expression levels of fatty acid synthase and fructokinase were increased and those of carnitine palmitoyltransferase-1 and peroxisome proliferator-activated receptor-α were decreased in the H-fruc group compared to the H-fat group. Liver regeneration after 70% partial hepatectomy (PHx) was evaluated by measuring the increase in postoperative liver mass and PCNA-positive hepatocytes, and was impaired in the H-fruc group compared to the H-fat and control groups on days 3 and 7. Serum levels of tumor necrosis factor-α, interleukin-6 and hepatocyte growth factor did not change significantly after PHx. In contrast, serum TGF-β1 levels were slightly but significantly lower in the control group on day 1 and in the H-fat group on day 3 compared to the level in each group on day 0, and then gradually increased. However, the serum TGF-β1 level did not change after PHx in the H-fruc group. These results indicate that impairment of liver regeneration after PHx in HS is related to the cause, rather than the degree, of steatosis. This difference may result from altered metabolic gene expression profiles and potential dysregulation of TGF-β1 expression.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common clinical problem that results from a variety of metabolic disorders, including obesity, diabetes mellitus, and dyslipidemia. Fatty liver is present in 9.6–29% of autopsy cases [1,2], and moderate hepatic steatosis (30–60% fatty hepatocytes) is found in 6% of autopsies after accidental deaths [2]. The 5-year survival rate of patients with nonalcoholic steatohepatitis (NASH) which is a progressive liver disease in NAFLD, is 75% [3]. Complications of NASH, including cirrhosis and hepatocellular carcinoma (HCC), are expected to increase with the growing epidemic of diabetes and obesity [4]. However, it is unclear which cases of NAFLD are likely to progress to severe fibrosis and HCC.

Obesity has escalated to epidemic proportions worldwide and many causes have been suggested, including dietary components. Excessive caloric intake has been related to high-fat foods and increased portion sizes. The increase in consumption of high-fructose corn syrup (HFCS) has a temporal relationship with the epidemic of obesity, and overconsumption of HFCS in calorically sweetened beverages may have played a role in the epidemic [5]. Excessive fructose and sucrose (which contains 50% fructose) intake is a risk factor for developing NAFLD and may be associated with NASH [6,7]. Therefore, abnormal metabolism may be a pathophysiological feature of hepatic steatosis.

Many growth factors and cytokines such as hepatocytes growth factor (HGF) stimulate liver regeneration. In contrast, transforming growth factor (TGF)-β is a potent inhibitor of hepatocyte proliferation [8] and is involved in growth arrest once the liver has reached an appropriate functional mass [9]. Liver regeneration requires orchestrated functions of these molecules in appropriate amounts and at appropriate locations and times [10]. Hepatic steatosis is associated with an increased incidence of complications and mortality after liver resection or transplantation [11], and these outcomes are associated with a decrease in the ability of the liver to regenerate in NAFLD [12]. However, direct experimental evidence of a relationship between a high fructose or high fat diet and cytokine expression in liver regeneration is lacking.

In this study, we investigated mechanistic differences in liver regeneration in fatty liver and metabolic abnormalities caused by

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dietary fructose or fat. The results may contribute to elucidation of the mechanisms that affect the outcome of NAFLD.

2. Materials and methods

2.1. Animals and diets

Male Sprague-Daley rats of age 7 weeks were obtained from Kyudo (Kumamoto, Japan). Rats were acclimatized to laboratory conditions for at least 7 days at a constant temperature of 24 °C with a 12-h light-dark cycle, and fed a standard rodent diet (CE-2; Kyudo, Kumamoto, Japan) and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee guideline of Kagoshima University.

After acclimatization, rats were placed in groups that were fed three different diets for 4 weeks ad libitum: control chow (10% of total calories as fructose, 12% as fat, and 19% as protein; control group), a high fat diet (8% of total calories as fructose, 54% as fat, and 15% as protein; H-fat group), and a high fructose diet (66% of total calories as fructose, 11% as fat, and 19% as protein; H-fruc group). The diets were obtained from Nosan Corporation (Kanagawa, Japan). All diets were prepared as a powder, and diet weights and intake energy were measured every other day.

A 70% partial hepatectomy (PHx) was performed after 4 weeks of intake of each diet. Rats were anesthetized by ether inhalation. After midline laparotomy, the median and left hepatic lobe were pushed out and ligated with 2.0 silk sutures, and resected as described previously [13]. Resected livers were weighed and used for RNA extraction, or thin slices were immersed in 10% formalin and embedded in paraffin to make 4-μm sections for routine staining with hematoxylin and eosin. After PHx, all rats were fed with a standard CE-2 diet and sacrificed on days 1, 3, and 7 after PHx. Blood was collected by vena cava puncture and centrifuged. The resulting serum was stored at −80 °C. The remaining right hepatic lobes were removed and weighed.

2.2. Evaluation of insulin sensitivity

Fasting blood glucose (FBG) and serum immunoreactive insulin (IRI) levels were determined by ELISA (Morinaga Institute of Biological Science, Kanagawa, Japan). The homeostasis model assessment-insulin resistance index (HOMA-IR) was calculated from FBG (mg/dl) and IRI (mU/ml) levels as FBG × IRI/405. After 4 weeks diet intake, rats in all groups underwent an intraperitoneal insulin tolerance test (ITT), which was performed after a 4-h fast by intraperitoneal injection of 0.5 U insulin per kg of body weight. Blood was drawn from a tail vein after injection for measurement of plasma glucose concentrations [14].

2.3. Assessment of hepatic steatosis

Hepatic steatosis was assessed using hepatic triglyceride levels and oil red O staining. Hepatic lipids were extracted with chloroform-methanol and measured enzymatically using a commercial kit (L-type Wako TGH, Wako Pure Chemical Industries, Osaka, Japan). Oil red O staining was performed to evaluate accumulation of fat droplets in hepatocytes in frozen liver sections. The ratio of the oil red O-stained area to the total area was determined using Image J software (http://rsb.info.nih.gov/ij/index.html).

2.4. Assessment of hepatic mRNA levels of genes associated with metabolism of lipids and fructose

The relative levels of specific mRNAs in resected liver on day 0 were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using Syber Premix Ex Taq (TaKaRa Bio, Shiga, Japan). Total RNA was extracted from each liver using Isogen (Nippon Gene, Tokyo, Japan). Expression levels of target genes were calculated relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control gene to normalize the target gene expression levels. All procedures were performed according to manufacturers' instruction. mRNA levels were determined for carnitine palmitoyltransferase (CPT)-1, peroxisome proliferator-activated receptor (PPAR)-α, AMP-activated protein kinase (AMPK), fatty acid synthase (FAS), fructokinase (FK), and cytochrome P450 2E1 (Cyp2E1). Primer sequences are listed in Table 1. The PCR primers were obtained from TaKaRa Bio Inc.

Table 1
Primers used for PCR.

Gene	Gene Bank Number	Primer sequences	
PPARα	NM_013196.1	Forward	5′-GACAAGGCCTCAGGATACCACTATG-3′
		Reverse	5′-TTGCAGCTTCGATCACACTTGTC-3′
AMPK	NM_019142.1	Forward	5′-GGCTCGCCCAATTATGCTG-3′
		Reverse	5′-AGAGTTGGCACGTGGTCATCA-3′
CPT-1	NM_031559.2	Forward	5′-CGCTCATGGTCAACAGCAACTAC-3′
		Reverse	5′-TCACGGTCTAATGTGCGACGA-3′
FK	NM_031855.3	Forward	5′-CCAGCTGTTCGGCTATGGAGA-3′
		Reverse	5′-CACAGCCAACCAGATGCTTCA-3′
FAS	NM_017332.1	Forward	5′-CACAGCATTCAGTCCTATCCACAGA-3′
		Reverse	5′-TCAGCCCAGGCACAGATGAG-3
Cyp2E1	NM_031543	Forward	5′-CCTACATGGATGCTGTGGTG-3′
		Reverse	5′-CTGGAAACTCATGGCTGTCA-3′
GAPDH	NM_017008.3	Forward	5′-GGCACAGTCAAGGCTGAGAATG-3′
		Reverse	5′-ATGGTGAAGACGCCAGTA-3′

2.5. Assessment of liver regeneration

The liver regeneration rate (%) was calculated as $100 \times \{C - (A - B)\}/A$, where A is the estimated total liver weight at the time of partial hepatectomy, B is the excised liver weight, and C is the weight of the regenerated liver at the final resection [13]. Liver samples were also stained for proliferating cell nuclear antigen (PCNA). After fixation with formalin and paraffin embedding, tissue was incubated with anti-PCNA (clone PC10; Dako, Tokyo, Japan). The PCNA proliferation index was determined in at least 1000 hepatocytes in each group. Data are expressed as the percentage of PCNA-stained hepatocytes out of the total number of hepatocytes examined.

2.6. Assessment of serum markers

Serum level of high sensitivity C-reactive protein (hs-CRP; Rat CRP ELISA, Life Diagnostics, West Chester, PA) was determined using commercially available kits. Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, TGF-β1 (all R&D Systems, Minneapolis, MN) and HGF (Institute of Immunology, Tokyo, Japan) were measured on days 0, 1, 3 and 7.

2.7. Statistical analysis

Statistical comparison among groups was performed using one-way ANOVA or repeated ANOVA and a post-hoc Fisher PLSD test. A Student t-test was used for comparison of two groups. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error (SE) or standard deviation (SD).

3. Results

3.1. Metabolic parameters in rats fed with a high fat or high fructose diet

Total energy intake for 4 weeks and body weight (mean body weight relative to basal body weight) did not differ between the H-fat and H-fruc groups, but were significantly higher in these groups compared to the control group ($P < 0.05$) (Table 2). The serum triglyceride level was higher in the H-fruc group than in the H-fat and control groups. There were no significant differences in serum adiponectin levels among the three groups, but serum leptin levels were significantly higher in the H-fat and H-fruc groups compared to the control group ($P < 0.05$). Serum hs-CRP was significantly higher in the H-fruc group compared to the H-fat group ($P < 0.05$). FBG was significantly higher in the H-fruc group than in the control group ($P < 0.05$). IRI and HOMA-IR showed a tendency to be higher in the H-fruc group (Table 2) and the H-fruc group had significantly more severe insulin resistance based on ITT results ($P < 0.001$) (Fig. 1A).

Table 2
Metabolic parameters and serum biochemical markers after 4 weeks of diet intake.

	Control	H-fat	H-fruc
Body weight (% basal BW)	140.3 (7.5)	170.7 (6.3)*	171.7 (16.4)*
Total energy intake (kcal)	2108.8 (248.4)	2530.1 (72.0)*	2419.3 (84.1)*
ALT (IU/L)	33.6 (4.5)	22.6 (1.8)	24.8 (5.0)
Triglyceride (mg/dl)	67.2 (23.36)	62.7 (21.82)	145.0 (48.19)**
Free fatty acids (mEq/L)	5.63 (3.43)	8.03 (2.55)	6.90 (3.25)
Fasting blood glucose (mg/dl)	108.0 (25.0)	116.8 (18.8)	144.8 (16.3)*
Insulin (ng/ml)	3.73 (2.37)	2.76 (1.05)	4.74 (2.84)
HOMA-IR	1.09 (0.90)	0.78 (0.21)	1.77 (1.14)
Adiponectin (μg/ml)	1.71 (0.61)	2.80 (0.62)	2.36 (0.51)
Leptin (ng/ml)	0.61 (0.15)	1.64 (0.62)*	1.45 (0.50)*
Hs-CRP (mg/ml)	0.13 (0.07)	0.12 (0.05)	0.17 (0.06) ***

Data are shown as mean (standard deviation) after 4-week intake of the three diets. ALT, alanine aminotransferase; HOMA-IR, homeostasis model of assessment for insulin resistance index; and Hs-CRP, high sensitivity C-reactive protein.
* $P < 0.05$ *vs.* Cont.
** $P < 0.01$ *vs.* Cont and H-fat.
*** $P < 0.05$ *vs.* H-fat.

3.2. Severity of hepatic steatosis in the H-fat and H-fruc groups

Micro and macrovesicular steatosis were clearly visible on HE staining of livers from H-fat and H-fruc rats (Fig. 1B). The hepatic fat area (%) calculated by oil red O staining and the hepatic triglyceride content were significantly higher in the H-fat group compared to the H-fruc and control groups (Fig. 1C and D).

3.3. Hepatic mRNA expression levels in the H-fat and H-fruc groups after PHx

Hepatic mRNA levels of FK and FAS significantly increased in the H-fruc group and significantly decreased in the H-fat group compared to the control group (Fig. 2). In contrast, mRNA levels for CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly lower in the H-fruc group compared to the H-fat group ($P < 0.05$) (Fig. 2).

3.4. Liver regeneration after PHx in the H-fat and H-fruc groups

Liver regeneration and the PCNA labeling index at 3 and 7 days after PHx were significantly lower in the H-fruc group than in the

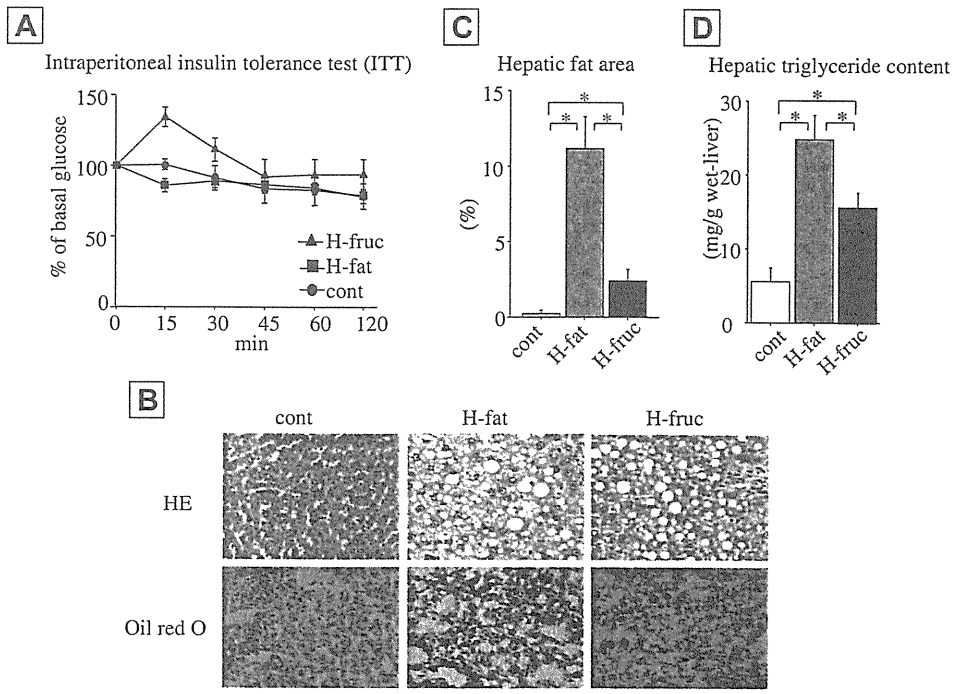

Fig. 1. Insulin tolerance test and histological evaluation of fatty liver in rats fed a chow control diet (Cont), a high-fat diet (H-fat) or a high-fructose diet (H-fruc). (A) An intraperitoneal insulin tolerance test (ITT) was performed after 4 weeks intake of each diet. After a 4-h fast, mice were injected intraperitoneally with 0.5 U of insulin per kg body weight. Glucose was measured in blood sampled from a tail vein at 0, 15, 30, 45, 60, and 120 min. H-fruc rats had significantly more severe insulin resistance compared to control and H-fat rats ($n = 5$–6, $P < 0.001$, repeated ANOVA). (B–D) Fatty liver was evaluated by hematoxilin-eosin staining (B), image analysis using oil red O staining (C), and hepatic triglyceride content (D). Fatty infiltration of the liver was most severe in the H-fat group ($n = 6$), and that in the H-fruc group ($n = 6$) was higher than in the control group ($n = 5$). *$P < 0.01$ (one way ANOVA).

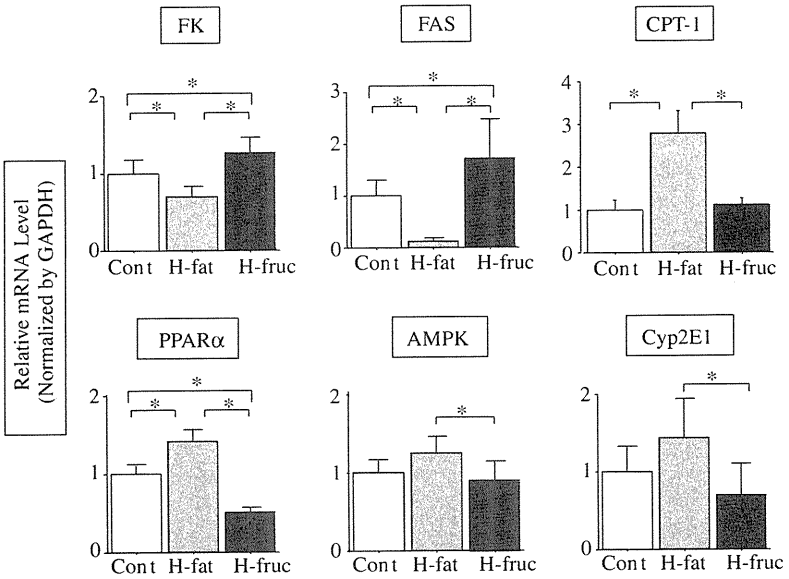

Fig. 2. Hepatic mRNA expression of genes involved in metabolism assessed by RT-qPCR (each $n = 5–6$) after 4 weeks of intake of the three diets. Expression levels of CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly suppressed in the H-fruc group compared to the H-fat group. FK and FAS were significantly increased in the H-fruc group compared to the H-fat or control group. In contrast, FAS and FK were significantly decreased in the H-fat group compared to the control group. $^{*}P < 0.05$ (one way ANOVA).

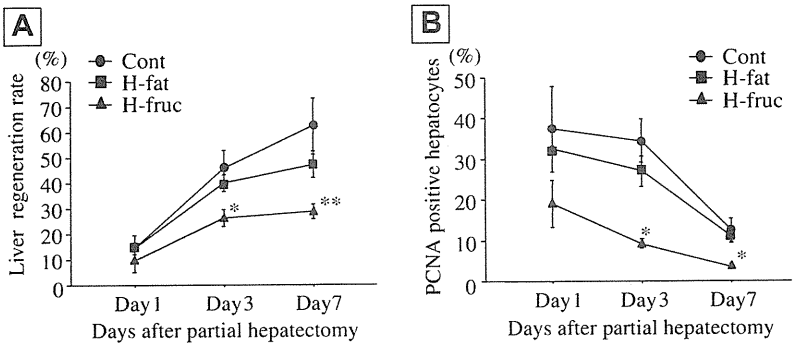

Fig. 3. Liver regeneration after 70% partial hepatectomy in rats fed three different diets (each $n = 4–6$). (A) The liver regeneration rate (%) was lowest in the H-fruc group on days 3 and 7. This rate was also lower in the H-fat group compared to the control group, but the difference was not significant. (B) PCNA labeling showed that the mitotic index was significantly lower in the H-fruc group compared to the other two groups. The PCNA proliferation index was determined in at least 1000 hepatocytes. Values are shown as the mean ± standard error ($^{*}P < 0.05$ *vs.* control and H-fat group, $^{**}P < 0.05$ *vs.* control group).

H-fat and control groups (Fig. 3A and B), but only showed a tendency to be lower in the H-fat group compared to the control group.

3.5. Serum biochemistry and cytokine levels just before and after PHx

The serum ALT level in the H-fruc group on day 1 after PHx was significantly higher than that in the H-fat group, but similar to the control group, and there was no difference among the three groups on days 0, 3 and 7 (Fig. 4A). Serum AST, ALP and albumin did not differ significantly before and after PHx. The serum TNF-α level just before PHx showed a tendency to be higher in the H-fruc group, and serum IL-6 and HGF did not differ significantly among the three groups throughout the course of the study (Fig. 4B). In contrast, the serum TGF-β1 level in the control group on day 1 and in the H-fat group on day 3 were significantly lower than the respective levels on day 0, and then gradually increased (Fig. 4B). However, serum TGF-β1 levels were not suppressed after PHx in the H-fruc group.

4. Discussion

Studies in animal models and patients with fatty liver have shown impaired liver regeneration that worsens in severe fatty liver or NASH [14–16]. However, the mechanism of impaired liver regeneration in fatty liver is not fully elucidated. In our study, we found that liver regeneration was more impaired in rats given a high fructose diet (H-fruc group) compared to those given a high fat diet (H-fat group), although the severity of hepatic steatosis in the H-fruc group was lower than that in the H-fat group. These results provide the first evidence that liver regeneration is affected by the cause, rather than the severity, of hepatic steatosis. In addition, insulin resistance, altered metabolic gene expression profiles, and abnormal expression of TGF-β1 might be involved in the mechanism of the delay in liver regeneration.

Sucrose, the major component of sugar, is hydrolyzed to glucose and fructose, and increased consumption of sugar and fructose is related to the increase in the overweight population. Fructose in soft drinks and sweetened beverages may be strongly related to

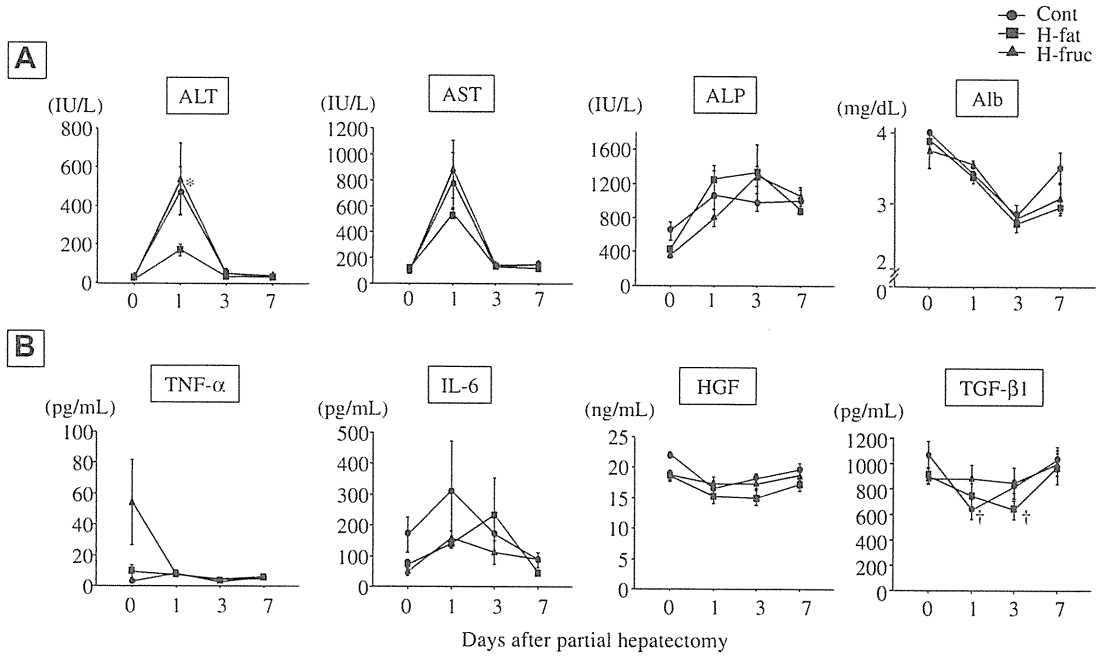

Fig. 4. Course of serum chemistry and cytokine levels during liver regeneration after partial hepatectomy (each $n = 4$–6). (A) Hepatocellular damage assessed by serum ALT in the H-fruc and control group on day 1 was higher than that in the H-fat group. However, AST, ALP and albumin did not differ among the three groups during the course of the study. (B) The serum TNF-α level just before partial hepatectomy tended to be higher in the H-fruc group. In contrast, serum TGF-β1 levels in the control group on day 1 and the H-fat group on day 3 were lower than the respective levels on day 0. Serum TGF-β1 levels in the H-fruc group did not change significantly over the course of the study. Values are shown as the mean ± standard error. *$P < 0.05$ *vs.* H-fat group.

obesity [17,18], and excessive intake of sugar and fructose also cause insulin resistance [19]. An overweight status, obesity and insulin resistance are important risk factor for NAFLD, and fructose consumption has been associated with increased hepatic fibrosis in patients with NAFLD [7]. Collectively, these results suggest that sucrose, sugar and fructose are associated with the pathogenesis of NAFLD. Fructose intake is 2- to 3-fold higher in patients with NAFLD compared with gender, age and body mass index-matched controls, and hepatic mRNA expression of FK and FAS is increased in patients with NAFLD [6]. Fructose is absorbed through glucose transporter 5 (GLUT5) in the jejunum. Excess fructose is taken to the liver and phosphorylated by FK for use in gluconeogenesis and lipid production. However, since lipid production from fructose is not regulated, excessive fructose induces production of a large amount of TG [17,18]. The serum TG level and hepatic mRNA levels of FK and FAS in this study were higher in the H-fruc group compared to those in the H-fat or control groups (Table 2 and Fig. 2A). Thus, our experimental model resembles human NAFLD in these respects.

Excessive intake of sugar and fructose may have more adverse effects than excessive intake of a high fat diet. The mortality of rats with hypertension was greater after intake of a high fructose diet compared to a high fat diet [20], and a high sugar diet has been shown to induce cardiac depression and increase mortality [21]. We have also found that liver regeneration was more impaired in the H-fruc group than in the H-fat group for the first time, although these groups did not differ in total energy intake and increase of body weight. Pioglitazone, a drug that improves insulin resistance, has been shown to improve delayed liver regeneration after hepatectomy in KK-Ay mice, which develop insulin resistance [22]. In our study, insulin resistance in the H-fruc group was higher than that in the H-fat group before PHx (Fig. 1A). Thus, the different rates of liver regeneration in the two groups might have been caused by a difference in insulin resistance.

In the liver, ATP is produced as an energy source by carbohydrate metabolism. ATP is also produced by β-oxidation of fatty acids. Fatty acids are oxidized by mitochondria or peroxisomes and are finally transformed to acetyl-CoA. In β-oxidation, ATP is produced from fatty acid. In this process of fatty acid oxidation, CPT-1 is the rate-controlling enzyme, and nuclear receptor transcription factors such as PPAR-α and AMPK regulate expression of CPT-1. In our study, expression of CPT-1 was decreased in rats fed a high fructose diet, and expression of PPARα and AMPK also decreased. The decreased expression of PPAR-α, AMPK and CPT-1 may reduce ATP production [23,24]. On the other hand, expression of FK in the liver increased in H-fruc rats, and this increases consumption of ATP and leads to ATP depletion in the liver [6,25]. Thus, a high fructose diet induced a decrease in ATP production due to a decrease in fatty acid β-oxidation and an increase in ATP consumption due to increased FK expression. These changes produce an overall decrease in ATP, which is essential for liver regeneration, compared to high-fat diet. Delayed liver regeneration has also been shown in PPAR-α null mice [26] and may involve decreased expression of cyclin D1 and c-Myc, which are G1/S phase regulators, and increased expression of IL-1β. Thus, the greater impairment of liver regeneration in the H-fruc group compared to the H-fat group was due to alteration of gene expression profiles.

Liver regeneration after administration of carbon tetrachloride is impaired via abnormal expression of IL-6 and TNF in leptin-deficient ob/ob mice [27]. Picard et al. examined liver regeneration after PHx in methionine-low, choline-deficient diet-fed rats and Zucker fatty rats, and suggested that abnormal function of the leptin receptor might be related to impaired liver regeneration, rather than fatty liver itself [28]. We did not examine leptin receptor expression in this study, but the serum leptin and adiponectin levels did not differ between the H-fruc and H-fat groups. This suggests that changes in the leptin pathway were

not involved in the different rates of liver regeneration in the two groups.

Serum TGF-β1 levels were slightly but significantly lower on day 1 in control rats and on day 3 in H-fat rats, compared to the respective levels just before PHx. However, serum TGF-β1 in the H-fruc group did not change in liver regeneration from day 0 to day 7 (Fig. 4). TGF-β1 is a potent inhibitor of cell proliferation. In a small-for-size liver graft model, hepatic TGF-β1 increased slightly after transplantation of full-size grafts and then decreased rapidly. TGF-β1 progressively increased after transplantation of quarter-size grafts and hepatocyte proliferation was markedly inhibited [10]. TGF-β1 enhanced by LPS also plays an important role in the mechanism of hepatic failure due to infection after hepatectomy, especially in inhibition of liver regeneration [29]. In contrast, inhibition of TGF-β/Smad signaling improves regeneration of small-for-size rat liver grafts, and a single dose of anti-TGF-β1 monoclonal antibody enhances liver regeneration after partial hepatectomy in biliary-obstructed rats [30]. It is possible that dysregulation of TGF-β expression was one of the causes of delayed liver regeneration in the H-fruc rats. However, there were only small differences in serum TGF-β1 levels in control and H-fat rats after PHx. In addition, we did not examine the TGF-β/Smad signaling pathway in the liver of these rats. Therefore, a further study is needed to elucidate the association of dysregulation of TGF-β expression and delayed liver regeneration in the H-fruc rat.

In conclusion, fatty liver was less severe in the H-fruc group than in the H-fat group, but liver regeneration was more impaired in the H-fruc group. These results suggest that impaired liver regeneration in fatty liver is related to the cause, but not necessarily to the degree, of hepatic steatosis. Dysregulation of genes associated with metabolism or ATP production and potential dysregulation of TGF-β1 expression may contribute to impairment of liver regeneration after PHx in hepatic steatosis induced by a fructose diet.

Acknowledgments

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period

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Abstract
Background Metabolic syndrome, which includes obesity, hyperglycemia, dyslipidemia, and hypertension, is a major risk factor for the development of nonalcoholic fatty liver disease (NAFLD). Cigarette smoking is a well-known risk factor for metabolic syndrome, but the epidemiological impact of cigarette smoking on development of NAFLD is unclear.
Methods In this retrospective study, 2,029 subjects underwent a complete medical health checkup in 1998 and again in 2008. Those who were positive for hepatitis B surface antigen or hepatitis C virus antibody, or had an alcohol intake of >20 g/day as assessed by questionnaire, were excluded. Fatty liver was diagnosed by abdominal ultrasonography. Independent risk factors associated with the development of NAFLD were determined by multiple logistic regression analysis. Smoking status was expressed using the Brinkman index (BI), which was calculated as the number of cigarettes smoked per day multiplied by the number of years of smoking.
Results Of 1,560 subjects without NAFLD in 1998, 266 (17.1%) were newly diagnosed with NAFLD in 2008. Multiple logistic analysis identified age [adjusted odds ratio (AOR) 0.95, 95% confidence interval (95% CI) 0.94–0.97], male sex (AOR 1.46, 95% CI 1.01–2.10), body mass index ≥25 (AOR 3.08, 95% CI 2.20–4.32), dyslipidemia (AOR 1.79, 95% CI 1.25–2.58) and cigarette smoking (AOR 1.91, 95% CI 1.34–2.72) as risk factors associated with the development of NAFLD. Smoking status at baseline was also associated with the development of NAFLD (BI 1–399: AOR 1.77, 95% CI 1.02–3.07, BI ≥400: AOR 2.04, 95% CI 1.37–3.03).
Conclusion Cigarette smoking is an independent risk factor for onset of NAFLD.

Keywords NAFLD · Risk factor · Brinkman index · Body mass index · Metabolic syndrome

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a liver disorder characterized by fatty changes of the liver with no apparent history of habitual alcohol intake. NAFLD was initially considered to be a reversible chronic liver disease with a favorable prognosis. However, some NAFLD patients show evidence of nonalcoholic steatohepatitis (NASH), which may progress to hepatic cirrhosis and hepatocellular carcinoma, with a resultant unfavorable prognosis [1, 2]. There are also racial differences in the prevalence of NAFLD. In Japan, the prevalence is reported to be 9–30% [3]. The prevalence of visceral fat-type obesity is high in Asian populations, and this may lead to insulin resistance and an increased incidence of NAFLD, even though the body mass index (BMI) of Asians is

generally lower than that of African-Americans and Caucasians [4–6]. A high rate of NAFLD also occurs concomitantly with obesity, hyperglycemia, dyslipidemia, and hypertension (collectively referred to as metabolic syndrome) [3, 4, 7–9], but few large-scale long-term studies of the risk factors involved in the development of NAFLD have been reported.

Cigarettes contain more than 4,000 toxic chemicals, including tar, nicotine, and carbon monoxide. Cigarette smoking is a risk factor for the prevalence of and mortality from malignant cancers such as lung and esophageal cancers, lung diseases such as chronic obstructive pulmonary disease (COPD), and circulatory diseases [10–12]. An association of cigarette smoking with risk factors for NAFLD, such as insulin resistance, diabetes, and dyslipidemia, has also been reported [13–18]. However, a large-scale long-term study of the association between cigarette smoking and NAFLD has not been performed. Therefore, in this study, we investigated the factors involved in the development and cure of NAFLD in a follow-up study of a 10-year interval, and examined the association of smoking with the development of NAFLD.

Subjects and methods

Study design

We designed a retrospective follow-up study of a 10-year interval to investigate the effects of cigarette smoking on the development or cure of NAFLD. A total of 3,365 subjects underwent a complete medical health checkup including abdominal ultrasonography at the Kagoshima Kouseiren Medical Healthcare Center in both 1998 and 2008. Subjects positive for hepatitis B virus surface antigen (HBsAg) and hepatitis C virus antibody (HCV Ab) and those who did not undergo virus marker measurements were excluded. Alcohol intake was investigated by questionnaire, and the ethanol equivalent of alcohol consumption per day was calculated from the frequency of alcohol intake per month. Subjects who drank >20 g/day of ethanol were excluded from the study.

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was performed by trained technicians. Fatty liver was diagnosed when hepatorenal echo contrast and liver brightness were observed [19, 20]. The diagnosis of fatty liver was subsequently confirmed by a specialist physician independently without reference to other data.

Although abdominal obesity (abdominal circumference >85 cm for men and >90 cm for women) is a necessary variable according to the Japanese criteria for diagnosing metabolic syndrome [21], waist measurements were not available for all the subjects in this study. In addition, a BMI of ≥25 has been proposed as a cutoff for the diagnosis of obesity in Asian people [19–22]. Therefore, we defined obesity as a BMI ≥25 and included it as one of the metabolic syndrome risk factors in this study. BMI was calculated by dividing body weight (kg) by the square of height (m^2). Patients with hypertension were defined as those with a systolic blood pressure of ≥130 mmHg, those with a diastolic blood pressure of ≥85 mmHg, or those who were undergoing medical treatment for hypertension in 1998. Patients with dyslipidemia were defined as those with triglycerides of ≥150 mg/dL, those with HDL <40 IU/L, or those who were undergoing medical treatment of dyslipidemia in 1998. Patients with dysglycemia, including diabetes mellitus, were defined as those who had a fasting plasma glucose of ≥110 mg/dL or who were under medical treatment for diabetes in 1998. Thus, hypertension, dyslipidemia and dysglycemia were defined as risk factors for metabolic syndrome in this study according to the Japanese criteria for diagnosing this disorder [20, 21].

Cigarette smoking was investigated by questionnaire, and the Brinkman index (BI) was calculated as the number of cigarettes smoked per day multiplied by the number of years that the subjects had smoked. Subjects who stopped smoking before 1998 (former smokers) or started smoking after 1998 (new smokers) were classified as nonsmokers in 1998, and those who stopped smoking after 1998 and before 2008 (new quitters) were classified as smokers in 1998. We performed further subanalysis using two groups (subjects who continued to smoke between 1998 and 2008, and those who did not smoke at all during this time) or three groups (the previous two groups in addition to new quitters). For alcohol consumption per day, the subjects were divided into 2 groups: those who did not drink alcohol (consumption 0 g/day) and light drinkers (mean consumption ≤20 g/day).

The study was approved by the ethics committees of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare and the Kagoshima University Graduate School of Medical and Dental Sciences.

Statistical analysis

All analyses were performed using SPSS v.18 (SPSS, Inc., Chicago, IL, USA), with the significance level set at <5%. Continuous variables are shown as mean ± standard deviations (SD). Between-group comparison was performed by unpaired *t* test and Fisher's exact test. Potential factors involved in the development or cure of NAFLD were analyzed by logistic regression analysis. Unadjusted and adjusted odds ratios (OR) and 95% confidence interval (95% CI) were calculated.

Table 1 Baseline characteristics of subjects enrolled in 1998

Characteristic	NAFLD (n = 469)	Non-NAFLD (n = 1,560)	P value[a]
Mean age, year ± SD	49.2 ± 8.9	51.1 ± 9.3	<0.001
Men, n (%)	342 (72.9)	772 (49.5)	<0.001
BMI ≥25 kg/m^2, n (%)	276 (58.8)	251 (16.1)	<0.001
Hypertension, n (%)[b]	198 (42.2)	422 (27.1)	<0.001
Dyslipidemia, n (%)[b]	202 (43.1)	216 (13.8)	<0.001
Dysglycemia, n (%)[b]	185 (39.4)	322 (20.6)	<0.001
Current smoker, n (%)	130 (27.8)	315 (20.2)	0.001
Light alcohol drinker, n (%)[b]	310 (66.1)	905 (58.0)	<0.01

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

[a] Calculated by Fisher's exact test for categorical variables or unpaired t test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

Results

Subjects' baseline characteristics in 1998

Of the initial 3,365 subjects, 76 were positive for HBsAg, 90 were positive for HCV Ab, and 2 were positive for both. Four hundred thirty-nine subjects were not tested for HBsAg or HCV Ab. In addition, 729 subjects drank >20 g/day of ethanol. On the basis of these data, 2,029 subjects were eligible for the study.

In 1998, 469 subjects (342 men and 127 women) and 1,560 subjects (772 men and 788 women) were included in the NAFLD and non-NAFLD groups, respectively. There was a significantly higher number of men in the NAFLD group, and the mean age in the NAFLD group was significantly lower than that in the non-NAFLD group (Table 1). The frequencies of obesity (BMI ≥25), hypertension, dyslipidemia, dysglycemia including diabetes mellitus, current cigarette smoking, and light alcohol drinkers were significantly higher in the NAFLD group compared to the non-NAFLD group (Table 1).

Comparison of subjects who developed NAFLD with non-NAFLD subjects

Two hundred sixty-six (17.1%) patients from the non-NAFLD group in 1998 were newly diagnosed with NAFLD in 2008 (164 men 21.2%, 102 women 12.9%) (Fig. 1). The baseline characteristics in 1998 were compared between the new-NAFLD and non-NAFLD groups. Age, frequency of male gender, obesity, dyslipidemia, and cigarette smoking differed significantly between the two groups (Table 2). These factors also had an independent association with NAFLD development (all subjects in Table 3), indicating that smokers were likely to develop NAFLD.

Furthermore, in a limited group of subjects that excluded former smokers (before 1998), new smokers after 1998 and those who quit between 1998 and 2008 (new quitters), cigarette smoking tended to be a risk factor for NAFLD development [adjusted odds ratio (AOR) 1.44, 95% CI 0.86–2.42 among the limited group of subjects in Table 3].

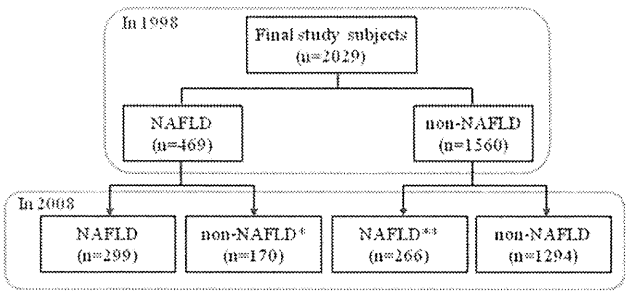

Fig. 1 Study flow diagram. A total of 2,029 subjects were enrolled in the study. * One hundred seventy subjects had apparent nonalcoholic fatty liver disease (NAFLD) in 1998, but not in 2008. ** Two hundred sixty-six subjects were newly diagnosed with NAFLD in 2008

Association between cigarette smoking and the development of NAFLD

The association between smoking patterns and NAFLD was analyzed by classifying the subjects into 3 groups: BI = 0 (non-smokers), BI = 1–399, and BI ≥400. Of the 1,553 subjects in the non-NAFLD group in 1998 (excluding 7 subjects whose BI was not calculated because of lack of data), the risk of developing NAFLD correlated positively with BI in multivariate analysis adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia, and alcohol intake (Table 4).

Association of metabolic syndrome risk factors and cigarette smoking with NAFLD development

The association of obesity, hypertension, dyslipidemia, and dysglycemia (four metabolic syndrome risk factors) with the incidence of NAFLD was analyzed. As shown in Fig. 2, the incidence of NAFLD was 13.2% in subjects with no metabolic syndrome risk factors, and 19.0, 22.5, and 24.7% in those with 1, 2 and ≥3 factors, respectively. The risk of NAFLD was significantly correlated with number of metabolic syndrome risk factors (Table 5).

Table 2 Comparison of baseline characteristics in subjects with development or regression of nonalcoholic fatty liver disease

Characteristic	Non-NAFLD at baseline and NAFLD at follow-up (n = 266)	Non-NAFLD at baseline and follow-up (n = 1,294)	P value[a]	NAFLD at baseline and non-NAFLD at follow-up (n = 170)	NAFLD at baseline and follow-up (n = 299)	P value[a]
Mean age, year ± SD	48.0 ± 8.0	51.8 ± 9.4	<0.001	51.6 ± 8.5	47.8 ± 8.8	<0.001
Men, n (%)	164 (61.7)	608 (47.0)	<0.001	116 (68.2)	226 (75.6)	0.10
BMI ≥25 kg/m^2, n (%)	79 (29.7)	172 (13.3)	<0.001	92 (54.1)	184 (61.5)	0.12
Hypertension, n (%)[b]	65 (24.4)	357 (27.6)	0.32	81 (47.6)	117 (39.1)	0.08
Dyslipidemia, n (%)[b]	61 (22.9)	155 (12.0)	<0.001	64 (37.6)	136 (45.5)	0.12
Dysglycemia, n (%)[b]	57 (21.4)	265 (20.5)	0.74	72 (42.4)	113 (37.8)	0.38
Current smoker, n (%)	94 (35.3)	221 (17.1)	<0.001	38 (22.4)	92 (30.8)	0.05
Light alcohol drinker, n (%)[b]	162 (60.9)	743 (57.4)	0.31	110 (64.7)	200 (66.9)	0.68

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

[a] Calculated by Fisher's exact test for categorical variables or an unpaired t test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

Table 3 Risk factors for the development of nonalcoholic fatty liver disease

Variable	All subjects (n = 1,560)				Limited group of subjects (n = 1,174)[a]			
	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]
Age	0.95	0.94–0.97	0.95	0.94–0.97	0.94	0.92–0.96	0.93	0.91–0.95
Male sex	1.81	1.38–2.38	1.46	1.01–2.10	1.62	1.18–2.24	1.28	0.79–2.08
Obesity[c]	2.76	2.03–3.75	3.08	2.20–4.32	3.42	2.38–4.91	4.03	2.69–6.06
Hypertension[c]	0.85	0.63–1.15	0.90	0.64–1.27	0.87	0.60–1.27	0.87	0.56–1.36
Dyslipidemia[c]	2.19	1.57–3.05	1.79	1.25–2.58	2.76	1.84–4.13	2.67	1.70–4.21
Dysglycemia[c]	1.06	0.77–1.46	1.04	0.73–1.48	1.28	0.87–1.89	1.41	0.92–2.16
Cigarette smoking	2.65	1.98–3.54	1.91	1.34–2.72	2.29	1.57–3.35	1.44	0.86–2.42
Light alcohol intake[c]	1.16	0.88–1.51	0.75	0.54–1.04	1.03	0.75–1.41	0.61	0.41–0.92

CI Confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

[c] Definitions are provided in "Subjects and methods"

Table 4 Incidence of nonalcoholic fatty liver disease based on smoking status in 1998

Brinkman index[a]	Newly diagnosed NAFLD (n = 265), n (%)[b]	Non-NAFLD (n = 1,288) n (%)[b]	P value[c]	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)[d]
0	172 (64.9)	1,072 (83.2)		1.0	1.0
1–399	24 (9.1)	57 (4.4)	<0.001	2.62 (1.59–4.34)	1.77 (1.02–3.07)
≥400	69 (26.0)	159 (12.3)		2.70 (1.95–3.74)	2.04 (1.37–3.03)

NAFLD nonalcoholic fatty liver disease, *CI* confidence interval

[a] Calculated as the number of cigarettes smoked per day multiplied by the number of years that the subject smoked

[b] Seven subjects whose Brinkman indices were not calculated because of a lack of data were excluded from this analysis

[c] Calculated by Fisher's exact test for categorical variables

[d] Adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia and alcohol intake

Cigarette smoking at baseline was also found to be an independent risk factor for NAFLD development in this model (all subjects in Table 5).

The incidence of NAFLD increased as the number of metabolic syndrome risk factors increased in nonsmokers (Fig. 2). In contrast, the incidence in smokers with one or more metabolic syndrome risk factors (≥35%) was higher than in those with none, but did not differ among those with 1, 2, or ≥3. The incidence of NAFLD was significantly higher in smokers than in nonsmokers, regardless of the number of metabolic syndrome risk factors.

Furthermore, in the limited group of subjects including continuing smokers and nonsmokers only, cigarette smoking at baseline was a risk factor for NAFLD development, although it did not achieve statistical significance (<0.05) in multivariate analysis (AOR 1.64, 95% CI 0.99–2.72 among the limited group of subjects in Table 5).

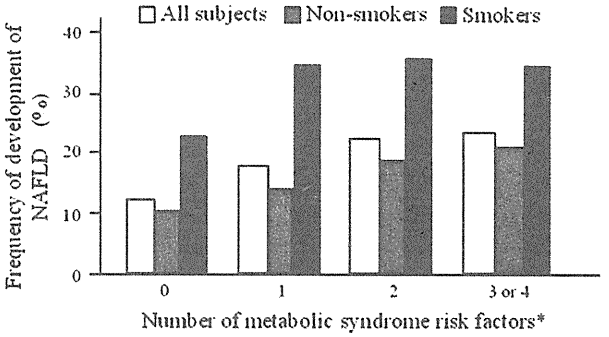

Fig. 2 Frequency of the development of nonalcoholic fatty liver disease (NAFLD) for subjects with different numbers of metabolic syndrome risk factors in smokers and nonsmokers. The incidence of NAFLD was higher in smokers than in nonsmokers. The incidence of NAFLD in smokers with one or more metabolic syndrome risk factor was higher than in those with none, but did not differ among those with 1, 2, or ≥3 factors. * Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

Comparison of subjects in whom NAFLD was cured with those with persistent NAFLD

Of the 469 subjects in the NAFLD group in 1998, NAFLD was cured in 170 (36.2%) in 2008 (116 men 33.9%, 54 women 42.5%) (Fig. 1). A comparison of baseline characteristics in 1998 between the NAFLD-cured and NAFLD-persistent groups showed a significant difference in age, but not in sex, obesity, hypertension, dyslipidemia, dysglycemia, cigarette smoking, or light alcohol intake (Table 2). In multivariate analysis using these factors, only age had an independent association with cure of NAFLD. The frequency of NAFLD cure was 31.7% in subjects with no metabolic syndrome risk factors, compared with 36.1, 42.7, and 31.4% in those with 1, 2 and ≥3 factors, respectively, showing no association between NAFLD cure and the number of metabolic syndrome risk factors ($P = 0.21$).

Table 5 Association of the development of nonalcoholic fatty liver disease and the number of metabolic syndrome risk factors

Variables	Newly diagnosed NAFLD vs. non-NAFLD							
	All subjects (n = 1,560)				Limited group of subjects (n = 1,174)[a]			
	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]
Age	0.95	0.94–0.97	0.95	0.93–0.96	0.94	0.92–0.96	0.92	0.90–0.94
Male sex	1.81	1.38–2.38	1.28	0.90–1.83	1.62	1.18–2.24	1.17	0.73–1.86
MS risk factors								
0	1.0		1.0		1.0		1.0	
1	1.55	1.14–2.11	1.86	1.34–2.58	1.93	1.32–2.82	2.47	1.65–3.70
2	1.92	1.33–2.77	2.63	1.77–3.92	2.70	1.73–4.21	4.65	2.83–7.64
3 or 4	2.16	1.22–3.83	2.99	1.62–5.50	3.15	1.55–6.41	5.20	2.41–11.22
Cigarette smoking	2.65	1.98–3.54	2.11	1.49–3.00	2.29	1.57–3.35	1.64	0.99–2.72
Light alcohol intake	1.16	0.88–1.51	0.71	0.51–0.97	1.03	0.74–1.41	0.55	0.37–0.81

Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

MS metabolic syndrome, *NAFLD* nonalcoholic fatty liver disease, *CI* confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

Table 6 The frequency of NAFLD in three groups categorized by smoking status

	Continuing nonsmokers (n = 1,237)[a]	Cigarette smoker in 1998		P value
		New quitters (n = 238)[b]	Continuing smokers (n = 263)	
In 1998, n (%)	246 (19.9)	61 (25.6)	80 (30.4)	<0.001[c]
In 2008, n (%)	275 (22.2)	91 (38.2)	106 (40.3)	<0.001[c]
1998–2008, n (%)				
NAFLD–NAFLD	145 (11.7)	37 (15.5)	59 (22.4)	
NAFLD–non NAFLD	101 (8.2)	24 (10.1)	21 (8.0)	<0.001[d]
Non NAFLD–NAFLD	130 (10.5)	54 (22.7)	47 (17.9)	[0.16][e]
Non NAFLD–non NAFLD	861 (69.6)	123 (51.7)	136 (51.7)	

NAFLD nonalcoholic fatty liver disease

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Calculated by Fisher's exact test for categorical variables

[d] Calculated by Pearson's chi-square test for categorical variables

[e] Calculated among new quitters and continuing smokers by Pearson's chi-square test for categorical variables

Table 7 Development of NAFLD in the three groups categorized

	Newly diagnosed NAFLD vs. non-NAFLD		
	Continuing nonsmokers[a] Odds ratio	New quitters[b] Odds ratio (95% CI)	Continuing smokers Odds ratio (95% CI)
Unadjusted	1.0	2.91 (2.01–4.21)	2.29 (1.57–3.35)
Model 1[c]	1.0	2.96 (2.00–4.38)	1.61 (1.07–2.43)
Model 2[d]	1.0	2.77 (1.75–4.40)	1.50 (0.92–2.44)
Model 3[e]	1.0	2.73 (1.71–4.36)	1.47 (0.90–2.42)
Model 4[f]	1.0	1.94 (1.30–2.90)	1.91 (1.28–2.84)

NAFLD nonalcoholic fatty liver disease, *CI* confidence interval

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Adjusted for age, obesity, and dyslipidemia

[d] Adjusted for all factors in model 1 plus sex

[e] Adjusted for all factors in model 2 plus hypertension, dysglycemia and light alcohol intake

[f] Adjusted for increase of body mass index (BMI) during the 10-year period

Influence of smoking cessation on NAFLD status or NAFLD development

The association between smoking cessation and risk of NAFLD was analyzed using subjects who never smoked, those who smoked consistently from 1998 to 2008, and new quitters who were smokers in 1998 but had stopped by 2008. The frequency of NAFLD cure in new quitters was similar (10.1%) to those in continuing smokers (8.0%) and nonsmokers (8.2%, Table 6). In contrast, the frequency of the development of NAFLD in new quitters (22.7%) was higher than that in nonsmokers (10.5%) and was similar to that in continuing smokers (17.9%). Furthermore, after adjusting for age, obesity, dyslipidemia, sex, hypertension, dysglycemia and alcohol intake, compared with nonsmokers, the odds ratios of the development of NAFLD among new quitters and continuing smokers were 2.73 (95% CI 1.71–4.36) and 1.47 (95% CI 0.90–2.42), respectively (Table 7). In addition, after adjusting for an increase in BMI from 1998 to 2008 (Table 7, model 4), the odds ratio in new quitters decreased more compared to that in continuing smokers (2.91–1.94 vs. 2.29–1.91).

Discussion

During the 10-year period of the study, 17.1% of the subjects developed NAFLD. Cigarette smoking was an

independent risk factor for NAFLD, in addition to age, obesity, dyslipidemia, and the total number of metabolic syndrome risk factors. The Brinkman index (a smoking index) was also associated with NAFLD development. Metabolic syndrome risk factors are known to be related to NAFLD, but this is the first follow-up study over a 10-year period to show that cigarette smoking is an independent risk factor for NAFLD development, as well as for metabolic syndrome risk factors. However, in multivariate analysis, the association between cigarette smoking and NAFLD development did not reach statistical significance in the limited group of subjects, which may be due in part to the modest sample size. In addition, subanalysis using subjects who quit smoking demonstrated that smoking cessation seems to be a risk for NAFLD development, a result which can be partially explained by an increase in BMI.

Cigarette smoking had been previously associated with chronic liver diseases such as chronic hepatitis C and B, primary biliary cirrhosis, and alcoholic liver diseases [23–25], but the association between NAFLD and cigarette smoking had not been fully elucidated. Suzuki et al. [26] reported that initiation of cigarette smoking in patients with NAFLD was associated with ALT elevation in a 1-year follow-up survey, but the association between the development of NAFLD and cigarette smoking was not fully investigated. Chavez-Tapia et al. [27] showed that smoking was not associated with NAFLD in univariate regression analysis, but found that the risk of NAFLD tended to increase in subjects who smoked $\geq$10 (OR = 1.16 [95% CI 0.76–1.64]) and $\geq$20 (OR = 1.54 [95% CI 0.94–2.52]) packs per year compared to nonsmokers. These results may depend on the number of subjects and the duration of the study, and require confirmation in larger long-term longitudinal studies.

Increases in BMI partially explained the excess risk of NAFLD development in smokers in 1998, especially in new quitters (Table 7). Other mechanisms are also speculated, for instance smoking-induced fatty changes and fibrosis in the liver [28–40]. H_2O_2 and nicotine produced by smoking reduce adiponectin expression [30, 31]. Smoking also promotes the production of activated NADPH oxidase-induced reactive oxygen species, which enhances oxidative stress and lipid peroxidation due to impaired antioxidative action [32–34]. Yuan et al. [35] reported that cigarette smoking inactivated 5′-adenosine monophosphate-activated protein kinase (AMPK) by dephosphorylation and promoted triglyceride accumulation in hepatocytes via activation of sterol regulatory element-binding protein-1 (SREBP-1), inducing fatty liver in mice fed a high-fat diet. In heavy smokers, tissue becomes hypoxic due to elevation of carbon monoxide and hemoglobin levels and impairment of oxygen transport by red blood cells, which induces erythropoietin production and promotes iron absorption in the intestines [36]. Excess iron is thought to be deposited in the liver and to eventually induce hepatocellular damage and fibrosis [36]. In addition, oxidative stress produces necrotizing inflammation in fatty liver [37]. In obese rats, cigarette smoking elevated ALT and caused hepatocellular ballooning and lobular inflammation [38]. Smoking also promotes the production of inflammatory cytokines and hepatic fibrosis-associated molecules [38–40]. Further investigation of the mechanism whereby smoking influences development or progression of NAFLD in humans is required.

There is a sex difference in the incidence of NAFLD, with men being more likely to develop fatty liver compared to women [7, 41, 42]. A similar result was obtained in this study. The prevalence of fatty liver has been shown to be about 25% in men in their 30s–60s, while it gradually increases with age in women and reaches a similar prevalence after 60 years of age [7]. Sex hormones are involved in this change, and postmenopausal reduction of estrogen levels is thought to promote visceral fat accumulation and induce insulin resistance [43]. The involvement of smoking in increasing testosterone levels has been proposed [44], suggesting that sex hormones are involved in cigarette smoking-induced NAFLD [45]. Therefore, smoking and changes in sex hormones may both be related to the development or progression of NAFLD.

Many cross-sectional studies have shown that metabolic syndrome risk factors are associated with NAFLD [3, 4, 7–9], but causal relationships cannot be proven by cross-sectional studies alone. Associations of changes in body weight and metabolic syndrome with the development and cure of NAFLD have been demonstrated in longitudinal studies [19, 26, 46, 47]. Hamaguchi et al. [19] followed 4,401 healthy subjects for an average period of 414 days and found that new NAFLD developed in 308 subjects (10% of the non-NAFLD subjects). The presence of metabolic syndrome was most strongly associated with newly developed NAFLD, and body weight gain was also an independent risk factor. NAFLD was cured in 113 subjects (16% of the NAFLD patients) during the observation period, with body weight loss being the most important factor, indicating that weight loss is more important than the absence of metabolic syndrome. In our study, the incidence of NAFLD development after 10 years was investigated based on the number of metabolic syndrome risk factors in 1998, and was found to increase as the number of risk factors increased (Fig. 2; Table 5). In addition, the number of metabolic syndrome risk factors in 1998 was not associated with cure of NAFLD after 10 years. Body weight loss during the 10-year period was an independent factor contributing to NAFLD cure (data not shown), similar to the findings of Hamaguchi et al. [19]. In contrast, in

Hamaguchi et al., the Brinkman index did not differ between the NAFLD and non-NAFLD groups at baseline, which is inconsistent with our results. This may be because the mean period of 414 days in Hamaguchi et al. was not long enough to investigate the influence of smoking. In addition, the results of our analysis are very important because a significant association between cigarette smoking and the development of NAFLD was present in a population in which a strong association between the development of NAFLD and metabolic syndrome risk factors was evident.

Risk factor modification such as weight loss and medication for insulin resistance and dyslipidemia should cure or prevent NAFLD. In this study, we found that cigarette smoking is a risk factor for NAFLD. Therefore, smoking cessation is likely to decrease the risk of NAFLD among current smokers. However, in our subanalysis of three groups that included new quitters, and continuing smokers and nonsmokers, smoking cessation seemed to convey a higher risk for developing NAFLD than continuing to smoke (Table 7). It has been reported that smoking is a risk factor for diabetes mellitus, but smoking cessation is also associated with substantial weight gain and may lead to a higher short-term risk of type 2 diabetes [17, 29]. Despite the fact that we were unable to clarify the effect of smoking cessation on the treatment or development of NAFLD because (1) our study population was too small, (2) the date when smoking was stopped was not considered, and (3) metabolic syndrome risk factors that arose or were cured during the 10-year period and the effects of treatment on these diseases were not fully considered, we speculate that smoking cessation without weight gain is likely to be beneficial for patients with NAFLD.

Subjects who drank ≤20 g/day of alcohol were included in our study. In a cross-sectional study in Japanese men, low alcohol consumption (40–140 g/week) significantly reduced the incidence of fatty liver (AOR = 0.824 [95% CI 0.683–0.994]) [20]. We have also found that alcohol intake may inhibit the development of fatty liver through an association of alcohol drinking pattern with obesity [48]. In the current study, intake of a small amount of alcohol had an inhibitory effect on NAFLD development in multivariate analysis that included the number of metabolic syndrome risk factors as a variable (Table 5). In animal models, cigarette smoking and alcohol intake have been shown to contribute to the development and exacerbation of fatty liver [49]. Thus, further studies of the apparent synergistic effect of alcohol intake and cigarette smoking on NAFLD are required in humans.

There are several limitations in this study. Firstly, ultrasonography is effective for diagnosing fatty liver, but detecting fatty liver in patients with ≤30% liver fat or in obese patients is relatively difficult [4]. Moreover, as simple fatty liver and NASH cannot be distinguished by ultrasonography, an association of cigarette smoking with fatty liver severity could not be shown. Secondly, although the frequency of NAFLD development in continuing smokers was higher than that in continuing nonsmokers in the limited group of subjects, the difference was not statistically significant in multivariate analysis (Table 5). This may be due to the modest sample size. Finally, our study was a follow-up study of a 10-year interval in which the data was obtained at only two points, in 1998 and in 2008. Longitudinal studies such as those involving the evaluation of NAFLD incidence and smoking patterns over time using several points during a 10-year period may provide more convincing evidence of the contribution of cigarette smoking to NAFLD development.

In conclusion, metabolic syndrome risk factors increase the risk of NAFLD. In this retrospective study, we found that cigarette smoking, a risk factor for metabolic syndrome, was also a risk factor for NAFLD development independent of metabolic syndrome risk factors. In addition, although NAFLD development became more likely as the Brinkman index increased, smoking cessation was also a likely risk factor for NAFLD development, which is partially explainable by an increase in BMI. Therefore, having never smoked is important for the prevention of NAFLD in nonsmokers, and additional treatment or prevention of metabolic syndrome risk factors may be necessary to encourage the cessation of cigarette smoking to treat or prevent NAFLD in smokers.

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