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.).
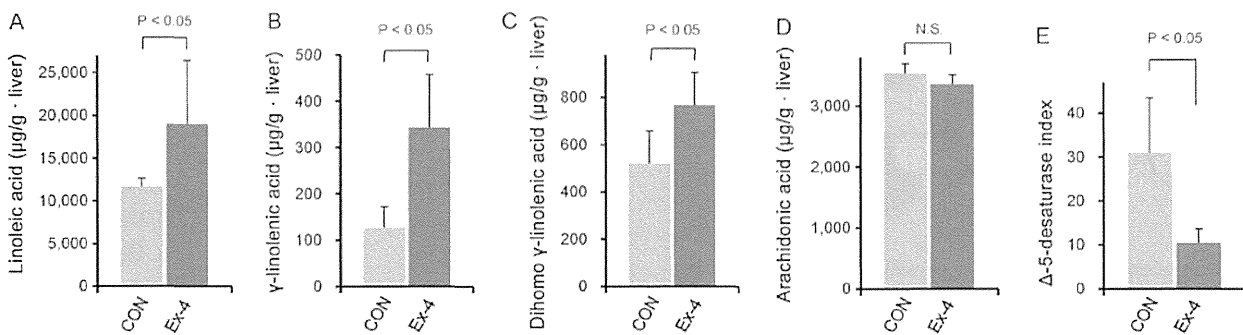

Figure 4. Effects of exendin-4 (Ex-4) on hepatic n-6 polyunsaturated fatty acids (PUFAs). (A) Linoleic acid, (B) γ-linolenic acid, (C) dihomo γ-linolenic acid, (D) arachidonic acid, and (E) Δ-5-desaturase index. P≤0.05 was considered statistically significant. Control (CON), not significant (N.S.).
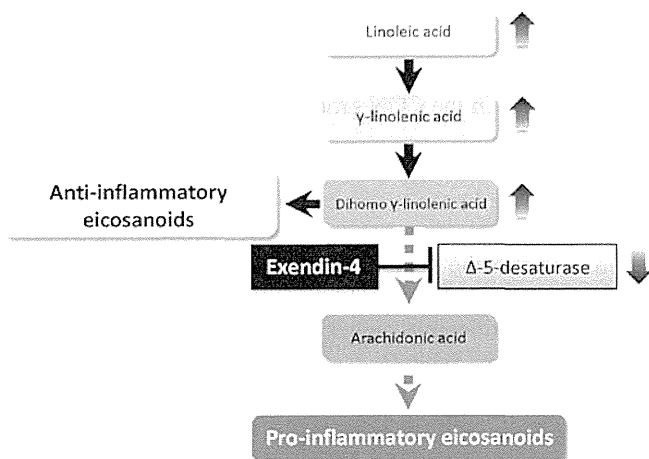

Figure 5. A scheme for exendin-4 (Ex-4)-caused alterations in lipid metabolism. Ex-4 may inhibit Δ-5-desaturase activity, resulting in arachidonic acid production and subsequent pro-inflammatory eicosanoids. Inhibition of Δ-5-desaturase activity also increases the hepatic content of dihomo γ-linolenic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

Acknowledgements

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VIRAL HEPATITIS

Independent factors associated with altered plasma active ghrelin levels in HCV-infected patients

Takumi Kawaguchi[1,2], Yumiko Nagao[1] and Michio Sata[1,2]

1 Department of Digestive Disease Information & Research, Kurume University School of Medicine, Kurume, Japan
2 Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan

Abstract
Background & Aims: Metabolic disorders are frequently seen in hepatitis C virus (HCV)-infected patients. Ghrelin, a gut hormone, regulates hepatic metabolisms, and must be activated to exert its biological effects. The aims of this study were to investigate changes in plasma active ghrelin levels and identify independent factors associated with plasma active ghrelin levels in HCV-infected patients. *Methods:* We enrolled patients with HCV infection ($n = 96$), hepatitis B virus (HBV) infection ($n = 49$), non-alcoholic fatty liver disease (NAFLD; $n = 20$) and healthy subjects (CON; $n = 16$). Plasma active ghrelin levels were measured using ELISA. Factors associated with plasma active ghrelin levels were assessed by multivariate and Spearman's correlation analyses. *Results:* Plasma active ghrelin levels were significantly lower in relation to the severity of liver disease in both the HBV and HCV groups. Furthermore, HCV infection was identified as an independent factor associated with decreased plasma active ghrelin levels in the multivariate analysis (OR -3.05; 95% CI -0.93 to -19.51; $P = 0.0192$). Plasma active ghrelin levels were significantly correlated with serum albumin levels in the HCV group ($\rho = 0.497$, $P < 0.0001$). *Conclusions:* We demonstrated that liver cirrhosis and HCV infection were independent factors associated plasma active ghrelin levels. Moreover, plasma active ghrelin levels were positively correlated with serum albumin levels among HCV-infected patients. Therefore, active ghrelin levels may be regulated by both progression of liver disease and HCV infection and could be involved in the regulation of serum albumin levels in HCV-infected patients.

Various metabolic disorders are more frequently seen in patients with chronic hepatitis C virus (HCV) infection than in those with other hepatobiliary diseases (1, 2). Metabolic disorders including insulin resistance and hypoalbuminemia are risk factors for the development of hepatocellular carcinoma and oesophageal varices, and thus, for increased mortality (3, 4). These metabolic disorders are seen even in early stage chronic liver disease (1, 5) and are partly caused by HCV-induced metabolic dysregulation in multiple organs including the liver, pancreas and intestine (6–8).

Ghrelin, a gut hormone, predominantly secreted from X/A-like stomach cells, was originally identified as a potent growth hormone secretagogue (9). Ghrelin also regulates appetite and its downregulation is involved in the development of malnutrition (10–12). Basic studies have demonstrated that ghrelin plays a significant role in glucose metabolism, fatty acid beta oxidation, hepatic fibrosis and cell proliferation (13–17). Therefore, changes in ghrelin could be associated with various metabolic disorders in HCV-infected patients.

Ghrelin is secreted as a preprohormone; therefore, it must be activated to exert its biological effects, which are tightly regulated by a unique mechanism. Preproghrelin is acylated by ghrelin O-acyltransferase in the serine-3 residue. The acylated peptide is then cleaved by a processing protease prohormone convertase 1/3 to produce active ghrelin (18, 19). Thus, ghrelin is classified into 2 isoforms: des-acyl ghrelin (the inactive form) and acylated ghrelin, so-called "active ghrelin." In healthy adults, approximately 90% of serum ghrelin exists in the inactive form and 10% exists in the active form (20). Although several previous studies have shown inconsistent results regarding serum total ghrelin levels among patients with chronic liver diseases (12, 21–24), changes in plasma active ghrelin level and their clinical association with metabolic disorders have not yet been investigated in HCV-infected patients.

The aims of this study were to investigate changes in plasma active ghrelin levels and identify independent factors associated with plasma active ghrelin levels in HCV-infected patients.

Subjects and methods

Ethics statement

The study protocol was approved by The Ethics Committee of Kurume University. All experiments were carried out in accordance with the Declaration of Helsinki. No subjects were institutionalized.

Materials

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

Patients

We enrolled consecutive patients with HCV-related liver disease (HCV; $n = 96$), hepatitis B virus (HBV)-related liver disease (HBV; $n = 20$), non-alcoholic fatty liver disease (NAFLD; $n = 49$) and healthy control subjects (CON; $n = 16$). All patients and controls were Asian. All diagnoses were based on clinical, serological and/or histological evidence. All data were collected on the same day as collecting blood. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in metres (kg/m^2).

Laboratory tests

Venous blood samples were taken in the morning after a 12-h overnight fast. Plasma glucose, serum aspartate aminotransferase (AST), alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyl transpeptidase, total protein, albumin, prothrombin activity, blood glucose, immunoreactive insulin (IRI), haemoglobin A1c, total cholesterol, free fatty acids, total bilirubin, type IV collagen, hyaluronic acid, blood urea nitrogen, creatinine, alpha-foetoprotein and protein induced by vitamin K absence or antagonist-II levels were measured using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital) as previously described (25). Insulin resistance was calculated on the basis of fasting levels of plasma glucose and IRI using the homoeostasis model assessment for insulin resistance (HOMA-IR) equation: fasting glucose (mg/dl) × fasting IRI (mU/ml)/405 (26).

Determination of HCV genotype and measurement of HCV viral load

HCV genotyping was performed using Okamoto's method (27), and genotypes were classified according to Simmonds' classification system (28). An Amplicor-HCV-Monitor 1.0 (Roche Diagnostics K.K., Tokyo, Japan) was used to quantify HCV RNA levels.

Diagnosis of cirrhosis

Cirrhosis was diagnosed by liver histology or AST to platelet ratio index (APRI): serum AST level (U/L)/ upper limit of normal AST (33 U/L) × 100/platelet count (× 10^4/ml). APRI is a non-invasive index and can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Patients with APRI values above 1.5 were diagnosed with cirrhosis as previously described (29). In this study, out of 96 patients in the HCV group, 18 patients were diagnosed by liver histology and the remaining 78 patients were diagnosed by APRI.

Plasma active ghrelin level assay

For measurement of plasma active ghrelin levels, 1 ml of blood was collected in a tube containing 1.25 mg of ethylenediaminetetraacetic acid and 500 KIU of aprotinin, a serine protease inhibitor. Each sample was immediately centrifuged at 1500g for 15 min at 4°C and treated with 10% vol/vol of 1 mM hydrochloric acid. Quantification of active ghrelin in plasma samples was accomplished with an Active Ghrelin ELISA Kit (SCETI K.K., Tokyo, Japan) that specifically quantifies active ghrelin. This ELISA kit consists of monoclonal antibodies for both the C-terminal and the acylated N-terminal of ghrelin. The absorbance (450 nm) of each well was then measured with a Bio-Rad Model 550 microplate reader (Bio-Rad, Hercules, CA, USA). Each serum sample was assayed in duplicate and the values were averaged.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical comparisons among multiple groups were performed by analysis of variance (ANOVA) followed by Scheffé's *post-hoc* test. Stepwise forward-selection multivariate linear regression analysis was used to identify any independent variables related to plasma active ghrelin levels, as previously described (30, 31). Spearman's correlation coefficient was calculated to test the relationship between different quantities in a bivariate regression model using JMP version 9.0 (SAS Institute, Cary, NC, USA). *P* values < 0.05 were considered significant.

Results

Patient characteristics

The characteristics of enrolled patients are summarized in Table 1. Although serum levels of AST and ALT were significantly higher in the NALFD, HBV and HCV groups than those in the CON group, these levels were not significantly different among the NAFLD, HBV and HCV groups. Serum albumin levels were significantly lower in the HBV and HCV groups than in the CON and NAFLD groups. Serum total cholesterol levels revealed significant depletion in the HCV group compared with the other groups. Significantly elevated

Table 1. Patient characteristics

	Reference value	CON	NAFLD	HBV	HCV
n	18.5–22	16	20	49	96
Age (yr)	N/A	67.5 ± 5.3	62.6 ± 10.8	57.3 ± 3.1*	63.5 ± 12.5
Sex (Female/Male)	N/A	7/9	12/8	29/20	58/38
BMI (m²/kg)	18.5–22	22.3 ± 0.9	26.8 ± 2.2*	22.3 ± 2.8	23.5 ± 3.5
AST (U/L)	13–33	10.6 ± 3.4	35.5 ± 22.6†	31.5 ± 23.8†	47.9 ± 28.5†
ALT (U/L)	8–42	12.7 ± 5.1	41.2 ± 24.4†	35.9 ± 27.8†	45.2 ± 34.6†
LDH (U/L)	119–229	192 ± 51	201 ± 35	201 ± 52	210 ± 42
ALP (U/L)	115–359	242 ± 53	262 ± 106	261 ± 125	281 ± 119
GGT (U/L)	10–47	31.5 ± 17.2	51.6 ± 53.4†	42.2 ± 28.6†	53.2 ± 68.1†
Total protein (g/dl)	6.70–8.30	8.1 ± 0.5	7.7 ± 0.3	7.3 ± 0.5†	7.1 ± 0.6†
Albumin (g/dl)	4.00–5.00	4.3 ± 0.3	4.3 ± 0.5	3.8 ± 0.7‡	3.7 ± 0.6‡
Prothrombin activity (%)	60–130	105.2 ± 13.3	108.7 ± 25.0	94.0 ± 8.8‡	91.6 ± 19.3*
Total bilirubin (mg/dl)	0.30–1.50	0.6 ± 0.2	0.9 ± 0.5	0.8 ± 0.7	1.1 ± 0.5†
C-reactive protein (mg/dl)	<0.40	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
Total cholesterol (mg/dl)	128–220	197 ± 41	251 ± 66*	199 ± 42	163 ± 42*
Free fatty acids (μEq/L)	100–540	286 ± 75	383 ± 186†	375 ± 131†	665 ± 362*
Fasting blood glucose (mg/dl)	80–109	96 ± 5	121 ± 35†	103 ± 12†	109 ± 31†
Haemoglobin A1c (%)	4.3–5.8	5.3 ± 0.4	6.0 ± 0.7*	5.6 ± 0.4	5.3 ± 0.9
IRI (μIU/ml)	5.0–20.0	6.1 ± 2.3	9.5 ± 6.5§	7.5 ± 4.4	14.3 ± 8.5§
HOMA-IR	<2.5	1.3 ± 0.4	2.6 ± 1.6§	2.0 ± 1.1	3.8 ± 1.5§
BUN (mg/dl)	8.0–22.0	14.5 ± 7.1	14.2 ± 4.3	17.2 ± 2.8	15.8 ± 4.3
Creatinine (mg/dl)	0.4–0.7	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.6 ± 0.2
Hyaluronic acid (ng/ml)	< 50	N/A	31.6 ± 12.5	98.5 ± 72.5‖	182.8 ± 155.2*
Type IV collagen (ng/ml)	< 140	N/A	88 ± 68	198 ± 121‖	252 ± 146*
AFP (ng/ml)	<8.7	N/A	3.4 ± 1.2	7.8 ± 2.1‖	14.8 ± 36.2*
PIVKA-II (mAU/ml)	<40	N/A	18.5 ± 12.2	21.5 ± 16.2	28.5 ± 18.8

Data are expressed as mean ± SD or number of patients.
*P < 0.05 compared to all of other groups.
†P < 0.05 compared to the CON group.
‡P < 0.05 compared to the CON and NAFLD groups.
§P < 0.05 compared to the CON and HBV groups.
‖P < 0.05 compared to the HBV group.
CON, healthy control subjects; NAFLD, non-alcoholic fatty liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus; N/A, not applicable; AST, aspartate transaminase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; IRI, Immunoreactive insulin; HOMA-IR, homoeostasis model assessment for insulin resistance; BUN, blood urea nitrogen; AFP, α-foetoprotein; PIVKA-II, protein induced by vitamin K absence.

serum insulin and HOMA-IR levels were observed in the HCV group compared with those in the CON and HBV groups. Serum levels of hyaluronic acid and type IV collagen were significantly higher in the HCV group than in the other groups.

Changes in plasma active ghrelin levels in chronic liver disease

There were no significant differences in plasma active ghrelin levels among the CON, NAFLD and HBV groups. However, plasma active ghrelin levels were significantly lower in the HCV group than in the other groups (Fig. 1).

Changes in plasma active ghrelin levels in relation to the progression of liver disease

In the HBV groups, plasma active ghrelin levels were significantly decreased in relation to the severity of liver

disease (compare with dotted lines in Fig. 2). Similarly, significant decreases in plasma active ghrelin levels in relation to severity of liver disease were seen in the HCV group (compare with dotted lines in Fig. 2).

The association of plasma active ghrelin levels with virological factors and disease severity in HCV-infected patients

No significant differences in plasma active ghrelin levels were seen among HCV genotypes 1b, 2a and 2b. There was no significant correlation between plasma active ghrelin levels and HCV viral load (ρ = 0.589, P < 0.396).

Stage-matched comparisons of plasma active ghrelin levels

In patients with chronic hepatitis, plasma active ghrelin levels were significantly lower in the HCV group than in the HBV or NAFLD group (compare with solid lines in
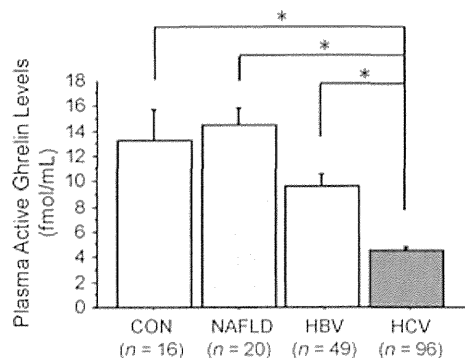
Fig. 1. Changes in plasma active ghrelin levels in the CON, NAFLD, HBV and HCV groups. Values are expressed as mean ± SD. Comparisons among groups were made using analysis of variance with Scheffé's *post-hoc* test. *, *P* < 0.01.

Table 2. Multivariate analysis of plasma active ghrelin levels

Variable	Odds ratio	95% confidence interval	*P* value
Liver cirrhosis	−1.82	−0.37 to −4.33	0.0359
Serum albumin levels	2.39	1.32 to 7.95	0.0251
HCV infection	−3.05	−0.93 to −19.51	0.0192

HCV, hepatitis C virus.

Correlations between plasma active ghrelin levels and metabolic parameters in HCV-infected patients

A significant negative correlation was observed between plasma active ghrelin levels and BMI ($\rho = -0.137$, $P = 0.0171$) (Table 3). Although plasma active ghrelin levels were not significantly correlated with the parameters indicated in Table 3, plasma active ghrelin levels tend to be correlated with total cholesterol, total bilirubin, type IV collagen and creatinine levels. A significant positive correlation was seen only between plasma active ghrelin levels and serum albumin levels ($\rho = 0.497$, $P < 0.0001$) (Table 3 and Fig. 3).
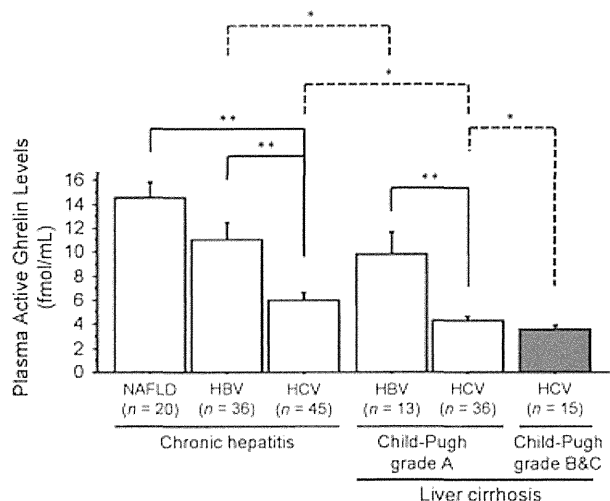

Fig. 2. Changes in plasma active ghrelin levels stratified by aetiology and progression of liver disease. Values are expressed as mean ± SD. Comparisons among the groups were made using analysis of variance with Scheffé's *post-hoc* test. *, *P* < 0.05; **, *P* < 0.01.

Fig. 2). In cirrhotic patients with Child-Pugh grade A, a significant decrease in plasma active ghrelin levels was also seen in the HCV group compared with the HBV group (compare with solid lines in Fig. 2).

Multivariate analysis of plasma active ghrelin levels

In the multivariate analysis, the presence of cirrhosis and low serum albumin levels were independent factors associated with decreased plasma active ghrelin levels (Table 2). In addition, the presence of HCV infection was identified as the most significant independent factor associated with lowering of plasma active ghrelin levels (Table 2).

Discussion

In this study, we demonstrated that plasma active ghrelin levels were significantly decreased in relation to the progression of liver disease in both HBV- and HCV-infected patients. In addition, HCV infection was identified as the most significant factor associated with decreased plasma active ghrelin levels, independent of the presence of cirrhosis and serum albumin levels. In HCV-infected patients, plasma active ghrelin levels were positively correlated with serum albumin levels. Taken together, these findings suggest that HCV itself, along with cirrhosis, may be involved in decreasing plasma active ghrelin levels, which could be linked to serum albumin levels.

In this study, plasma active ghrelin levels were significantly lower in the HCV group than in the other groups. To assess the effect of advanced liver disease on the plasma ghrelin levels, we performed stratified analyses based on the severity of liver disease. The results indicated that plasma active ghrelin levels significantly decreased in relation to the severity of liver disease in the HBV and HCV groups. The presence of cirrhosis was also identified as an independent factor associated with plasma active ghrelin levels in multivariate analysis. Although changes in total ghrelin levels in cirrhosis remain a controversial subject (12, 21, 24), we have demonstrated that active ghrelin was decreased in patients with cirrhosis. The causal relationship between advanced cirrhosis and changes in active ghrelin levels remains unclear. Plasma active ghrelin levels are regulated by production of preproghrelin or activation of ghrelin (11, 32, 33). Therefore, it is likely that chronic liver injury may change X/A-like stomach cells and

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Table 3. Correlations between plasma active ghrelin levels and variables

Variables	ρ	P
Age	0.048	0.6972
BMI	−0.137	0.0171
AST	−0.068	0.5083
ALT	0.021	0.8371
LDH	0.093	0.3660
ALP	−0.139	0.1788
GGT	0.142	0.1674
Total protein	−0.049	0.6323
Albumin	0.497	<0.0001
Prothrombin activity	0.163	0.1621
Total bilirubin	−0.210	0.0533
Total cholesterol	0.194	0.0595
Free fatty acids	−0.190	0.4469
Fasting blood glucose	−0.142	0.1682
Haemoglobin A1c	0.162	0.1201
IRI	0.160	0.2591
HOMA-IR	0.120	0.3968
BUN	0.077	0.4508
Creatinine	0.182	0.0766
Type IV collagen	0.378	0.0639
Hyaluronic acid	0.273	0.2910
AFP	0.106	0.3022
PIVKA-II	0.044	0.6835

BMI, body mass index; AST, aspartate transaminase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; IRI, Immunoreactive insulin; HOMA-IR, homoeostasis model assessment for insulin resistance; BUN, blood urea nitrogen; AFP, α-foetoprotein; PIVKA-II, protein induced by vitamin K absence.
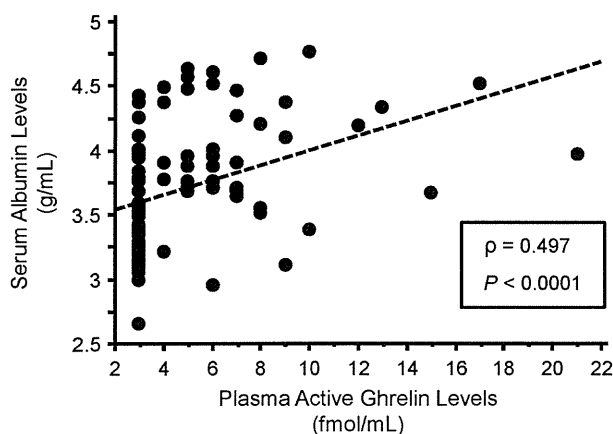
Fig. 3. The association between plasma active ghrelin levels and serum albumin levels. This association was analysed using the Spearman's correlation coefficient in a bivariate regression model.

ghrelin-O-acyl transferase and/or prohormone convertase 1/3.

We also examined the effect of HCV infection on plasma ghrelin levels. HCV genotype and HCV viral load were not associated with plasma active ghrelin

levels, however; plasma active ghrelin levels were significantly lower in the HCV group than in the NAFLD or HBV group in stage-matched analyses. Moreover, HCV infection was identified as the most significant factor associated with lowering of plasma active ghrelin levels, independent of the presence of cirrhosis. These findings suggest that HCV itself may be involved in decreasing plasma active ghrelin levels. Although the mechanisms of HCV-related decreases in plasma ghrelin levels remain unclear, serum leptin levels are known to be high in HCV-infected patients (34, 35). Given that leptin is a potent inhibitor of ghrelin secretion (36, 37), HCV may suppress ghrelin secretion through up-regulation of leptin.

Various metabolic disorders are frequently seen in cirrhotic patients with HCV infection (38, 39). Therefore, we investigated the correlation between plasma active ghrelin levels and various metabolic parameters in the HCV group. Consistent with the previous reports (40, 41), in this study, plasma active ghrelin levels were found to be negatively correlated with BMI. These changes in plasma active ghrelin levels may represent a physiological adaptation to the positive energy balance associated with obesity. Furthermore, albumin was the only serum parameter, which significantly positively correlated with plasma active ghrelin levels in this study. Serum albumin is a representative marker for both protein metabolism and the severity of liver disease (42); however, other protein metabolism parameters such as total protein level and prothrombin activity were not correlated with plasma active ghrelin levels in this study. Ghrelin is a potent stimulator for secretion of growth hormone (33) and also elicits marked up-regulation of the mammalian target of rapamycin (mTOR) signalling pathway (43). Both growth hormone and activation of mTOR signalling up-regulate albumin synthesis (39, 44). Taken together, these findings suggest that active ghrelin has the novel biological effect on specifically regulating albumin synthesis in hepatocytes.

One limitation of this study is that we did not investigate changes in HCV or HBV carriers with persistently normal ALT levels. As these data may further elucidate the mechanisms underlying the decrease in plasma active ghrelin in patients with liver disease, further study should be conducted while bearing these points in mind.

In conclusion, this study showed that plasma active ghrelin levels were significantly decreased with the progression of liver disease in both HBV- and HCV-infected patients. Moreover, HCV infection was identified as the most significant factor associated with decreased plasma active ghrelin levels, independent of cirrhosis. Furthermore, plasma active ghrelin levels were positively correlated with serum albumin levels in HCV-infected patients. These findings indicated that active ghrelin may be regulated by both the progression of liver disease and HCV infection, and could be related to serum albumin levels.

Acknowledgements

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Short Communication

Lipid profile is associated with the incidence of cognitive dysfunction in viral cirrhotic patients: A data-mining analysis

Eitaro Taniguchi,[1] Takumi Kawaguchi,[1,2] Masahiro Sakata,[1] Minoru Itou,[1] Tetsuharu Oriishi[1] and Michio Sata[1,2]

[1]*Division of Gastroenterology, Department of Medicine, and* [2]*Department of Digestive Disease Information and Research, Kurume University School of Medicine, Kurume, Japan*

Aim: Cognitive dysfunction (CD) is frequently observed in cirrhotic patients. However, the biochemical profiles associated with CD remain unclear. We investigated the biochemical profiles associated with the incidence of CD in cirrhotic patients by using multivariate analyses, including a decision-tree algorithm.

Methods: In this study, 27 viral cirrhotic patients were enrolled. All subjects underwent neuropsychiatric tests; two or more abnormal results were defined as CD. A logistic regression model was used for multivariate stepwise analysis. A decision-tree algorithm was constructed, and the categorical differences based on the decision-tree model were analyzed by χ^2-tests.

Results: Multivariate stepwise analysis showed the levels of total bilirubin, triglycerides and free fatty acids (FFA) as independent bioparameters associated with the incidence of CD in cirrhotic patients. The decision-tree algorithm showed that among patients with FFA of 514 mEq/L or more, 77.8% had CD. Meanwhile, among patients with FFA of less than 514 mEq/L and triglycerides of 106 mg/dL or more, 20.0% had CD. The sensitivity, specificity and accuracy for the incidence of CD using the lipid profile (FFA >514 mEq/L or triglycerides <106 mg/dL) were 85.7% (12/14), 61.5% (8/13) and 74.1% (20/27), respectively.

Conclusion: The levels of total bilirubin, FFA and triglycerides are independently associated with the incidence of CD in cirrhotic patients. In addition, a decision-tree algorithm revealed that FFA of more than 514 mEq/L or triglycerides of less than 106 mg/dL is a profile associated with the incidence of CD. Thus, this lipid profile could be a possible screening bioparameter for CD in cirrhotic patients.

Key words: decision-tree algorithm, fatty acid, minimal hepatic encephalopathy, neuropsychiatric test

INTRODUCTION

CIRRHOSIS IS FREQUENTLY accompanied by various complications, including esophageal varices

and hepatocellular carcinoma.[1,2] Cognitive dysfunction (CD) is another frequent complication in patients with chronic liver disease and is known as minimal hepatic encephalopathy or subclinical hepatic encephalopathy.[3,4] CD predicts the development of hepatic encephalopathy and poor prognosis.[5,6] Moreover, CD itself is associated with impaired health-related quality of life[7–9] and serious social issues such as falls and motor vehicle accidents.[10–16] Therefore, CD is one of the critical complications of chronic liver disease.

Because bacterial overgrowth in the intestine and delayed gastrointestinal transit time are associated with the development of CD,[17] ammonia and pro-inflammatory cytokines derived from enteric bacterial flora are thought to be pathogenic factors of CD. In fact, treatment with gut-specific agents such as lactulose and rifaximin can improve CD in cirrhotic patients.[18–22]

However, CD is not always correlated with the severity of liver disease, blood levels of ammonia or inflammation.[6] CD can also be caused by malnutrition, cerebrovascular disease secondary to diabetes mellitus and psychoactive agents.[4,12,23,24] These previous reports suggest that complicated interactions between various factors underlie the development of CD in cirrhotic patients.

Data-mining analysis is a set of statistical techniques used to reveal complex interactions within a dataset.[25,26] A decision-tree algorithm is an exploratory data-mining analysis technique that is a series of rules for classification by identifying priorities.[26] This is a quantitative systematic approach that allows clinicians to maximize the net benefit to patients.[27] Decision-tree algorithms are now clinically applied to predict the following issues: response to interferon treatment of hepatitis C virus (HCV);[28] severity of hepatic fibrosis;[29] progression of hepatocellular carcinoma;[29] safety of hepatic resection;[30] outcome of patients with acute liver failure;[31] and dietary factors for normalizing serum alanine aminotransferase levels in patients with HCV infection.[26]

The aim of this study is to investigate the profiles associated with the incidence of CD by using multivariate analyses, including the decision-tree algorithm in cirrhotic patients.

METHODS

Subjects

CIRRHOTIC PATIENTS WHO were followed up at Kurume University Hospital were enrolled in this study. The inclusion criteria were viral liver cirrhosis, aged less than 70 year and able to undergo neuropsychiatric (NP) tests. The exclusion criteria were a history of overt hepatic encephalopathy and treatment for transjugular intrahepatic portosystemic shunt or esophagogastric varices. Finally, 27 subjects were enrolled in this study.

Informed consent was obtained from all the subjects. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in prior approval by the Ethics Committee of the Kurume University School of Medicine. None of the participants was institutionalized.

NP tests and definition of CD

The subjects underwent NP tests, including the block design test, digit symbol test, and number connection tests A and B. Patients with two or more abnormal results in these tests were defined as having CD, as described previously.[32]

Measurement of biochemical parameters

Venous blood samples were collected in the morning after overnight fasting. Biochemical parameters were measured by conventional clinical methods (Department of Clinical Laboratory, Kurume University Hospital), as described previously.[33,34]

Statistical analysis

Data are expressed as mean ± standard deviation. Nonparametric multiple comparisons were made by the Mann–Whitney U-test. Categorical comparisons were made by Fisher's exact test. A logistic regression model was used for multivariate stepwise analysis. A decision-tree algorithm was constructed, and the categorical differences in the decision-tree model were analyzed by χ^2-tests, as described previously.[25,26] The level of statistical significance was set at $P < 0.05$.

RESULTS

Analysis of bioparameters associated with CD

THE CHARACTERISTICS OF patients with and without CD are shown in Table 1. Univariate analysis revealed no significant differences between cirrhotic patients with and without CD in age, sex or Child–Pugh grade. No significant differences were observed between patients with and without CD in biochemical parameters such as the ammonia level, branched-chain amino acid/tyrosine ratio, zinc level or Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR) value. In addition, fasting glucose and HOMA-IR were not significantly different between CD and non-CD patients with fasting glucose of less than 140 mg/dL ($P = 0.9096$ in fasting glucose and $P = 0.7055$ in HOMA-IR).

Multivariate stepwise analysis identified the levels of total bilirubin, triglycerides and free fatty acids (FFA) as independent bioparameters associated with the incidence of CD (Table 2).

Decision-tree algorithm for CD

The decision-tree algorithm showed that all the subjects were classifiable into three groups on the basis of two variables (Fig. 1). FFA was selected as the initial split variable with a cut-off value of 514 mEq/L. Among the nine patients with FFA of 514 mEq/L or more, seven

Table 1 Characteristics of all subjects

	CD	No CD	*P* value
n	14	13	
Age	59.8 ± 8.0	62.8 ± 6.1	N.S.
Sex (F/M)	5/9	7/6	N.S.
Child–Pugh (A/B/C)	12/2/0	7/5/1	N.S.
Aspartate aminotransferase (U/L)	68.8 ± 27.4	63.4 ± 24.7	N.S.
Alanine aminotransferase (U/L)	73.1 ± 74.8	56.0 ± 29.8	N.S.
Alkaline phosphatase (U/L)	416.0 ± 400.2	366.6 ± 187.6	N.S.
γ-Glutamyltransferase (U/L)	87.2 ± 128.5	56.2 ± 39.6	N.S.
Total bilirubin (mg/dL)	1.11 ± 0.49	1.53 ± 0.83	N.S.
Albumin (g/dL)	3.46 ± 0.41	3.32 ± 0.76	N.S.
Prothrombin time (%)	86.4 ± 10.2	74.5 ± 17.3	N.S.
Ammonia (μg/dL)	59.2 ± 25.2	58.1 ± 31.9	N.S.
Fasting glucose (mg/dL)	136.4 ± 60.1	127.8 ± 56.4	N.S.
Hemoglobin A1c (%)	5.8 ± 1.9	5.6 ± 1.3	N.S.
Fasting immunoreactive insulin (μU/mL)	16.8 ± 14.0	13.9 ± 6.8	N.S.
HOMA-IR	6.20 ± 7.41	4.92 ± 5.07	N.S.
Total cholesterol (mg/dL)	157.2 ± 31.3	152.5 ± 29.7	N.S.
Triglyceride (mg/dL)	96.6 ± 33.9	116.4 ± 47.2	N.S.
Free fatty acids (mEq/L)	567.9 ± 272.9	453.5 ± 229.1	N.S.
Iron (μg/dL)	274.8 ± 560.0	160.4 ± 63.0	N.S.
Ferritin (ng/mL)	194.6 ± 248.3	129.5 ± 115.5	N.S.
Zinc (μg/dL)	63.6 ± 9.7	59.8 ± 22.0	N.S.
Branched-chain amino acids/tyrosine ratio	3.54 ± 1.26	3.54 ± 1.60	N.S.

Data are expressed number of mean ± standard deviation.
CD, cognitive dysfunction; HOMA-IR, Homeostasis Model of Assessment – Insulin Resistance; N.S., not significant.

(77.8%) had CD. Meanwhile, among the 18 patients with FFA of less than 514 mEq/L, seven (38.9%) had CD.

Triglycerides were selected as the second split variable with a cut-off value of 106 mg/dL. Among patients with FFA of less than 514 mEq/L, five patients (62.5%) had

Table 2 Logistic regression analysis for CD

	OR	95% CI	*P*-value
Total bilirubin	0.002	5.708e-7–0.154	<0.05
Triglyceride	0.889	0.748–0.964	<0.05
Fee fatty acids	1.015	1.004–1.037	<0.05
Total cholesterol	1.119	1.024–1.323	N.S.
HOMA-IR	2.053	0.889–7.631	N.S.
Zinc	0.915	0.784–1.010	N.S.
Fasting glucose	1.033	0.989–1.102	N.S.
Ammonia	1.063	0.964–1.186	N.S.

CD, cognitive dysfunction; CI, confidence interval; HOMA-IR, Homeostasis Model of Assessment – Insulin Resistance; N.S., not significant; OR, odds ratio.
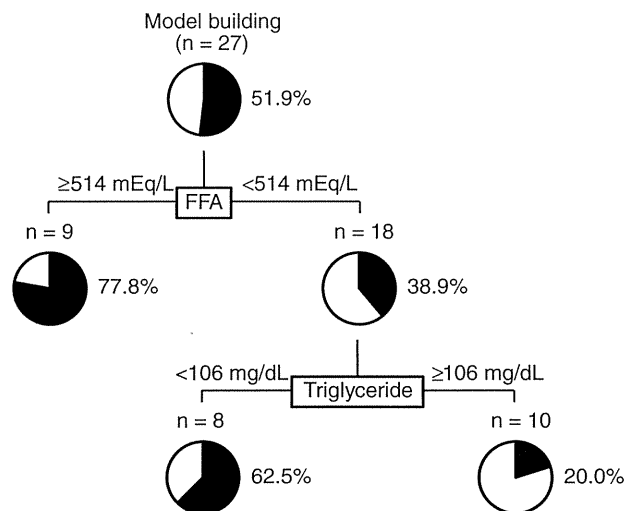

Figure 1 Decision-tree algorithm for cognitive dysfunction (CD). The subjects were classified according to the indicated cut-off values of the variables. The pie graphs indicate the percentage of CD (black)/no CD (white) in each group. FFA; free fatty acids.

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Table 3   Biochemical profiles and the incidence of CD

	CD	No CD
FFA ≥514 mEq/L or triglyceride <106 mg/dL	12	5
FFA <514 mEq/L and triglyceride ≥106 mg/dL	2	8

CD, cognitive dysfunction; FFA, free fatty acids.

CD among the eight patients with triglycerides of less than 106 mg/dL, while two patients (20.0%) had CD among the 10 patients with triglycerides of 106 mg/dL or more.

The distribution of CD differed significantly between the groups ($P = 0.0325$).

Categorical differences according to the decision-tree algorithm for CD

According to the results of the decision-tree algorithm, all subjects were classified into two groups: one group with FFA of 514 mEq/L or more or triglycerides of less than 106 mg/dL ($n = 17$), and another group with FFA of less than 514 mEq/L and triglycerides of 106 mg/dL or more ($n = 10$). The distribution of CD was significantly different between the groups ($P = 0.0183$) (Table 3). The sensitivity, specificity and accuracy using the cut-off values of FFA and triglycerides were 85.7% (12/14), 61.5% (8/13) and 74.1% (20/27), respectively.

DISCUSSION

THE RESULTS OF this study show that FFA and triglycerides were independent risk factors for CD in cirrhotic patients. Furthermore, data-mining analysis revealed that FFA of more than 514 mEq/L or triglycerides of less than 106 mg/dL is a profile associated with the incidence of CD in cirrhotic patients.

Hyperammonemia and inflammation are known to occur in the pathogenesis of CD in cirrhotic patients.[35-38] However, CD is not always correlated with the severity of liver disease, blood levels of ammonia or inflammation,[6] suggesting the presence of other pathogenic factors. In this study, we demonstrated that FFA and triglycerides are associated with the incidence of CD in cirrhotic patients. Although higher serum FFA levels and lower serum triglyceride levels can be thought to reflect hepatic insufficiency, serum albumin levels and blood ammonia were not identified as risk factors for CD in this study. Moreover, FFA and triglycerides were identified as independent risk factors. Although the precise

causal relationship between these factors and CD remains unclear, FFA and triglycerides vary with starvation and meal uptake. Malnutrition is associated with CD,[24] and eating breakfast is known to improve CD in cirrhotic patients.[39] Taken together, changes in lipid metabolism caused by starvation or meal uptake may pleiotropically affect the development of CD in cirrhotic patients.

Data-mining analysis provided FFA of more than 514 mEq/L as the initial classification, suggesting that FFA is the most closely related factor to the incidence of CD in cirrhotic patients. Although the relationship between FFA and CD is unclear, there are some possible explanations. Serum albumin is a carrier protein for various substances, including FFA and tryptophan.[40,41] An increase in FFA–albumin binding results in the dissociation of tryptophan from albumin and a subsequent increase in serum-free tryptophan levels.[41-43] Tryptophan can be transported into the brain across the blood–brain barrier and converted to 5-hydroxytryptamine, which is a neurotransmitter known to be associated with hepatic encephalopathy[44,45] as well as cognitive function.[46]

In patients with FFA of less than 514 mEq/L, triglycerides of less than 106 mg/dL was identified as the secondary classification. Contrary to our results, hypertriglyceridemia is a previously established risk factor for CD.[47,48] Although the reason for this discrepancy remains unclear, it can be speculated that hypertriglyceridemia may cause CD via the micro-impairment of cerebrovascular circulation.[49] Meanwhile, triglycerides, particularly medium-chain triglycerides, are structured lipids and good sources of energy in the brain.[50] In fact, patients with hypotriglyceridemia develop complications such as neurological manifestations with structural changes in the nerves.[51,52] Moreover, treatment with medium-chain triglycerides is reported to improve cognitive functioning in older adults with memory disorders.[53] Thus, lower FFA levels may cause CD via structural or functional nerve impairment.

Cognitive dysfunction occurs in up to 80% of patients at any stage of chronic liver disease[24] and is related not only to poor prognosis[54] but also to social issues, including falls and motor vehicle accidents.[10,13,14,16,55,56] Although NP tests are reliable tools for diagnosing CD in cirrhotic patients,[3,57] they are time-consuming (~30 min) and are affected by the educational status. Both electroencephalogram and critical flicker frequency are rapid tests for diagnosing CD. However, these tests require a trained personnel and

specialized equipment.[6,58] Therefore, a simple screening tool for CD is required for patients with chronic liver disease. In this study, we demonstrated that the lipid profile of FFA of more than 514 mEq/L or triglycerides of less than 106 mg/dL is associated with the incidence of CD with high sensitivity. Because evaluating serum FFA and triglyceride levels are simple objective assessments, further studies will focus on the significance of serum FFA and triglyceride levels as a screening bioparameter set for the presence of CD in patients with chronic liver disease.

The limitation of this study would be related to inclusion criteria. Contrary to expectation, a lower level of total bilirubin was identified as a risk factor. In this study, only the cirrhotic patients with no incidence of overt hepatic encephalopathy were enrolled. Because overt hepatic encephalopathy is generally seen in patients with decompensated cirrhosis or liver failure, the odds ratio of total bilirubin may be influenced by inclusion criteria. CD is also seen in cirrhotic patients with the treatment for overt hepatic encephalopathy; these patients should be included in further study.

In conclusion, the results of this study show that the serum levels of FFA and triglycerides are independently associated with the incidence of CD in cirrhotic patients. In addition, data-mining analysis revealed that FFA of more than 514 mEq/L or triglycerides of less than 106 mg/dL is a profile associated with the incidence of CD in cirrhotic patients. Thus, the lipid profile could be involved in the development of CD and could be considered as a possible screening bioparameter for CD in cirrhotic patients.

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Original Article

Fish to meat intake ratio and cooking oils are associated with hepatitis C virus carriers with persistently normal alanine aminotransferase levels

Momoka Otsuka,[1] Yuki Uchida,[2] Takumi Kawaguchi,[3,4] Eitaro Taniguchi,[4]
Atsushi Kawaguchi,[6] Shingo Kitani,[7] Minoru Itou,[4] Tetsuharu Oriishi,[4] Tatsuyuki Kakuma,[6]
Suiko Tanaka,[2] Minoru Yagi[1,5] and Michio Sata[3,4]

Departments of [1]Clinical Nutrition and [2]Nutrition, Kurume University Hospital, Departments of [3]Digestive Disease
Information & Research, [4]Medicine and [5]Pediatric Surgery, Kurume University School of Medicine, [6]Biostatistics
Center, Kurume University, and [7]Department of Biostatistics, Kurume University Graduate School of Medicine,
Kurume, Japan

Aim: Dietary habits are involved in the development of chronic inflammation; however, the impact of dietary profiles of hepatitis C virus carriers with persistently normal alanine transaminase levels (HCV-PNALT) remains unclear. The decision-tree algorithm is a data-mining statistical technique, which uncovers meaningful profiles of factors from a data collection. We aimed to investigate dietary profiles associated with HCV-PNALT using a decision-tree algorithm.

Methods: Twenty-seven HCV-PNALT and 41 patients with chronic hepatitis C were enrolled in this study. Dietary habit was assessed using a validated semiquantitative food frequency questionnaire. A decision-tree algorithm was created by dietary variables, and was evaluated by area under the receiver operating characteristic curve analysis (AUROC).

Results: In multivariate analysis, fish to meat ratio, dairy product and cooking oils were identified as independent variables associated with HCV-PNALT. The decision-tree algorithm

was created with two variables: a fish to meat ratio and cooking oils/ideal bodyweight. When subjects showed a fish to meat ratio of 1.24 or more, 68.8% of the subjects were HCV-PNALT. On the other hand, 11.5% of the subjects were HCV-PNALT when subjects showed a fish to meat ratio of less than 1.24 and cooking oil/ideal bodyweight of less than 0.23 g/kg. The difference in the proportion of HCV-PNALT between these groups are significant (odds ratio 16.87, 95% CI 3.40–83.67, $P = 0.0005$). Fivefold cross-validation of the decision-tree algorithm showed an AUROC of 0.6947 (95% CI 0.5656–0.8238, $P = 0.0067$).

Conclusion: The decision-tree algorithm disclosed that fish to meat ratio and cooking oil/ideal bodyweight were associated with HCV-PNALT.

Key words: data-mining, diet, hepatitis C virus carriers with persistently normal alanine transaminase levels, nutrition

INTRODUCTION

APPROXIMATELY 30% OF hepatitis C virus (HCV) carriers show persistently normal aminotransferase levels (HCV-PNALT).[1,2] HCV-PNALT is associated with less likelihood of developing hepatic fibrosis and hepatocellular carcinoma (HCC) than in patients with chronic hepatitis C (CHC).[3-5] Thus, the natural course of

HCV-PNALT is different from that of patients with CHC and this difference is considered to be caused by less severe hepatic inflammation in HCV-PNALT.[6,7]

Various epigenetic factors regulate inflammation.[8,9] Consumption of fish and vegetables is associated with decreased inflammation in healthy adults,[10,11] while meat consumption is associated with greater risk of inflammation in patients with metabolic syndrome.[12] Thus, dietary habit is closely involved with the development of inflammation. However, an association of the dietary profiles with HCV-PNALT remains unclear.

Data-mining, a set of statistical techniques, uncovers meaningful patterns from a data collection.[13] The decision-tree algorithm is an exploratory technique of data-mining that represents a series of rules for

classification by identifying priorities.[14-16] This is an explicit, quantitative and systematic approach, and allows clinicians to maximize the net benefit to patients.[17] Recently, a decision-tree algorithm has been used to reveal the clinical feature profiles associated with sustained virological response to antiviral therapy against HCV[18,19] and disease progression of HCC.[20] The aim of this study is to investigate dietary profiles associated with HCV-PNALT using a decision-tree algorithm.

METHODS

Ethics statement

THE STUDY PROTOCOL conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the approval of the Ethics Committee of Kurume University School of Medicine. None of the patients were institutionalized. We obtained written informed consent from all participants involved in our study.

Subjects

In this study, we enrolled 27 HCV-PNALT who had been regularly followed up at Kurume University School of Medicine (median observation period 9.3 years). HCV-PNALT was defined when the subjects met all of the following five criteria: (i) positive result of serum HCV RNA; (ii) never treated by interferon therapy; (iii) no medication for hepatic inflammation; (iv) no morphological changes in the liver assessed by an abdominal ultrasonography; and (v) less than upper normal limit of serum alanine aminotransferase (ALT) levels on two or more occasions over the 12-month period following diagnosis. As a comparison to HCV-PNALT, we enrolled a consecutive 41 patients with CHC, who showed elevated serum ALT levels on two or more occasions over the 12-month period following the diagnosis and were consulted for interferon treatment. All of the diagnoses were based on clinical, serological and/or histological evidence. Cases complicated with other causes of liver disease including hepatitis B virus infection, alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis or hemochromatosis were excluded. Although intake of statins is known to reduce serum ALT levels,[21] none of the subjects were taking statins in this study.

Body mass index (BMI) and body composition

Anthropometry and body composition were evaluated by a direct segmental multifrequency bioelectrical impedance analyzer (DSM-BIA) (InBody720; Biospace, Tokyo, Japan). BMI was calculated as bodyweight in kilograms divided by the square of height in meters (kg/m^2). Percent body fat was evaluated using the software supplied with the DSM-BIA (Lookin'body 2.0; Biospace). The accuracy and precision of the DSM-BIA has been reported previously.[22-24]

Laboratory determinations

Venous blood samples were taken in the morning after a 12-h overnight fast and were used for measurement of platelet count and serum level of aspartate aminotransferase (AST), ALT, total bilirubin, albumin, low-density lipoprotein (LDL) cholesterol and triglyceride. Hepatic fibrosis was evaluated by FIB-4, a non-invasive index calculated by the following formula: age (years) $\times$ AST [U/L] / (platelets [10^9/L] $\times$ [ALT [U/L]]$^{1/2}$). An FIB-4 index higher than 3.25 indicates the existence of significant hepatic fibrosis.[25] We also evaluated HCV genotype and viral load.

Assessment for dietary habits

Dietary habits were assessed using semiquantitative food frequency questionnaire (SFFQ) tests of a dietary questionnaire for 3 days as previously described.[26] The SFFQ was developed to assess recent Japanese food habits by asking the frequency of intake of 67 food items. The nutrient intakes of the SFFQ are reported to be significantly correlated with the diet record.[26] Each dietary variable was normalized by ideal bodyweight (IBW).

Caffeine consumption is known to reduce serum ALT levels.[27] Because green tea was a main source of caffeine consumption in this study, caffeine consumption was classified as "green tea and coffee".

Univariate and multivariate stepwise analyses, and decision-tree algorithm

A logistic regression model was used for univariate and multivariate stepwise analysis to identify any independent variables that are related to HCV-PNALT. A decision-tree algorithm was constructed to disclose dietary profiles associated with HCV-PNALT. Then, each profile was compared by odds ratio, 95 % confidence interval (CI) and *P*-value of the Wald test from the logistic regression model. Finally, predictive accuracy of the decision-tree model was validated by area under the receiver operating characteristic curve analysis (AUROC) using fivefold cross-validation as previously

described.[20] All statistical analyses were conducted using SAS ver. 9.2 and JMP ver. 9.0 (SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of all subjects

CHARACTERISTICS OF ALL subjects are summarized in Table 1. There was no significant difference in BMI and percent body fat between the CHC and HCV-PNALT groups (Table 1). Although serum levels of AST, ALT, albumin and ferritin were significantly higher in the CHC group compared to those in the HCV-PNALT group, total bilirubin level and platelet count were not significantly different between the groups (Table 1). Neither was any significant difference seen in HCV genotype and viral load between the groups (Table 1).

Univariate and multivariate analyses for being HCV-PNALT

Dietary characteristics in the CHC and HCV-PNALT groups are shown in Table 2 and results of univariate logistic regression analysis for being HCV-PNALT are presented in Table 3. None of the significant dietary variables including iron, green tea and coffee consumption were associated with HCV-PNALT (Table 3). In

multivariate stepwise analysis, fish to meat ratio, dairy product (g/IBW) and cooking oils (g/IBW) were identified as independent dietary variables associated with HCV-PNALT (Table 4).

Decision-tree algorithm

A decision-tree algorithm was created, in which two variables were able to classify all subjects into three groups (Fig. 1). Fish to meat ratio was selected as the variable of initial split with a cut-off value of 1.24. When subjects showed a fish to meat ratio of 1.24 or more, 68.8% of the subjects were HCV-PNALT (group 1 in Fig. 1). However, 30.7% of the subjects were HCV-PNALT when subjects had a fish to meat ratio of less than 1.24.

A cooking oils/IBW was selected as the second split with a cut-off value of 0.23 g/kg. When subjects showed a fish to meat ratio of less than 1.24 and a cooking oils/IBW of 0.23 g/kg or more, 50.0% of the subjects were HCV-PNALT (group 2 in Fig. 1). On the other hand, 11.5% of the subjects were HCV-PNALT when subjects showed a fish to meat ratio of less than 1.24 and a fat/IBW of less than 0.23 g/kg (group 3 in Fig. 1).

In comparisons among the three groups in the decision-tree algorithm, the proportion of HCV-PNALT in group 3 was significantly lower than that in groups 1

Table 1 Characteristics of all subjects

Variable	CHC	HCV-PNALT	P
n	41	27	
Age (years)	54 ± 9	57 ± 14	N.S.
Sex (female/male)	26/15	19/8	N.S.
BMI	22.5 ± 3.0	21.3 ± 2.0	N.S.
Percent body fat (%)	26.2 ± 6.4	27.1 ± 6.2	N.S.
Aspartate aminotransferase (U/L)	50 ± 55	25 ± 5	<0.0001
Alanine aminotransferase (U/L)	67 ± 78	20 ± 5	<0.0001
Total bilirubin (mg/dL)	0.80 ± 0.26	0.71 ± 0.26	N.S.
Albumin (g/dL)	4.34 ± 0.26	4.15 ± 0.19	<0.005
Platelet count ($\times 10^4$/µL)	19.7 ± 3.4	21.9 ± 5.5	N.S.
FIB-4 index	1.72 ± 0.72	1.62 ± 0.80	N.S.
Ferritin (ng/mL)	165.1 ± 29.5	76.7 ± 63.8	<0.01
HCV genotype (1b/2a/2b/not classified)	31/4/3/3	16/7/3/1	N.S.
Viral load (Log IU/mL)	6.3 ± 0.9	6.1 ± 0.8	N.S.
LDL cholesterol (mg/dL)	103.8 ± 28.5	105.1 ± 25.4	N.S.
Triglyceride (mg/dL)	109.5 ± 46.3	92.3 ± 36.8	N.S.

Data 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 significant.
CHC, chronic hepatitis C; HCV, hepatitis C virus; HCV-PNALT, hepatitis C virus carriers with persistently normal alanine transaminase levels; LDL, low-density lipoprotein; N.S., not significant.