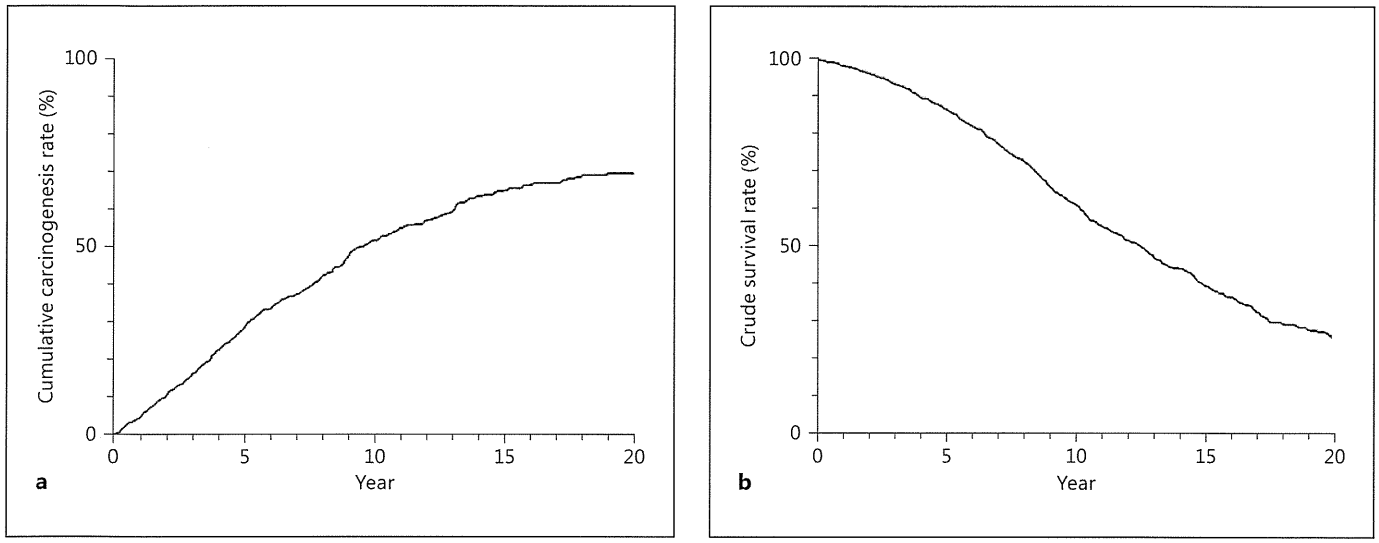

Fig. 3. HCV-positive chronic hepatitis patients with cirrhosis were retrospectively analyzed for hepatocellular carcinogenesis and mortality. **a** Cumulative hepatocellular carcinogenesis rate in the entire group of patients with cirrhosis. **b** Crude survival rate in the entire group of patients with cirrhosis.

Table 2. One-year state-transition probability matrix of the entire study group (n = 10,501 person years)

	Cirrhosis	HCC	Death
Liver cirrhosis (n = 8,273)	7,554 (91.3)	562 (6.8)	157 (1.9)
HCC (n = 2,228)		1,805 (81.0)	423 (19.0)

Figures in parentheses are percentages.

received continuous interferon therapy [5.5% (39/714) and 0.7% (5/714)] than in patients with NR or no interferon therapy [7.2% (542/7,494) and 2.0% (151/7,494); $\chi^2 = 7.59$, $p = 0.0059$].

Probabilities for Transition among the Remaining Patients with High ALT

Among 376 patients without SVR/BR effect and continuous interferon injection and with a high ALT value of 75 IU/l or more, 264 patients (70.2%) received glycyrrhizin injection as anti-inflammatory therapy. Among 692 patients without SVR/BR effect and continuous interferon injection and with relatively low ALT of less than 75 IU/l, glycyrrhizin injection was performed only in 253 patients (36.6%).

We evaluated the transition probabilities among the three states in the remaining patients with high ALT levels of 75 IU/l or more. In the matrix of patients without glycyrrhizin injection therapy, the transition probability from liver cirrhosis to HCC was 6.8% (85/1,245), and the probability of transitioning from cirrhosis to death was 2.0% (25/1,245). In the patients who received glycyrrhizin injection therapy, the transition probability from liver cirrhosis to HCC was 5.9% (45/764), and the probability of transitioning from cirrhosis to death was 0.8% (6/764). Glycyrrhizin injection therapy slightly improved the transition probability both from liver cirrhosis to HCC and from liver cirrhosis to death, but statistical significance was not observed ($\chi^2 = 5.5$, $p = 0.06$; table 4).

Disease Control Rates (Annual Non-Progression Probability) of Anti-Viral and Anti-Inflammatory Treatment

The disease control rates depended on the probabilities for transition between progression and non-progression of disease at a specific time interval, which was set at 1 year. The yearly transition probabilities were calculated based on the data of 10,501 person years of the 1,280 study patients with HCV-positive liver cirrhosis.

The disease control rate of the patients with SVR or BR (874/910, 96.0%) was significantly higher than that of the

Table 3. One-year state-transition probability matrices according to initial treatment

	Cirrhosis	HCC	Death
Patients with SVR or BR (n = 910 person years)			
Liver cirrhosis (n = 778)	753 (96.8)	20 (2.6)	5 (0.6)
HCC (n = 132)		121 (91.7)	11 (8.3)
Patients with no response or no interferon therapy (n = 9,590 person years)			
Liver cirrhosis (n = 7,494)	6,801 (90.8)	542 (7.2)	151 (2.0)
HCC (n = 2,096)		1,684 (80.3)	412 (19.7)
Patients with continuous interferon therapy (n = 856 person years)			
Liver cirrhosis (n = 714)	670 (93.8)	39 (5.5)	5 (0.7)
HCC (n = 142)		132 (93.0)	10 (7.0)

Figures in parentheses are percentages.

Table 4. One-year state-transition probability matrices according to glycyrrhizin injection therapy for patients with high ALT values

	Cirrhosis	HCC	Death
Patients without glycyrrhizin therapy (n = 1,637 person years)			
Liver cirrhosis (n = 1,245)	1,135 (91.2)	85 (6.8)	25 (2.0)
HCC (n = 392)		305 (77.8)	87 (22.2)
Patients with glycyrrhizin therapy (n = 913 person years)			
Liver cirrhosis (n = 764)	713 (93.3)	45 (5.9)	6 (0.8)
HCC (n = 149)		130 (87.2)	19 (12.8)

Figures in parentheses are percentages.

Table 5. One-year non-progression probability matrix of anti-viral and anti-inflammatory treatment

	Non-progression	Progression
Entire study group (n = 10,501)	9,359 (89.1)	1,142 (10.9)
Patients with SVR or BR (n = 910)	874 (96.0)	36 (4.0)
Patients with NR or no interferon therapy (n = 9,590)	8,485 (88.5)	1,105 (11.5)
Patients without glycyrrhizin therapy (n = 1,637)	1,440 (88.0)	197 (12.0)
Patients with glycyrrhizin therapy (n = 913)	843 (92.3)	70 (7.7)

Figures in parentheses are percentages.

patients with NR or the patients without interferon therapy (8485/9590, 88.5%; $\chi^2 = 49.1$, $p < 0.0001$).

We also evaluated disease control rates according to glycyrrhizin injection therapy in the subgroups of patients who either failed or did not receive interferon therapy with a high ALT of 75 IU/l or more. Anti-inflammation therapy with glycyrrhizin injections significantly increased the disease control rates, as shown by the rate of 92.3% (843/913) in the patients who received glycyrrhizin injection therapy versus 88.0% (1,440/1,637) in the patients without glycyrrhizin therapy ($\chi^2 = 11.9$, $p < 0.0001$; table 5).

Discussion

Based on our epidemiological data obtained from long-term observations of patients with chronic hepatitis [34] and patients with cirrhosis [35], we found that the life expectancy of patients with HCV-related liver cirrhosis heavily depends on the development of HCC. The probability of patients with HCV-related liver cirrhosis eventually developing HCC is staggeringly high at 75% [35]. In the present study, interferon administration significantly decreased the probability for transition from liver cirrhosis to HCC in the patients who achieved SVR or BR. However, there were some background varieties between the patients with SVR or BR and NR or no interferon therapy with respect to stage of fibrosis, sex, platelet count and age, which can affect the carcinogenesis rate.

From the standpoint of anti-inflammatory effects and cancer prevention [8–10, 13, 14, 19], interferon is effective in patients with chronic liver disease caused by HCV. Although the carcinogenesis rate is noticeably reduced when the ALT level becomes normal with or without HCV RNA eradication [10, 13, 14] after therapy, ALT levels become normal after interferon therapy in approximately half of the patients with a high viral load and group 1 HCV subtype. Furthermore, the anti-carcinogenic capacity of interferon has been demonstrated not only in patients with persistent ALT normalization, but also in patients with transient normalization of ALT for at least 6 or 12 months [20].

Many authors have already described that the activity of interferon in suppressing the development of HCC in patients with HCV RNA clearance (SVR) is similar to that in patients with ALT normalization in the absence of elimination of HCV RNA (BR) [13, 36–38]. Based on these compelling lines of evidence, the anti-carcinogenic activity of interferon is ascribed to the suppression of in-

flammatory and regenerative processes in hepatocytes. Moreno and Muriel [39] reported that interferon reverses liver fibrosis and, therefore, control of the necro-inflammatory process can suppress the growth of HCC.

An SVR improves clinical symptoms in decompensated cirrhosis [40], but interferon often induces severe complications, even in young patients with decompensated cirrhosis [41]. A patient with compensated cirrhosis can be a candidate for interferon therapy if careful, close hematologic monitoring is performed.

Because patients with liver cirrhosis generally experience some difficulties with interferon treatment, our present study demonstrated practical information about carcinogenesis and the life expectancy of patients with HCV-related liver cirrhosis and the order of priority in the management of interferon for these patients. Interferon administration is considered and initiated in patients with HCV-related liver cirrhosis preferably to reduce the probability for the transition from liver cirrhosis to HCC.

Because carcinogenesis is not a single-step event, but rather a complex, multi-step process, the exact mechanism of the role of glycyrrhizin in suppressing liver carcinogenesis remains unknown. One of the principal functions of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate is considered to be anti-inflammation, which blocks the active carcinogenic process of continuous hepatic necro-inflammation and cell damage. In the treated group of the present study, the median ALT values markedly decreased after initiation of the glycyrrhizin injections, suggesting that the pathological process of hepatocyte necrosis or apoptosis was significantly suppressed by glycyrrhizinic acid. The actions of the amino acids, glycine and cysteine contained in SNMC have not been completely explained, but these substances have been demonstrated to suppress increased aldosterone levels that are induced by glycyrrhizinic acid. Tarao et al. [42] reported that a high ALT level resulted in an increased HCC recurrence rate in patients with HCC. From the standpoint of these anti-inflammatory activities, SNMC may be considered to only postpone the time of HCC appearance in the clinical course of cirrhosis. Since the entire process of hepatocellular carcinogenesis from the initial transformation of a hepatocyte to a detectable growth of cancer is considered to take at least several years, the influence of glycyrrhizin on the carcinogenesis rate cannot be evaluated over a short period.

Because the data in the present study were obtained from a retrospective cohort analysis, glycyrrhizin doses, times of injection per week and duration of therapy varied in each patient in the treated group. In order to elucidate the cancer preventive effect of glycyrrhizin therapy in active HCV-related liver disease, we should further stratify the treated patients or perform much more detailed statistical procedures. Future studies should aim at defining the basic oncogenic mechanisms and roles of long-term administration of glycyrrhizin in carcinogenesis in chronic hepatitis patients with cirrhosis caused by HCV.

In conclusion, the results of the present study demonstrated that long-term intermittent glycyrrhizin (SNMC) therapy for a few years or more successfully reduced disease progression probability (progression to carcinogenesis plus progression to death) in patients with HCV-related cirrhosis. A randomized controlled trial with a larger number of cases, with or without glycyrrhizin therapy, is expected to confirm the effectiveness of this therapy.

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Exendin-4, a glucagon-like peptide-1 receptor agonist, modulates hepatic fatty acid composition and Δ-5-desaturase index in a murine model of non-alcoholic steatohepatitis

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Abstract. Glucagon-like peptide-1 (GLP-1) is involved in the development of non-alcoholic steatohepatitis (NASH), which is characterized by fatty acid imbalance. The aim of this study was to investigate the effects of the GLP-1 receptor (GLP-1R) agonist, exendin-4 (Ex-4), on hepatic fatty acid metabolism and its key enzyme, Δ-5-desaturase, in a murine model of NASH. NASH was induced in db/db mice fed a methionine-choline deficient (MCD) diet. Ex-4 (n=4) or saline [control (CON); n=4] was administered intraperitoneally for 8 weeks. Steatohepatitis activity was evaluated by non-alcoholic fatty liver disease (NAFLD) activity score. Hepatic fatty acid composition and Δ-5-desaturase index were analyzed by gas chromatography. Ex-4 treatment significantly reduced body weight and the NAFLD activity score. Hepatic concentrations of long-chain saturated fatty acids (SFAs) were significantly higher in the Ex-4 group compared to the CON group (23240±955 vs. 31710±8436 μg/g•liver, P<0.05). Ex-4 significantly reduced hepatic n-3 polyunsaturated fatty acid (PUFA)/n-6 PUFA ratio compared to the CON group (13.83±3.15 vs. 8.73±1.95, P<0.05). In addition, the hepatic Δ-5-desaturase index was significantly reduced in the Ex-4 group compared to the CON group (31.1±12.4 vs. 10.5±3.1, P<0.05). In conclusion, the results showed that Ex-4 improved steatohepatitis in a murine model of NASH. Furthermore, Ex-4 altered hepatic long-chain saturated and PUFA composition and reduced the Δ-5-desaturase index. Thus, Ex-4 may improve NASH by regulating hepatic fatty acid metabolism.

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Abbreviations: GLP-1, glucagon-like peptide-1; NASH, non-alcoholic steatohepatitis; Ex-4, exendin-4; GLP-1R, GLP-1 receptor; NAFLD, non-alcoholic fatty liver disease; PUFA, polyunsaturated fatty acid; MCD, methionine-choline deficient; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid

Key words: incretin, exenatide, steatosis, fatty acid composition, fatty acid desaturase

Introduction

The incidence of non-alcoholic steatohepatitis (NASH) is rapidly increasing worldwide (1-4). NASH can be caused by various pathogenic mechanisms, including overeating, physical inactivity, diabetes mellitus, and medications (5,6). The gut directly links to the liver through the portal vein and is involved in the development of NASH (7,8). The gut secretes various hormones in the portal vein and regulates hepatic metabolism (9-11). Glucagon-like peptide-1 (GLP-1) is a gut hormone and is known to affect lipid metabolism in hepatocytes (9,11).

Exendin-4 (Ex-4) is a long-acting GLP-1 receptor (GLP-1R) agonist. GLP-1R occurs in the pancreatic islets, kidney, lung, heart, stomach, intestine, thyroid gland, and numerous regions of the peripheral and central nervous system (12-14). GLP-1R also occurs in hepatocytes, and treatment with Ex-4 substantially reduces triglyceride stores in hepatoma cells (15). Similarly, GLP-1R agonist reduces steatosis severity in certain animal models of NASH (16-19). Findings of previous studies have also shown that reduced hepatic accumulation of triglycerides is mediated by GLP-1R agonist upregulation of hepatic 3-phosphoinositide-dependent kinase-1 activity, protein kinase C ζ activity, peroxisome proliferator-activated receptor α activity, and fatty acid β-oxidation (15-19).

Fatty acids are an important triglyceride component. Fatty acids are a substrate of β-oxidation and yield large quantities of adenosine 5'-triphosphate (20). In addition, some polyunsaturated fatty acids (PUFAs) are a source of eicosanoids, which are biologically active substances. n-3 PUFAs are precursors of anti-inflammatory eicosanoids, including leukotriene B5, prostaglandin E3, and thromboxane B3 (21). On the other hand, n-6 PUFA are precursors of pro-inflammatory eicosanoids, including leukotriene B4, prostaglandin E2, and thromboxane B2 (21). A reduced n-3/n-6 PUFA ratio is a risk factor for chronic inflammatory diseases such as cardiovascular disease, inflammatory bowel disease, rheumatoid arthritis, and

NASH (22-24). Thus, besides quantitative abnormality in fatty acids, qualitative abnormality in fatty acids is an important pathogenesis of NASH.

The production of pro- and anti-inflammatory eicosanoids is regulated by desaturases, which are rate-limiting enzymes of n-3 and n-6 PUFA cascades (25). Δ-5-desaturase, also known as fatty acid desaturase 1, removes two hydrogen atoms from dihomo γ-linolenic acid and synthesizes arachidonic acid. Upregulation of Δ-5-desaturase activity promotes the production of pro-inflammatory eicosanoids (26). Notably, single-nucleotide polymorphisms in the Δ-5-desaturase gene are associated with circulating high sensitivity C-reactive protein levels in healthy young adults (27). Moreover, Δ-5-desaturase activity is associated with aging (28), development of type 2 diabetes mellitus (29), and NASH (30). However, the effects of Ex-4 on hepatic fatty acid composition and Δ-5-desaturase activity remain unclear.

The aim of this study was to investigate the effects of Ex-4 on severity of steatohepatitis, hepatic fatty acid composition, and Δ-5-desaturase index in a murine model of NASH.

Materials and methods

Materials. Reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated.

Animals. NASH was induced in db/db mice fed a methionine-choline deficient (MCD) diet (31). Briefly, 5-week-old male db/db mice (BKS.Cg- + Leprdb/+Leprdb/Jcl®) weighing 15-20 g were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed individually in an air-conditioned room at 22±3˚C and 55±10% humidity with a 12-h light/dark cycle. The mice were fed a normal diet during a 1-week quarantine and acclimatization period, followed by the MCD diet (CLEA Japan, Inc.) and water *ad libitum* throughout the experimental period. All the rat experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee.

Treatment. Ex-4 (20 μg/kg; no. 24463, AnaSpec, Inc., Fremont, CA, USA) (Ex-4 group; n=4) or saline [control (CON) group; n=4] was administered intraperitoneally under anesthesia every morning for 8 weeks. At week 14, the mice were sacrificed by using ether anesthesia and the livers were obtained under anesthesia.

Measurement of body weight. Body weight was measured weekly, in the morning, through week 14.

Liver histology. Random histological sampling was performed throughout this study as previously described (32,33). Liver samples were fixed overnight in 10% buffered formalin and embedded in paraffin. All sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) (34,35).

Hepatic triglyceride content. Liver samples were fixed overnight in 10% buffered formalin. Sections were transferred to 70% ethanol and stained with Sudan IV (0.1% Sudan IV dissolved in equal parts acetone and 70% ethanol) to evaluate triglyceride content (36).

Non-alcoholic fatty liver disease (NAFLD) activity. NAFLD activity was evaluated by the NAFLD activity score, in which the following findings were evaluated semi-quantitatively: steatosis (0-3 points), lobular inflammation (0-2 points), hepatocellular ballooning (0-2 points), and fibrosis (0-4 points) (37).

Fatty acid composition. Total liver fatty acids were extracted according to Folch *et al* (38). Fatty acid methyl esters were isolated and quantified by gas chromatography furnished with a flame-ionization detector. The fatty acids measured (and expressed as μg/g•liver) were: lauric, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, γ-linolenic, linolenic, arachidic, eicosenoic, eicosadienoic, 5,8,11-eicosatrienoic, dihomo γ-linolenic, arachidonic, eicosapentaenoic, behenic, erucic, docosatetraenoic, docosapentaenoic, lignoceric, docosahexaenoic, and nervonic acid.

Classification of fatty acids. Fatty acids were classified as follows: saturated fatty acids (SFAs), the sum of all identified SAFs; atherogenic SFAs, the sum of lauric, myristic, and palmitic acids; thrombogenic SFAs, the sum of myristic, palmitic, and stearic acids; medium SFAs, the sum of SFAs containing 11-16 carbon atoms; long SFAs, the sum of SFAs containing ≥16 carbon atoms; monounsaturated fatty acids (MUFAs), the sum of all identified MUFAs; PUFAs, the sum of all identified PUFAs; n-3 PUFAs, the sum of n-3 series PUFAs; n-6 PUFAs, the sum of n-6 series PUFA; Δ-5-desatulase index, arachidonic acid/γ-linolenic acid.

Statistical analysis. Data were expressed as mean ± SD. Differences between two groups were analyzed by the Wilcoxon test (JMP version 10.0.2, SAS Institute, Inc., Cary, NC). $P \leq 0.05$ was considered statistically significant.

Results

Effects of Ex-4 on body weight, appearance, and macroscopic appearance of the liver. In the CON group, body weight gradually increased to ~50 g at week 14 (Fig. 1A). In the Ex-4 group, body weight gain stopped 1 week after the Ex-4 treatment and reached a plateau at ~40 g at week 7 (Fig. 1A). Ex-4 significantly suppressed weight gain in MCD-fed db/db mice.

Representative mice from the CON and Ex-4 groups are shown in Fig. 1B. The mouse from the Ex-4 group was smaller and had a good coat of fur in comparison to the mouse from the CON group (Fig. 1B).

A representative macroscopic image of the liver of CON and Ex-4 mice is shown in Fig. 1C. CON livers exhibited xanthochromia with swelling, while the Ex-4 livers were brown, with no swelling (Fig. 1C).

Effects of Ex-4 on hepatic histology, hepatic triglyceride content, and the NAFLD activity score. Representative images of hepatic histology and Sudan IV staining are shown in Fig. 2A. Steatosis, lobular inflammation, and hepatocyte ballooning were milder in the Ex-4 group compared to the CON group (Fig. 2A). Obvious hepatic fibrosis was not evident

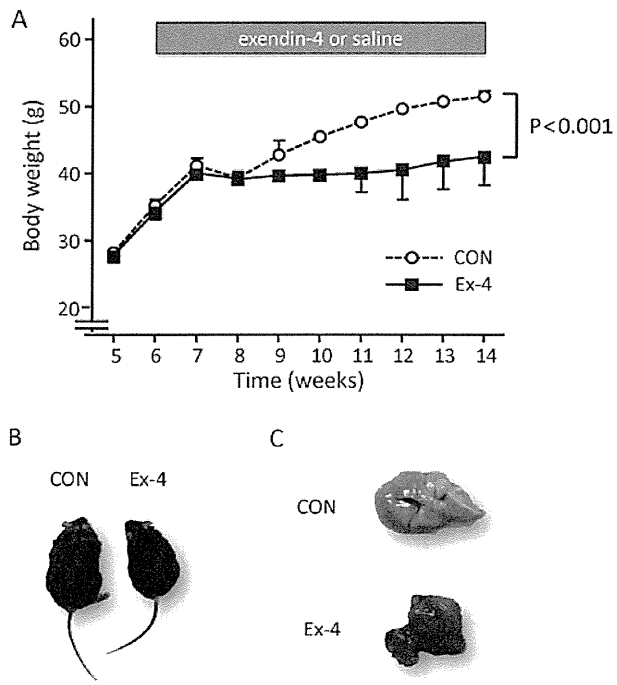

Figure 1. Effects of exendin-4 (Ex-4) on body weight, appearance, and macroscopic appearance of liver. (A) Changes in body weight. (B) Representative mice in the control (CON) and Ex-4 groups. (C) Representative livers from the CON and Ex-4 groups. P≤0.05 was considered statistically significant.

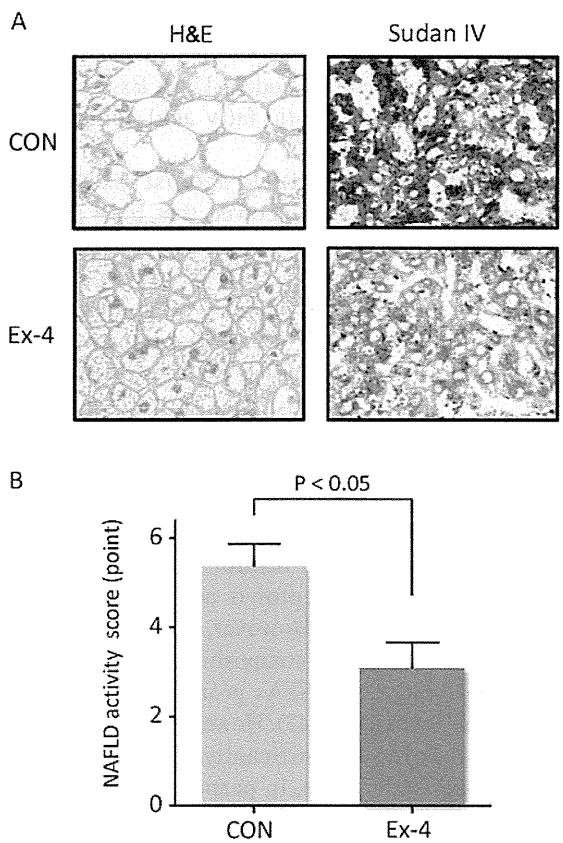

Figure 2. Effects of exendin-4 (Ex-4) on hepatic histology, hepatic triglyceride content, and non-alcoholic fatty liver disease (NAFLD) activity score. (A) Representative images of hepatic histology and Sudan IV staining. (B) The difference in the NAFLD activity score between the Ex-4 and control (CON) groups. P≤0.05 was considered statistically significant. Hematoxylin and eosin staining (H&E).

Table I. Effects of Ex-4 on hepatic SFA.

SFA type	Unit	CON	Ex-4	P
SFA	μg/g•liver	17838±3248	27541±9273	N.S.
Atherogenic	μg/g•liver	13414±2981	22457±8670	N.S.
Thrombogenic	μg/g•liver	17605±3244	27210±9260	N.S.
Medium-chain	μg/g•liver	15233±3554	25186±9799	N.S.
Long-chain	μg/g•liver	23240±955	31710±8436	<0.05

Ex-4, exendin-4; SFA, saturated fatty acid; CON, control; N.S., not significant.

Table II. Effects of Ex-4 on hepatic MUFAs and PUFAs.

Acid type	Unit	CON	Ex-4	P
MUFA	μg/g•liver	20355±6701	34965±14485	N.S.
PUFA	μg/g•liver	18410±791	25986±8050	<0.05
n-3 PUFA	μg/g•liver	2218.5±415.8	1992.4±288.7	N.S.
n-6 PUFA	μg/g•liver	16166±943	23937±7845	<0.05
n-3 PUFA/ n-6 PUFA	Ratio	13.83±3.15	8.73±1.95	<0.05

Ex-4, exendin-4; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; CON, control; N.S., not significant.

in either group. Hepatic triglyceride content was depleted in the Ex-4 group in comparison to the CON group (Fig. 2A).

The NAFLD activity score was significantly lower in the Ex-4 group than in the CON group (Fig. 2B).

Effects of Ex-4 on hepatic SFA. There was no significant difference in the hepatic SFA content of the CON and Ex-4 groups (Table I). No significant difference between the groups was observed in the hepatic content of atherogenic, thrombogenic, and medium-chain SFA. However, long-chain SFA content was significantly higher in the Ex-4 group compared to the CON group (Table I).

We also examined the hepatic content of each long-chain SFA component and found no significant differences in the palmitic, stearic, behenic, and lignoceric acid. However, hepatic arachidic acid was significantly higher in the Ex-4 group compared to the CON group (Fig. 3A-E).

Effects of Ex-4 on hepatic MUFAs and PUFAs. Hepatic MUFA content did not significantly differ between groups (Table II). However, hepatic PUFA content was significantly higher in the Ex-4 group compared to the CON group. Similarly, hepatic n-6 PUFA content and the n-3 PUFA/n-6 PUFA ratio were significantly higher in the Ex-4 group compared to the CON group (Table II).

We also assessed the hepatic content of each n-6 PUFA component and found no significant difference in arachidonic acid. However, the hepatic content of linoleic acid, γ-linolenic acid, and dihomo γ-linolenic acid was significantly higher in the Ex-4 group compared to the CON group (Fig. 4A-D). By contrast, hepatic Δ-5-desaturase index in the Ex-4 group

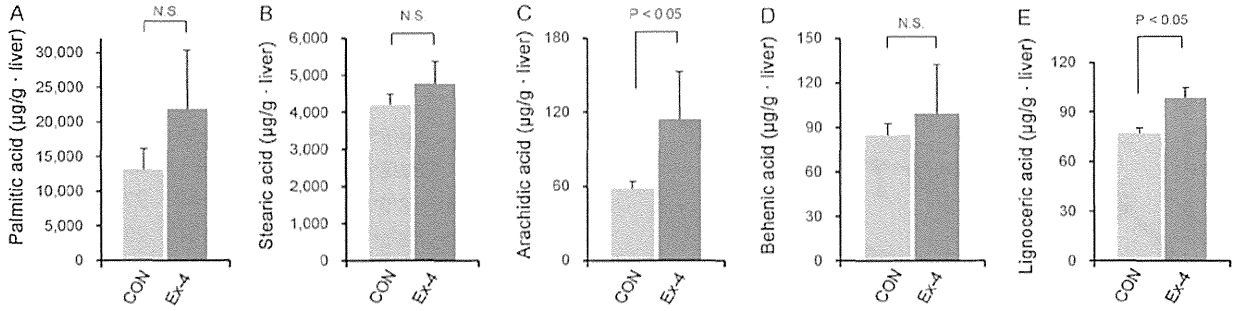

Figure 3. Effects of exendin-4 (Ex-4) on hepatic long-chain saturated fatty acids (SFAs). (A) Palmitic acid, (B) stearic acid, (C) arachidic acid, (D) behenic acid, and (E) lignoceric acid. P≤0.05 was considered statistically significant. Control (CON), not significant (N.S.).

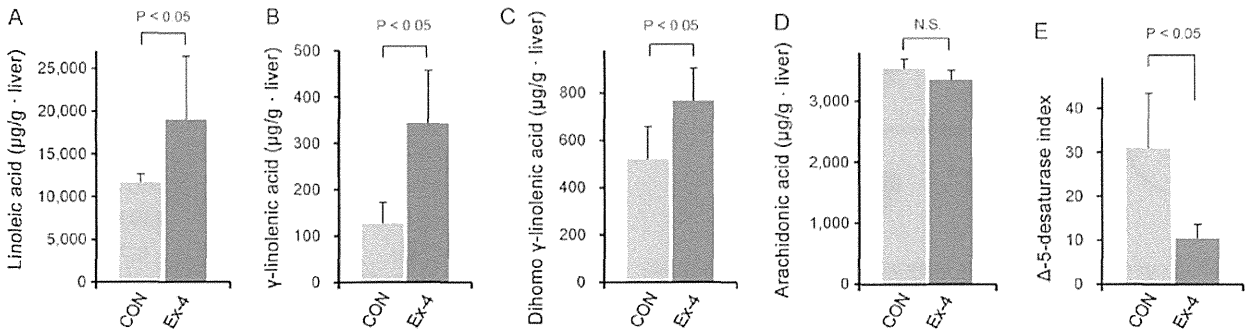

Figure 4. Effects of exendin-4 (Ex-4) on hepatic n-6 polyunsaturated fatty acids (PUFAs). (A) Linoleic acid, (B) γ-linolenic acid, (C) dihomo γ-linolenic acid, (D) arachidonic acid, and (E) Δ-5-desaturase index. P≤0.05 was considered statistically significant. Control (CON), not significant (N.S.).

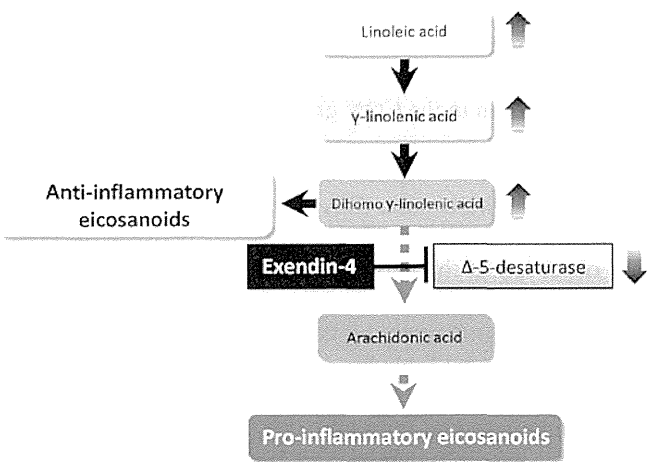

Figure 5. A scheme for exendin-4 (Ex-4)-caused alterations in lipid metabolism. Ex-4 may inhibit Δ-5-desaturase activity, resulting in arachidonic acid production and subsequent pro-inflammatory eicosanoids. Inhibition of Δ-5-desaturase activity also increases the hepatic content of dihomo γ-linolenic acid and subsequent anti-inflammatory eicosanoids.

was approximately one-third of that in the CON group. Ex-4 treatment significantly reduced hepatic Δ-5-desaturase index compared to the CON group (Fig. 4E).

Discussion

Results of this study have shown that Ex-4 inhibited body weight gain and improved NASH in MCD diet-fed db/db mice. Ex-4 also altered hepatic fatty acid composition with a decrease in Δ-5-desaturase index. Thus, Ex-4 may improve NASH by altering the hepatic fatty acid composition in a murine model of NASH.

The effects of the GLP-1R agonist Ex-4 on NASH were examined. The results showed that Ex-4 significantly suppressed body weight gain and the NAFLD activity score in MCD diet-fed db/db mice. GLP-1R expression is down-regulated in a NASH rat model as well as in patients with NASH (16). Moreover, GLP-1R agonist improves NASH in various animal models, including high-fat diet-fed rats (16), ob/ob mice (17,18), and diabetic male ApoE(-/-) mice (19). The GLP-1R agonist also reduced body weight and the NAFLD activity score in patients with NASH (39). Thus, our results are consistent with previous reports in this regard. Possible mechanisms for GLP-1R agonist-induced NASH improvement include the upregulation of insulin sensitivity, peroxisome proliferator-activated receptor α activity, and fatty acid β-oxidation (15,16,40,41). However, the effects of GLP-1R agonist in hepatic fatty acid composition remain unclear.

In general, long-chain SFAs promote inflammation and progression of NAFLD (42,43). However, results of this study have shown that Ex-4 significantly increased the hepatic content of long-chain SFAs, in particular the arachidic and lignoceric acids. Although the reason for the discrepancy between previous reports and our findings remains unclear, certain SFAs, including arachidic and lignoceric acids are not correlated with insulin resistance, a feature of NASH (44). Furthermore, arachidic acid improves lipid metabolism by enhancing apoB secretion (45). Lignoceric acid is a precursor of ceramide, thus an increase in hepatic lignoceric

acid content indicates a decrease in ceramide synthesis. Recently, Kurek *et al* showed that inhibition of ceramide synthesis reduces hepatic lipid accumulation in a rat model of NAFLD (46). This finding suggests that Ex-4 improves lipid metabolism through alterations in arachidic and lignoceric acids in a murine model of NASH.

Although hepatic MUFA content was not altered by Ex-4 treatment, hepatic PUFA content was increased. Ex-4 increased the hepatic content of n-6 PUFAs such as linoleic acid, γ-linolenic acid, and dihomo γ-linolenic acid. These n-6 PUFAs are precursors of pro-inflammatory eicosanoids and are involved in the development of NASH (22,47). Thus, our findings are different from those of previous studies. However, a possible explanation for the discrepancy is an Ex-4-induced alteration in n-6 PUFA metabolism. Δ-5-desaturase is a rate-limiting enzyme of n-6 PUFA metabolism that increases the production of pro-inflammatory eicosanoids (48). An oligonucleotide microarray analysis using human liver tissue showed that Δ-5-desaturase is upregulated in patients with NASH (30). In this study, we have found that the Δ-5-desaturase index was significantly reduced by Ex-4 treatment, indicating that Ex-4 inhibits Δ-5-desaturase activity and subsequently suppresses the production of pro-inflammatory eicosanoids (Fig. 5). In addition, the inhibition of Δ-5-desaturase activity increases hepatic contents of dihomo γ-linolenic acid, which is a precursor of anti-inflammatory eicosanoids (Fig. 5). López-Vicario *et al* recently showed that a Δ-5-desaturase inhibitor, CP-24879, significantly reduces intracellular lipid accumulation and inflammatory injury in hepatocytes *in vitro* (30), supporting our hypothesis. Thus, our findings together with those of previous studies suggest that suppression of Δ-5-desaturase activity could be a new therapeutic strategy for NASH.

In conclusion, the results of the present study have shown that Ex-4 suppressed body weight gain and improved steatohepatitis in a murine model of NASH. Ex-4 also altered hepatic fatty acid composition with a decrease in Δ-5-desaturase index. These findings suggest that Ex-4 improves NASH by modulating hepatic fatty acid metabolism.

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Hepatology Research

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Original Article

Serum albumin level is a notable profiling factor for non-B, non-C hepatitis virus-related hepatocellular carcinoma: A data-mining analysis

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Aim: Various factors are underlying for the onset of non-B, non-C hepatitis virus-related hepatocellular carcinoma (NBNC-HCC). We aimed to investigate the independent risk factors and profiles associated with NBNC-HCC using a data-mining technique.

Methods: We conducted a case-control study and enrolled 223 NBNC-HCC patients and 669 controls from a health checkup database (n = 176 886). Multivariate analysis, random forest analysis and a decision-tree algorithm were employed to examine the independent risk factors, factors distinguishing between the case and control groups, and to identify profiles for the incidence of NBNC-HCC, respectively.

Results: In multivariate analysis, besides γ-glutamyltransferase (GGT) levels and the Brinkman index, albumin level was an independent negative risk factor for the incidence of NBNC-HCC (odds ratio = 0.67; 95% confidence interval = 0.60–0.70; $P < 0.0001$). In random forest analysis, serum albumin level was the highest-ranked variable for distinguishing between the case and control groups (98 variable importance). A decision-tree algorithm was created for albumin and GGT levels, the aspartate aminotransferase-to-platelet ratio index (APRI) and the Brinkman index. The serum albumin level was selected as the initial split variable, and 82.5% of the subjects with albumin levels of less than 4.01 g/dL were found to have NBNC-HCC.

Conclusion: Data-mining analysis revealed that serum albumin level is an independent risk factor and the most distinguishable factor associated with the incidence of NBNC-HCC. Furthermore, we created an NBNC-HCC profile consisting of albumin and GGT levels, the APRI and the Brinkman index. This profile could be used in the screening strategy for NBNC-HCC.

Key words: lifestyle, metabolism, non-viral-related hepatoma, smoking

INTRODUCTION

LIVER CANCER IS the sixth most frequently diagnosed cancer worldwide and was the third most frequent cause of cancer-related death in 2008.[1] Although the incidence of liver cancer is increasing worldwide, the highest rate is found in East Asia. Hepatocellular carcinoma (HCC) accounts for 70–85% of the cases of primary liver cancer. The most significant risk factors for HCC are hepatitis C virus (HCV) and hepatitis B virus (HBV) infection. Although the incidence of HCV-related HCC has recently been decreasing,[2] the incidence of non-B, non-C hepatitis-related HCC (NBNC-HCC) in Japan has risen to 27.6% from 7.6% in the last 15 years.[2]

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Non-B, non-C hepatitis-related HCC can be caused by various non-viral chronic liver injuries, including those associated with alcoholic liver disease, non-alcoholic fatty liver disease, autoimmune liver diseases and hemochromatosis.[3] In addition, diabetes mellitus, use of exogenous insulin and smoking have been reported as risk factors for the development of HCC.[4–6] As NBNC-HCC is often diagnosed at an advanced stage, an efficient strategy for early detection is required.[7] Thus far, no case-control studies have been conducted to investigate the risk factors for NBNC-HCC; therefore, unidentified risk factors may exist. Moreover, the combined effect of these risk factors has not been examined. NBNC-HCC is thought to be caused by complicated interactions between multiple risk factors; hence, the identification of a risk profile for NBNC-HCC may aid the establishment of a novel strategy for the early detection of NBNC-HCC. The prevalence of NBNC-HCC may increase further due to the combined effects of more effective vaccine and treatment strategies for HBV and HCV coupled with the increasing prevalence of non-alcoholic fatty liver disease.

Two popular approaches to developing a risk profile for development of screening strategies are random forest analysis and decision-tree algorithms. Both approaches require identification of carefully matched cases and controls to avoid selection bias and to balance covariates.[8,9] Statistical matching techniques have been developed to facilitate this process. Genetic matching (GenMatch) is used to search the best pair on the basis of genetic Mahalanobis distance values. This process involves a multidimensional search to provide the near-optimal value of a fitness function in an optimization problem.[10,11] GenMatch is increasingly being employed in clinical practice and served to identify factors associated with gestational diabetes and multiple protein biomarkers for head and neck squamous cell cancer.[12,13] GenMatch has consistently been found to be more accurate than existing matching methods, such as propensity score.[14,15]

Random forest analysis is a data-mining technique that identifies the factors distinguishing between the case and control groups with an ordinal scale. A decision-tree algorithm is a data-mining technique that reveals a series of classification rules by identifying priorities, and therefore allows clinicians to choose an option that maximizes benefit for the patient. It has been used to identify the profiles associated with response to interferon therapy for chronic hepatitis C,[16,17] incidence of subclinical hepatic encephalopathy,[18] HCV carriers with persistently normal alanine aminotransferase (ALT) levels,[19] and the progression of NBNC-HCC.[7] Neither random forest analysis nor decision-tree algorithms have been applied to identify the clinical feature profile associated with NBNC-HCC incidence.

The aim of this study is to investigate independent risk factors associated with the incidence of NBNC-HCC by comparing cases to controls matched by GenMatch from a health checkup database. In addition, we also investigated a profile associated with NBNC-HCC incidence by using random forest analysis and a decision-tree algorithm.

METHODS

Ethics

THE STUDY PROTOCOL conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the prior approval given by each institutional review board. None of the subjects were institutionalized.

Subjects

We conducted a case–control study to examine NBNC-HCC risk factors. From 1995 to 2010, 1769 patients were diagnosed with HCC at Kurume University Hospital and all of the patients diagnosed with NBNC-HCC ($n = 223$) were enrolled in this study (no liver disease, $n = 109$; alcoholic liver disease, $n = 80$; schistosomiasis japonica, $n = 13$; autoimmune hepatitis, $n = 8$; non-alcoholic fatty liver disease, $n = 5$; hemochromatosis, $n = 2$; primary sclerosing cholangitis, $n = 2$; primary biliary cirrhosis, $n = 1$; sarcoidosis, $n = 1$; von Gierke disease, $n = 1$; Budd–Chiari syndrome, $n = 1$). NBNC-HCC patients were defined as those who were initially diagnosed with primary liver cancer with negative results for both serum hepatitis B surface antigen (HBsAg) and anti-HCV antibody. NBNC-HCC was diagnosed by a combination of tests for serum tumor makers such as α-fetoprotein and des-γ-carboxy prothrombin, and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging and angiography. In addition, 48.9% (109/223) of the patients were pathologically diagnosed with HCC following ultrasonography-guided fine-needle tumor biopsy. No patients had malabsorption syndrome, protein-losing gastroenteropathy, and chronic kidney disease including nephrotic syndrome.

Control data from the period 1996–2007 were obtained from a health checkup database ($n = 176\,886$) at St Mary's Hospital, which is located in the same city as

Table 1 Genetic matching for case : control ratio

Case : control	*P*-value
1:1	0.3173
1:2	0.6834
1:3	0.7857
1:4	0.0460
1:5	0.1430

GenMatch was employed to investigate the proper ratio of the case : control number. *P*-value is the highest in "case : control = 1:3", indicating that the most matched control number is threefold the number of cases.

Kurume University Hospital, and selected using the following criteria: (i) no HCC; and (ii) negative results for both the serum HBsAg and anti-HCV antibody. In a case–control study, selection of controls is an important step. Because age and sex are well-known risk factors for hepatocarcinogenesis,[20,21] control subjects were matched to the cases by age and sex. The case : control ratio can also affect the results of a study.[22] To evaluate the case : control ratio, the smallest *P*-values obtained from all the matching balance tests, including Student's *t*-test and Kolmogorov–Smirnov test, were used. GenMatch was employed and demonstrated that the highest *P*-value was obtained for "case : control = 1:3", indicating that the most matched control number is threefold the number of cases in this study (Table 1). Thus, we randomly selected 669 subjects from 176 886 non-HCC subjects who underwent a medical checkup as the control group.

Clinical characteristics, lifestyle, complications and biochemical parameters

Data for clinical characteristics, lifestyle, complications and biochemical parameters were obtained at the time of HCC diagnosis and data from the health examination included information regarding the following variables: age; sex; height; weight; habitual intake of alcohol; cumulative cigarette consumption; history of fatty liver, hypertension and diabetes mellitus; use of antidiabetic agents (regardless of drug type); serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT) and albumin; hemoglobin level; platelet count; levels of blood glucose, hemoglobin A1c (HbA1c), total bilirubin, total cholesterol, triglyceride and HBsAg; and HCV antibody status. Biochemical parameters were measured using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital or St Mary's Hospital).

Assessment of body constitution, daily alcohol intake and cumulative cigarette consumption

Body mass index (BMI) was calculated as bodyweight in kilograms divided by the square of height in meters (kg/m^2). Daily alcohol intake was categorized as none, less than 60 g, 60–100 g and more than 100 g, as previously described.[23] The cumulative cigarette consumption was estimated by the Brinkman index (number of cigarettes consumed/day × years of smoking).

Assessment of fatty liver and hepatic fibrosis

Fatty liver was diagnosed by the presence of at least two out of three abnormal findings on abdominal ultrasonography: (i) diffusely increased hepatic echogenicity ("bright") that was greater than that for the kidney; (ii) vascular blurring; and (iii) deep attenuation of the ultrasound signal, as previously described.[24] Hepatic fibrosis was evaluated by the AST-to-platelet ratio index (APRI): serum AST level (U/L) / upper limit of normal AST level (33 U/L) × 100 / platelet count ($\times 10^4$/mL).[25] Liver cirrhosis is excluded by APRI of less than 1.5 and 61.9% (85/223) showed APRI of less than 1.5.

Diagnosis of hypertension and diabetes mellitus

Hypertension was diagnosed by a systolic blood pressure of more than 140 mmHg and/or diastolic blood pressure of more than 90 mmHg,[26] or by prescription of antihypertensive agents. Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels of more than 126 mg/dL or HbA1c levels of more than 6.5% according to the Diagnostic Criteria for Diabetes Mellitus[27] or by use of antidiabetic agents.

Statistical analysis

Descriptive statistics were expressed as a number or mean ± standard deviation. GenMatch was used to determine the proper case : control ratio. Differences between the two groups were analyzed using the Mann–Whitney *U*-test. Variables or profiles associated with the incidence of NBNC-HCC were analyzed by data-mining techniques. The statistical methods are described in detail below.

Multivariate stepwise analysis

A logistic regression model was used for multivariate stepwise analysis to identify any independent variables associated with the incidence of NBNC-HCC as previ-

ously described.[7] Data were expressed as odds ratio (OR) and 95% confidence interval (CI) values.

Random forest analysis

Random forest analysis was used to identify the factors distinguishing between the case and control with an ordinal scale, as previously described.[28] The procedure employed for building the random forest was as follows: First, n tree-models were created using bootstrap samples that were randomly chosen from the original dataset. Second, each classification or regression tree model was grown with no pruning. Instead of determining the best split among all potential predictors, we chose a random sample of these variables (one-third of the variables) to consider as potential splitting variables. Thus, the best split variable was chosen from among those variables. Third, new data were predicted by aggregating the predictions of the n trees. Finally, the error rate was estimated by predicting the data not in the bootstrap sample (out-of-bag) by using the tree grown with the bootstrap sample. The variable importance value reflecting the relative contribution of each variable to the model was estimated by randomly permuting its values and recalculating the predictive accuracy of the model, and was expressed as the mean difference of the Gini index.

Decision-tree algorithm

A decision-tree algorithm was constructed to reveal profiles associated with the incidence of NBNC-HCC. Predictive accuracy of the decision-tree model was validated by the area under the receiver–operator curve (AUROC) analysis using 10-fold cross-validation, as previously described.[7]

All P-values were two-tailed, and a level of less than 0.05 was considered to be statistically significant. Multivariate stepwise analysis was conducted using SAS ver. 9.2 (SAS Institute, Cary, NC, USA). GenMatch, random forest analysis and decision-tree analysis were conducted using the R packages (URL http://www.r-project.org/index.html).[29]

RESULTS

Characteristics of all subjects

THE CHARACTERISTICS OF the 223 patients and 669 control subjects are summarized in Table 2. There was no significant difference between the case and control groups in BMI, serum triglyceride levels and comorbidity with hypertension. The case group showed significantly higher comorbidity with fatty liver and serum levels of AST, ALT, GGT and total bilirubin, with reference to the corresponding values in the control group (Table 2). The case group also showed significantly higher alcohol intake, Brinkman index, comorbidity with diabetes mellitus, use of antidiabetic agents, fasting blood glucose levels, HbA1c value and APRI (Table 2). The case group showed significantly lower blood hemoglobin levels, platelet counts and serum levels of total cholesterol and albumin (Table 2).

Multivariate stepwise analysis for the incidence of NBNC-HCC

Multivariate stepwise analysis was performed to identify independent variables for the incidence of NBNC-HCC. The APRI and HbA1c values and platelet counts were not significant variables. However, GGT levels, the Brinkman index and use of antidiabetic agents were identified as independent positive risk factors for the incidence of NBNC-HCC (GGT, OR = 1.17, 95% CI = 1.08–1.21, $P < 0.0001$; Brinkman index, OR = 1.17; 95% CI = 1.05–1.30; $P = 0.0047$; use of antidiabetic agents, OR = 7.42, 95% CI = 2.42–22.76, $P = 0.0005$) (Table 3). On the other hand, total cholesterol, hemoglobin and albumin levels were identified as independent negative risk factors for the incidence of NBNC-HCC (total cholesterol, OR = 0.88, 95% CI = 0.79–0.98, $P = 0.0155$; hemoglobin, OR = 0.95, 95% CI = 0.93–0.97, $P < 0.0001$; albumin, OR = 0.67, 95% CI = 0.60–0.70, $P < 0.0001$) (Table 3).

Random forest analysis for distinguishing between the case and control groups

The results of random forest analysis are summarized in rank order in Figure 1. The analysis demonstrated that serum albumin level is the highest-ranked variable for distinguishing between case and control (Fig. 1). This is followed by APRI, GGT level, platelet count, hemoglobin level, AST level, HbA1c value, total cholesterol level and the Brinkman index (Fig. 1).

Decision-tree algorithm for the incidence of NBNC-HCC

With the dataset ($n = 892$), a decision-tree algorithm was created by using four variables to classify five groups of subjects (Fig. 2). The serum level of albumin was selected as the initial split variable with an optimal cut-off of 4.01 g/dL. When subjects showed albumin levels of 4.01 g/dL or more, 17.5% (39/223) of subjects were found to have NBNC-HCC. When the albumin

Table 2 Characteristics of the subjects

	Case	Control	*P*-value
n	223	669	
Sex (M/F)	169/54	507/162	Matched
Age	68.0 ± 9.4	68.0 ± 9.4	Matched
BMI (kg/m^2)	23.6 ± 3.7	22.9 ± 3.2	0.2188
Daily alcohol intake (none/<60 g/60–100 g/>100 g)	61/82/34/46	194/433/35/7	<0.0001
Brinkman index	544.5 ± 639.3	140.2 ± 276.8	<0.0001
Comorbidity			
Fatty liver (yes/no)	30/193	150/519	0.0038
Hypertension (yes/no)	40/183	129/540	0.6940
DM (yes/no)	87/136	49/620	<0.0001
Hemoglobin (g/dL)	12.6 ± 2.1	14.4 ± 1.5	<0.0001
Platelet (× 10^4/mm^3)	16.5 ± 9.8	23.7 ± 5.3	<0.0001
AST (IU/L)	57.0 ± 55.9	23.1 ± 11.9	<0.0001
ALT(IU/L)	50.8 ± 50.3	24.5 ± 19.1	<0.0001
GGT (IU/L)	251.1 ± 307.8	43.8 ± 57.1	<0.0001
Albumin (g/dL)	3.55 ± 0.57	4.48 ± 0.25	<0.0001
Total bilirubin (mg/dL)	1.64 ± 2.65	0.89 ± 0.36	<0.0001
Total cholesterol (mg/dL)	169.1 ± 46.1	206.3 ± 34.2	<0.0001
Triglyceride (mg/dL)	108.2 ± 58.4	117.7 ± 89.6	0.3179
Fasting blood glucose (mg/dL)	131.2 ± 60.0	101.4 ± 18.9	<0.0001
HbA1c (%)	6.1 ± 1.4	5.3 ± 0.7	<0.0001
APRI	1.405 ± 1.472	0.313 ± 0.248	<0.0001
Use of antidiabetic agents (yes/no)	68/155	17/652	<0.0001

Descriptive statistics are expressed as the mean ± standard deviation or the number of patients. Differences between the two groups were analyzed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistical significant.
ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; GGT, γ-glutamyltransferase; HbA1c, hemoglobin A1c.

level was less than 4.01 g/dL, 82.5% (184/223) of the subjects had NBNC-HCC (Fig. 2).

Among the subjects with an albumin level of 4.01 g/dL or more, the APRI was selected as the variable for the second division with an optimal cut-off of 0.5. In addition, among the subjects with APRI of 0.5 or more, the Brinkman index was selected as the variable for third division with an optimal cut-off of 400 (Fig. 2). Thus, 2.3% (13/574) of subjects had NBNC-HCC when the subjects met the following criteria: albumin level of 4.01 g/dL or more and APRI of less than 0.5 (group 1 in Fig. 2). In contrast, 85.0% (17/20) of the subjects had

Table 3 Multivariate stepwise analysis for the incidence of NBNC-HCC

Variables	Unit	Odds ratio	95% confidence interval			*P* value
APRI	0.1	1.07	0.98	to	1.16	0.1283
HbA1c	0.1	1.03	0.99	to	1.07	0.1270
Platelet	1	0.95	0.89	to	1.01	0.0996
GGT	10	1.15	1.08	to	1.21	<0.0001
Brinkman index	100	1.17	1.05	to	1.30	0.0047
Use of antidiabetic agents	1	7.42	2.42	to	22.76	0.0005
Total cholesterol	10	0.88	0.79	to	0.98	0.0155
Hemoglobin	0.1	0.95	0.93	to	0.97	<0.0001
Albumin	0.1	0.67	0.60	to	0.76	<0.0001

APRI, aspartate aminotransferase-to-platelet ratio index; GGT, γ-glutamyltransferase; HbA1c, hemoglobin A1c.

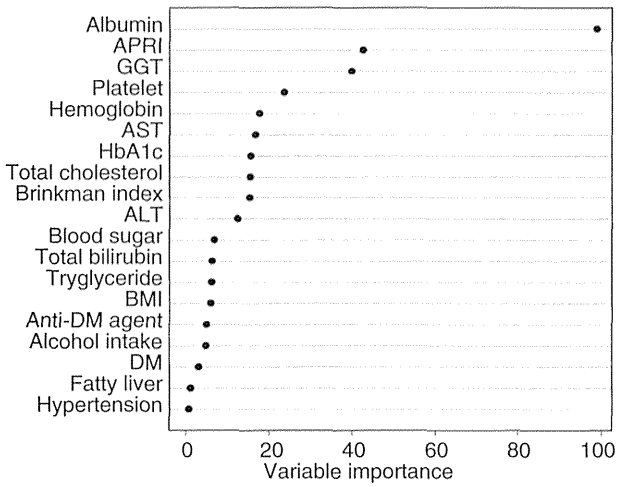

Figure 1 Random forest analysis for distinguishing between the case and control groups. Variable importance is a general measure of the contribution of each variable in distinguishing the classes. ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; GGT, γ-glutamyltransferase; HbA1c, hemoglobin A1c.

NBNC-HCC when they met the following criteria: albumin level of 4.01 g/dL or more, APRI of 0.5 or more and Brinkman index of 400 or more (group 3 in Fig. 2).

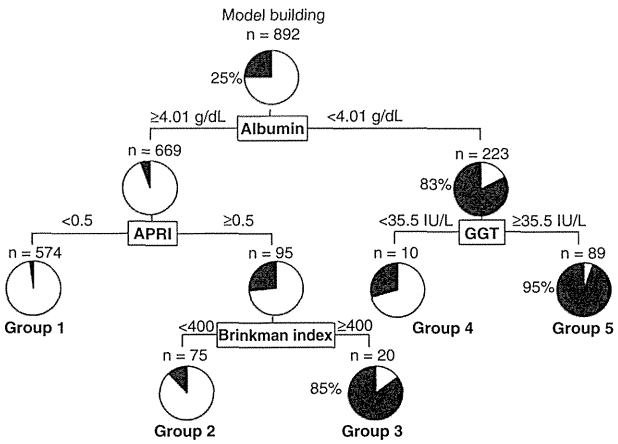

Figure 2 Decision-tree algorithm of NBNC-HCC predictive factors. The subjects were classified according to the indicated cut-off values of the variables. The pie graphs indicate the percentage of ordinary people (white)/NBNC-HCC patients (black) in each group. APRI, aspartate aminotransferase-to-platelet ratio index; GGT, γ-glutamyltransferase; NBNC-HCC, non-B, non-C hepatitis virus-related hepatocellular carcinoma.

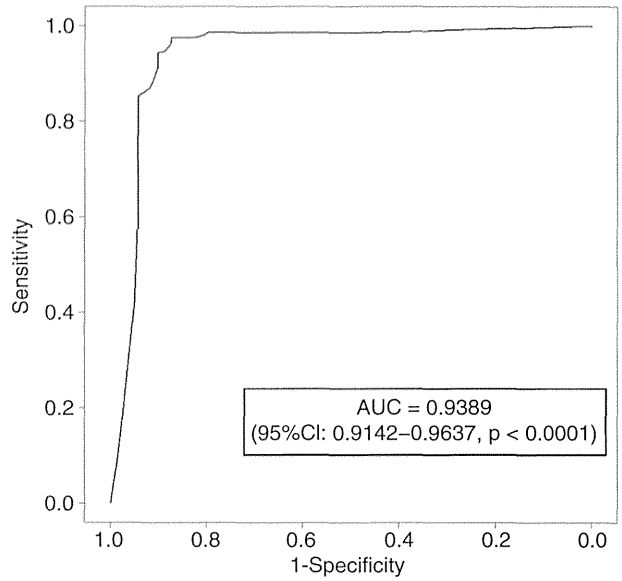

Figure 3 Predictive accuracy of the decision-tree model by area under the receiver–operator curve (AUC) using a 10-fold cross-validation. The black line indicates the decision-tree model. The gray line indicates the reference. CI, confidence interval.

In subjects with an albumin level less than 4.01 g/dL, however, the GGT level was selected as the variable for the second division with an optimal cut-off of 35.5 IU/L. We observed that 94.5% (172/182) of the subjects had NBNC-HCC when they met the following criteria: albumin level of less than 4.01 g/dL and GGT level of 35.5 IU/L or more (group 5 in Fig. 2). The predictive accuracy of the decision-tree model was validated by AUROC using a 10-fold cross-validation. The AUROC was 0.9389 (95% CI = 0.9142–0.9637, $P < 0.0001$) (Fig. 3).

DISCUSSION

IN THIS STUDY, we identified independent factors associated with the incidence of NBNC-HCC, including serum levels of albumin and GGT, APRI and the Brinkman index. Our data-mining using random forest analysis and a decision-tree algorithm demonstrated that serum albumin level is the most significant variable associated with the incidence of NBNC-HCC. These findings suggest that profiles consisting of albumin and GGT levels, APRI or the Brinkman index may be useful as screening tools for NBNC-HCC.

Previous studies have shown that obesity, fatty liver and diabetes mellitus are independent factors associated

with NBNC-HCC.[30,31] Our analysis makes the important new observation that serum albumin level is highly predictive of HCC among patients at risk for NBNC-HCC. Although the reason for the high predictivity of albumin for NBNC-HCC is unclear, there are some possible explanations. In the majority of previous studies, control data were obtained from patients with other diseases.[32,33] However, in this study, control data were obtained from a health examination database; thus, the nutritional status of this control group could be better than that of the controls in the other studies, which might have accentuated the difference in serum albumin levels. Another explanation could be the difference in statistical methods used. In this study, we employed a data-mining technique, which is suitable for investigating complex interactions of risk factors with no a priori hypothesis. Although we used three different statistical methods, namely, a random forest analysis, multivariate stepwise analysis and a decision-tree algorithm, the serum albumin level was an independent factor associated with the incidence of NBNC-HCC in all of these analyses. Thus, to our knowledge, our study is the first to show that serum albumin level is predictive of the incidence of NBNC-HCC.

In general, NBNC-HCC is diagnosed at advanced stages. It could be surmised that changes in serum albumin level may be linked to the progression of NBNC-HCC. However, in this study, no significant difference was seen in serum albumin levels among the tumor stages of NBNC-HCC (data not shown). The causal relationship between serum albumin levels and the development of NBNC-HCC remains unclear. However, an increased synthesis of reactive oxygen species is a relevant cause of cancer.[34] It has been established recently that albumin provides the first line of defense against reactive oxygen species in plasma.[35,36] Previous studies have shown that serum albumin levels predict the recurrence of colorectal cancer after elective colorectal resection.[37] Branched-chain amino acids, which increase serum albumin levels, also have been reported to suppress hepatocarcinogenesis.[38,39] Thus, changes in serum albumin level could be involved in the development of HCC.

A variety of factors are involved in hepatocarcinogenesis.[3,32,40]However, interactions between risk factors remain unclear. Therefore, a decision-tree algorithm was created and revealed that the following two profiles are associated with a high incidence of NBNC-HCC: (i) albumin level of less than 4.01 g/dL and GGT level of 35.5 or more; and (ii) albumin level of 4.01 g/dL or more, APRI of 0.5 or more and Brinkman index of 400 or more. The GGT level and APRI have been reported as independent risk factors for hepatocarcinogenesis in NBNC-HCC.[32,41] Using a decision-tree algorithm, we also showed that the GGT level and APRI were the second most significant risk factors after albumin levels. Although smoking is a significant risk factor for the development of several cancers including lung cancer, the impact of smoking on hepatocarcinogenesis remains controversial.[4,42,43] In this study, the decision-tree algorithm identified a Brinkman index of 400 or more as the third most significant factor in patients with an albumin level of 4.01 g/dL or more and APRI of 0.5 or more. Thus, smoking may exert hepatocarcinogenic activity under specific conditions.

Given the role of changes in serum albumin level and relative thrombocytopenia in identifying patients with NBNC-HCC, it may be that the basis of the high predictivity of albumin and APRI for NBNC-HCC is on the basis of consistently identifying patients with cirrhosis, and thus risk for HCC, in this population.

A limitation of this study is that we did not evaluate the impact of occult HBV infection on the incidence of NBNC-HCC because HBV DNA was not tested in the health screening examination. HBV is one of the most transmitted infectious diseases and a cryptogenic cause of HCC;[44] therefore, further studies will be focused on its effect on hepatocarcinogenesis. Another limitation is that we did not evaluate the serum amino acid level, because control data were obtained from a health screening database. Changes in amino acid levels, particularly changes in branched-chain amino acids to tyrosine ratio, are known to precede a reduction in serum albumin level.[45] Thus, the impact of amino acid imbalance on the incidence of NBNC-HCC is an issue which needs to be clarified.

In conclusion, this study has identified independent factors associated with the incidence of NBNC-HCC, such as the Brinkman index, use of antidiabetic agents, hemoglobin level, and serum levels of GGT, total cholesterol and albumin. In addition, random forest analysis showed that serum albumin levels were the most distinguishable factor, and a decision-tree algorithm revealed that it is the initial split variable for the incidence of NBNC-HCC. Thus, data-mining analyses revealed that serum albumin levels were a notable factor associated with the incidence of NBNC-HCC. Furthermore, we obtained a profile associated with the incidence of NBNC-HCC, which consists of albumin and GGT levels, APRI and the Brinkman index. This simple profile could be used in a possible screening strategy for NBNC-HCC.

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Amphipathic α-Helices in Apolipoproteins Are Crucial to the Formation of Infectious Hepatitis C Virus Particles

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Abstract

Apolipoprotein B (ApoB) and ApoE have been shown to participate in the particle formation and the tissue tropism of hepatitis C virus (HCV), but their precise roles remain uncertain. Here we show that amphipathic α-helices in the apolipoproteins participate in the HCV particle formation by using zinc finger nucleases-mediated apolipoprotein B (ApoB) and/or ApoE gene knockout Huh7 cells. Although Huh7 cells deficient in either ApoB or ApoE gene exhibited slight reduction of particles formation, knockout of both ApoB and ApoE genes in Huh7 (DKO) cells severely impaired the formation of infectious HCV particles, suggesting that ApoB and ApoE have redundant roles in the formation of infectious HCV particles. cDNA microarray analyses revealed that ApoB and ApoE are dominantly expressed in Huh7 cells, in contrast to the high level expression of all of the exchangeable apolipoproteins, including ApoA1, ApoA2, ApoC1, ApoC2 and ApoC3 in human liver tissues. The exogenous expression of not only ApoE, but also other exchangeable apolipoproteins rescued the infectious particle formation of HCV in DKO cells. In addition, expression of these apolipoproteins facilitated the formation of infectious particles of genotype 1b and 3a chimeric viruses. Furthermore, expression of amphipathic α-helices in the exchangeable apolipoproteins facilitated the particle formation in DKO cells through an interaction with viral particles. These results suggest that amphipathic α-helices in the exchangeable apolipoproteins play crucial roles in the infectious particle formation of HCV and provide clues to the understanding of life cycle of HCV and the development of novel anti-HCV therapeutics targeting for viral assembly.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files except for the cDNA array data of GSE32886 which is available from GEO (Gene Expression Omnibus) under the accession number GSE32886.

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Introduction

More than 160 million individuals worldwide are infected with hepatitis C virus (HCV), and cirrhosis and hepatocellular carcinoma induced by HCV infection are life-threatening diseases [1]. Current standard therapy combining peg-interferon (IFN), ribavirin (RBV) and a protease inhibitor has achieved a sustained virological response (SVR) in over 80% of individuals infected with HCV genotype 1 [2]. In addition, many antiviral agents targeting non-structural proteins and host factors involved in HCV replication have been applied in clinical trials [3,4].

In vitro systems have been developed for the study of HCV infection and have revealed many details of the life cycle of HCV. By using pseudotype particles bearing HCV envelope proteins and RNA replicon systems, many host factors required for entry and RNA replication have been identified, respectively [5,6]. In addition, development of a robust *in vitro* propagation system of HCV based on the genotype 2a JFH1 strain (HCVcc) has gradually clarified the mechanism of assembly of HCV particles [7,8]. It has been shown that the interaction of NS2 protein with structural and non-structural proteins facilitates assembly of the viral capsid and formation of infectious particles at the connection site between the ER membrane and the surface of lipid droplets (LD) [9]. On the other hand, very low density lipoprotein (VLDL) associated proteins, including apolipoprotein B (ApoB), ApoE, and microsomal triglyceride transfer protein (MTTP), have been shown to play crucial roles in the formation of infectious HCV particles [10–12]. Generally, ApoA, ApoB, ApoC and ApoE bind the surface of lipoprotein through the interaction between amphipathic α-helices and ER-derived membrane [13,14]. This