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Original Article

Survey of non-B, non-C liver cirrhosis in Japan

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Aim: The aim of this survey was to reveal clinical features for each etiology of non-B, non-C liver cirrhosis (NBNC LC) in Japan.

Methods: In a nationwide survey of NBNC LC in Japan at the 15th General Meeting of the Japan Society of Hepatology, 6999 NBNC LC patients were registered at 48 medical institutions. Epidemiological and clinical factors were investigated.

Results: The percentage of NBNC LC among LC patients was 26%. NBNC LC patients were categorized into 11 types according to etiological agents: non-alcoholic steatohepatitis (NASH), 14.5%; alcoholic liver disease (ALD), 55.1%; fatty liver disease (FLD), except NASH, ALD, and other known etiology, 2.5%; primary biliary cirrhosis, 8.0%; other biliary cirrhosis, 0.8%; autoimmune hepatitis, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5%. Compared with previous surveys, the percentage of ALD remained unchanged, whereas that of NASH increased. The mean age and percentage of females were significantly higher in NASH patients than in ALD and FLD patients. Prevalence of diabetes mellitus was significantly higher in NASH and FLD patients than in ALD ones. Prevalence of hepatocellular carcinoma (HCC) in NBNC LC patients was 35.9%. Among NASH, ALD and FLD patients, 50.9%, 34.3% and 54.5% had HCC, respectively. Positivity of hepatitis B core antibody was significantly higher in HCC patients than in those without HCC (41.1% vs 24.8%).

Conclusion: This survey determined the etiology of NBNC LC in Japan. These results should contribute new ideas toward understanding NBNC LC and NBNC HCC.

Key words: alcoholic liver disease, hepatocellular carcinoma, non-alcoholic steatohepatitis, non-B, non-C liver cirrhosis

INTRODUCTION

A NATIONWIDE SURVEY of liver cirrhosis (LC) for each etiology has been conducted as the main theme on four occasions at the national academic conference in Japan. Therefore, many registered patients have been surveyed on uniform diagnostic criteria.[1] The 15th General Meeting of the Japan Society of Hepatology was held in October 2011. In a featured session in this meeting, we conducted a nationwide survey of non-B, non-C LC (NBNC LC) in patients at medical institutions in Japan. NBNC LC was the main theme of the featured session in this meeting for two reasons. First, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing and has been recently reported to be approximately 20% in adults in Japan. Approximately 1% of adults in Japan are estimated to have non-alcoholic steatohepatitis (NASH).[2,3] Thus, NASH is the most common chronic liver disease not only in Western countries but also in Japan. NASH patients can develop LC and even hepatocellular carcinoma (HCC), although there have been few investigations concerning the incidence of LC associated with NASH (NASH LC)

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in Japan. Second, the number of NBNC HCC patients has been rapidly increasing, and it has been recently reported to account for approximately 15% of all HCC patients in Japan.[4] Most NBNC HCC patients seem to have LC with alcoholic liver disease (ALD LC); however, NASH LC has been noted as a high-risk group of NBNC HCC. Nevertheless, HCC complicated with NBNC LC of an unknown cause has been occasionally reported. Therefore, it is important to investigate the clinical features of NBNC LC, which will lead to the development NBNC HCC. Based on these backgrounds, we report the characteristics of NBNC LC in Japan. This was one of the programs of the 15th General Meeting of the Japan Society of Hepatology in 2011.

METHODS

Patient database

AT 48 MEDICAL institutions (all investigators are listed in Appendix I) (Table 1), 6999 subjects were diagnosed with NBNC LC based on the negative results for serum hepatitis B surface antigen (HBsAg), anti-hepatitis C antibody and hepatitis C virus (HCV) RNA. The patients registered in this study were clinically (laboratory examinations and imaging studies) and histologically diagnosed with LC based on the criteria proposed by a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008).[1] The NBNC LC patients were categorized into 11 types according to etiology: (i) NASH; (ii) ALD; (iii) fatty liver disease (FLD); (iv) primary biliary cirrhosis (PBC); (v) other biliary cirrhosis (such as primary sclerosing cholangitis [PSC] and secondary biliary cirrhosis); (vi) autoimmune hepatitis (AIH) (including AIH-PBC overlap syndrome); (vii) metabolic disease (such as Wilson's disease, hemochromatosis and glycogen storage disease); (viii) congestive disease (including Budd–Chiari syndrome); (ix) parasitic disease (such as Japanese schistosomiasis); (x) other known etiology (such as sarcoidosis and drug-induced liver injury); and (xi) unknown etiology. The diagnosis of NASH was based on the following criteria: (i) absence of clinically significant alcohol consumption (intake of ≤20 g ethanol/day); (ii) appropriate exclusion of other liver diseases; (iii) complications with risk factors of steatosis such as obesity (in particular, visceral obesity), metabolic syndrome and diabetes mellitus; and (iv) the presence of steatosis on liver histology (histological

Table 1 Forty-eight medical institutions registered at the 15th General Meeting of the Japan Society of Hepatology on 2011

Akita University Graduate School of Medicine
Asahikawa-Kosei General Hospital
Asahikawa Medical University
(Division of Gastroenterology and Hematology/Oncology)
(Division of Metabolism and Biosystemic Science)
Asahikawa Red Cross Hospital
Chiba University
Dokkyo Medical University
Ehime Prefectural Central Hospital
Ehime University Graduate School of Medicine
Fukushima Medical University School of Medicine
Gunma University Graduate School of Medicine
Hyogo College of Medicine
Iwate Medical University
Jikei University School of Medicine, Katsushika Medical Center
Juntendo University School of Medicine
Kagawa University
Kanazawa Medical University
Keio University School of Medicine
Kumamoto University
Kurume University School of Medicine
Kyoto Second Red Cross Hospital
Mie University Graduate School of Medicine
Musashino Red Cross Hospital
Nagano Red Cross Hospital
Nara Medical University
National Center for Global Health and Medicine
Nihon University School of Medicine
Niigata Prefectural Central Hospital
Niigata University Medical and Dental Hospital
Oji General Hospital
Osaka City University
Osaka Police Hospital
Osaka Red Cross Hospital
Saiseikai Suita Hospital
Saitama Medical University
Sapporo City General Hospital
Sapporo-Kosei General Hospital
Shinshu University School of Medicine
Teikyo University School of Medicine
Teine-Keijinkai Hospital
Tokyo Medical and Dental University
Tokyo Medical University Ibaraki Medical Center
Tokyo Women's Medical University
Tottori University School of Medicine
University of Tokyo
University of Yamanashi
(First Department of Internal Medicine)
(First Department of Surgery)
Yamagata University Faculty of Medicine

diagnosis) or imaging studies (imaging diagnosis). The diagnosis of ALD was based on the proposed Diagnostic Criteria for Alcoholic Liver Disease by a Japanese study group for ALD (the Takada group).[5] The diagnosis of FLD was based on the following criteria: (i) alcohol consumption between that for NASH and ALD (i.e. intake of >20 g and <70 g ethanol/day); (ii) appropriate exclusion of other liver diseases; and (iii) the presence of steatosis on liver histology or imaging studies.

The following variables were used to investigate the clinical features of NBNC LC: age; sex; body mass index (BMI); prevalence of diabetes mellitus (DM), impaired glucose tolerance, hypertension and dyslipidemia; Child–Pugh classification; prevalence of gastroesophageal varices and HCC; and presence of hepatitis B core antibody (anti-HBc). In addition, the percentage of NBNC LC was investigated among all LC patients at each institution and was compared with previous reports. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki (2000).

Statistical analyses

Statistical tests were performed using the IBM SPSS Statistics ver. 21. The statistical significance of difference was determined using the χ^2-test, Mann–Whitney *U*-test and multivariate Cox's proportional hazard model as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Percentage of NBNC LC among all LC patients

WE CALCULATED THE percentage of NBNC LC among all 25 020 LC patients at 37 registered institutions. The percentages of NBNC LC, hepatitis B virus (HBV)-related cirrhosis, HCV-related cirrhosis, and both HBV- and HCV-related cirrhosis were 26%, 12%, 60.9% and 1.1%, respectively. Compared with a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008),[1] there was no significant difference between them (Table 2).

Frequency of each etiology among NBNC LC patients

We determined the frequency and percentage of each etiology among all 6999 NBNC LC patients at 48 registered institutions. The percentages of each etiology were as follows: NASH, 14.5%; ALD, 55.1%; FLD, 2.5%; PBC, 8.0%; other biliary cirrhosis, 0.8%; AIH, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5% (Table 3). Among 1015 NASH patients, 309 (30.4%) were diagnosed histologically, 402 (39.6%) were diagnosed by imaging studies and the method of diagnosis of 304 patients (30%) was not described in detail. Among 60 patients with other biliary cirrhosis, 71.7% had PSC and the rest had cholestatic diseases, except PBC and PSC (such as congenital biliary atresia and secondary biliary cirrhosis). Among 39 metabolic disease patients, 66.7% had Wilson's disease, 25.6% had hemochromatosis (glycogen storage disease, amyloidosis and citrullinemia in one patient each). All 12 cases of parasitic disease were Japanese schistosomiasis. Of 11 patients with other known etiology, two patients sarcoidosis, two post-liver transplantation, two post-hepatectomy, one drug-induced liver injury, one systemic lupus erythematosus-related liver injury and the diagnosis of the remaining patients was not described in detail.

Compared with the survey at the 44th Annual Meeting of the Japan Society of Hepatology in 2008,[1] the percentage of ALD among all NBNC LC patients did

Table 2 Percentage of NBNC LC among all patients with liver cirrhosis compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 25\,020$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 33\,379$)	*P*-value
NBNC LC	26.0%	24.0%	N.S.
HBV-related cirrhosis	12.0%	13.9%	N.S.
HCV-related cirrhosis	60.9%	60.9%	N.S.
both HBV- and HCV-related cirrhosis	1.1%	1.2%	N.S.

P-values were analyzed by χ^2-test.
HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant.

Table 3 Frequency of each etiology among patients with NBNC LC compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 6999$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 8011$)	*P*-value
NASH	14.5%	8.7%	$P < 0.001$
ALD	55.1%	56.3%	N.S.
FLD	2.5%	–	–
PBC	8.0%	9.9%	$P < 0.001$
Other biliary cirrhosis	0.8%	1.2%	$P < 0.001$
AIH	6.8%	7.9%	$P = 0.018$
Metabolic disease	0.6%	1.2%	$P < 0.001$
Congestive disease	0.8%	1.2%	$P = 0.013$
Parasites	0.2%	0.4%	$P = 0.011$
Other known etiology	0.2%	0.8%	$P < 0.001$
Unknown etiology	10.5%	12.4%	$P < 0.001$

P-values were analyzed by χ^2-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

not change (55.1% vs 56.3%), whereas that of NASH increased (14.5% vs 8.7%; $P < 0.001$) (Table 3).

Clinical features of NBNC LC patients

The male : female ratio for the NBNC LC patients was 1.93. The percentages of each etiology among 4608 male and 2391 female patients were as follows: NASH (9.5% and 24%), ALD (73.4% and 19.8%), FLD (3.4% and 0.9%), PBC (1.9% and 20%), other biliary cirrhosis (0.8% and 0.9%), AIH (1.5% and 17.1%), metabolic disease (0.5% and 0.8%), congestive disease (0.8% and 0.8%), parasitic disease (0.2% and 0.1%), other known etiology (0.1% and 0.2%) and unknown etiology (7.9% and 15.4%), respectively (Fig. 1). The male : female ratio for each etiology among the NBNC LC patients was as follows: NASH, 0.77; ALD, 7.12; FLD, 6.86; PBC, 0.18; other biliary cirrhosis, 1.73; AIH, 0.17; metabolic disease, 1.29; congestive disease, 2.17; parasitic disease, 5; other known etiology, 0.83; and unknown etiology, 0.99 (Table 4). Thus, the NASH patients were predominantly female as opposed to the ALD and FLD patients who were predominantly male.

The mean age at clinical diagnosis in the NBNC LC patients for NASH, ALD, FLD, PBC, other biliary cirrhosis, AIH, metabolic disease, congestive disease, parasitic disease, other known etiology and unknown etiology was 66.9, 60.3, 64.2, 63.6, 51.3, 64.5, 42.6, 52.7, 77.4, 56.1 and 68.8 years, respectively. In the patients with NASH, AIH, congestive disease and unknown etiology, the mean ages at clinical diagnosis of the male patients were lower than those of the female patients ($P < 0.001$). In contrast, in the ALD, FLD, PBC and metabolic disease patients, the mean ages at clinical diagnosis of the female patients were lower than those of the male patients ($P < 0.001$) (Table 5).

Regarding the risk factors of NASH, the following variables were investigated in the NASH, ALD and FLD patients: BMI and the prevalence of DM, impaired glucose tolerance (IGT), hypertension and dyslipidemia. BMI in the NASH, ALD and FLD patients was 27, 23.4 and 25 kg/m^2, respectively, and the differences among them were statistically significant. The prevalence of DM and IGT in the NASH and FLD patients (63% and 57%, respectively) was significantly higher compared with that in the ALD patients (31%) ($P < 0.001$). The prevalence of dyslipidemia in the NASH and FLD patients (25% and 29%, respectively) was significantly higher compared with that in the ALD patients (14%) ($P < 0.001$). The prevalence of hypertension in the NASH patients (52%) was significantly higher compared with that in the ALD and FLD patients (28% and 35%, respectively) ($P < 0.001$) (Table 6).

The levels of hepatic functional reserve based on the Child–Pugh classification for each etiology are summarized in Table 7. The percentages of moderate-to-low hepatic reserve (Child–Pugh class B and C) in the ALD and AIH patients (52.9% in both) were significantly higher compared with those in the NASH and FLD patients (35.8% and 27%, respectively) ($P < 0.001$).

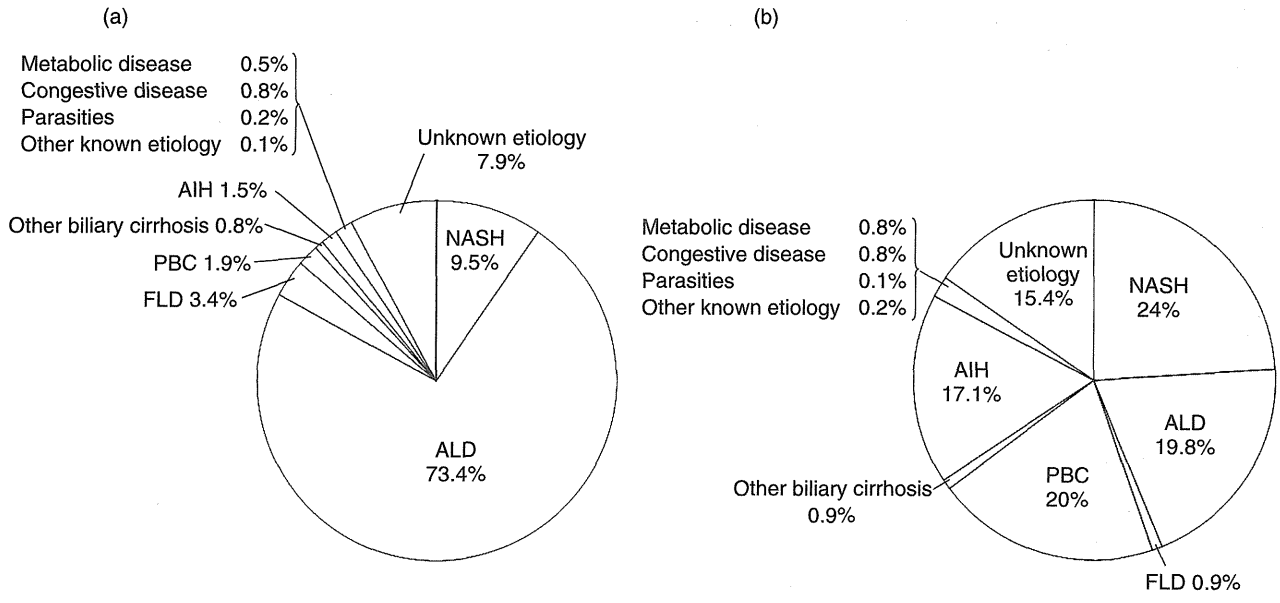

Figure 1 Frequency of each etiology among male or female patients with NBNC LC. (a) Male, (b) female. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

To determine the frequency of complicated portal hypertension patients, the prevalence of gastroesophageal varices was calculated. The prevalence in the ALD and PBC patients (54.5% and 61.9%, respectively) was significantly higher compared with that in the patients with NASH, FLD, AIH and unknown etiology (40.8%, 40.7%, 48.2% and 45.9%, respectively) ($P < 0.05$). Considering only patients with Child–Pugh class A, the prevalence of gastroesophageal varices in PBC patients was highest among all etiologies. ALD had significantly higher prevalence than NASH, the histology of which was very similar (Table 8).

Table 4 Male : female ratio of each etiology

	Male ($n = 4608$)	Female ($n = 2391$)	Male : female ratio
NASH	440	575	0.77
ALD	3381	475	7.12
FLD	151	22	6.86
PBC	87	477	0.18
Other biliary cirrhosis	38	22	1.73
AIH	69	409	0.17
Metabolic disease	22	19	1.29
Congestive disease	39	18	2.17
Parasites	10	2	5.00
Other known etiology	5	4	0.83

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

The prevalence of HCC in the NBNC LC patients was 35.9%. Among 2438 NBNC HCC patients, 51.9% were diagnosed with HCC simultaneously with the diagnosis of NBNC LC, 25.6% were diagnosed after, 1.4% were diagnosed before the diagnosis of NBNC LC and the diagnosis of the remaining patients was not described in detail. The male : female ratio for the NBNC HCC patients was 3.06. The percentage of each etiology among the HCC patients was as follows: NASH, 19.9%; ALD, 53.4%; FLD, 3.7%; PBC, 3.2%; other biliary cirrhosis, 0.2%; AIH, 4.9%; metabolic disease, 0.1%; congestive disease, 0.7%; parasitic disease, 0.1%; other known etiology, 0%; and unknown etiology, 13.8%. The percentage of NASH among the NBNC HCC patients was significantly higher than that among the NBNC LC patients (19.9% vs 14.5%, $P < 0.001$). The clinical diagnosis of HCC was made at a mean age of 67.2 years in all patients. The mean age of onset of HCC was 70.8, 64.8 and 68.4 years in the NASH, ALD and FLD patients, respectively, and the differences among them were significant ($P < 0.001$). The prevalence of

Table 5 The mean ages at clinical diagnosis in the patients with NBNC LC

	Total ($n = 6999$)	Male ($n = 4608$)	Female ($n = 2391$)	*P*-value (M vs F)
NASH	66.9 ± 11.6	64.8 ± 13.2	68.5 ± 9.8	$P < 0.001$
ALD	60.3 ± 11.0	60.9 ± 10.7	55.7 ± 12.1	$P < 0.001$
FLD	64.2 ± 11.8	64.7 ± 11.3	61.2 ± 15.0	$P < 0.001$
PBC	63.6 ± 12.1	66.0 ± 11.3	63.2 ± 12.0	$P < 0.001$
Other biliary cirrhosis	51.3 ± 20.7	52.0 ± 22.0	50.0 ± 19.0	$P < 0.001$
AIH	64.5 ± 12.2	63.3 ± 14.2	66.0 ± 11.7	$P < 0.001$
Metabolic disease	42.6 ± 18.2	44.0 ± 18.0	40.7 ± 19.0	$P < 0.001$
Congestive disease	52.7 ± 20.4	50.5 ± 20.7	57.4 ± 19.6	$P < 0.001$
Parasites	77.4 ± 5.9	76.5 ± 6.1	81.5 ± 2.1	$P < 0.001$
Other known etiology	56.1 ± 19.1	53.0 ± 18.7	58.7 ± 20.8	$P < 0.001$
Unknown etiology	68.8 ± 11.9	67.9 ± 13.0	69.8 ± 10.7	$P < 0.001$

All results are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

HCC in the patients with NASH, FLD and unknown etiology (50.9%, 54.5% and 47.5%, respectively) were significantly higher compared with that in the ALD, PBC and AIH patients (34.3%, 14.4% and 26.0%) ($P < 0.0001$). The percentage of moderate-to-low hepatic reserve (Child–Pugh class B and C) in HCC in AIH patients was significantly higher than those in the patients with NASH, FLD and unknown etiology

Table 6 Risk factors of NASH in the patients with NASH, ALD and FLD

Variable	NASH ($n = 1015$)	ALD ($n = 3856$)	FLD ($n = 173$)	*P*-value
Body mass index (kg/m^2)	27.0 ± 4.3	23.4 ± 6.4	25.0 ± 3.7	$P < 0.001$*,**
Diabetes mellitus or Impaired glucose tolerance	62.5%	37.5%	56.5%	$P < 0.001$*
Dyslipidemia	25.0%	13.5%	29.4%	$P < 0.001$* $P = 0.01$**
Hypertension	52.0%	28.2%	34.7%	$P < 0.001$*,**

ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.
Results of body mass index are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test and χ^2-test.
*NASH vs ALD, **NASH vs FLD.

Table 7 Levels of hepatic functional reserve based on the Child–Pugh classification

Child–Pugh classification	Class A	Class B	Class C	Percentages of both class B and C	*P*-value
NASH ($n = 783$)	503	222	58	35.8%	
ALD ($n = 2710$)	1276	867	567	52.9%	$P < 0.001$*
FLD ($n = 89$)	65	18	6	27.0%	
PBC ($n = 355$)	204	105	46	42.5%	
AIH ($n = 295$)	139	106	50	52.9%	$P < 0.001$** $P = 0.01$***
Unknown etiology ($n = 515$)	300	150	65	41.7%	

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.
P-values were analyzed by χ^2-test.
*vs NASH, FLD, PBC and unknown etiology; **vs NASH and FLD; ***vs PBC and unknown etiology.

Table 8 Prevalence of patient with gastroesophageal varices

	Total	Child–Pugh classification		
		Class A	Class B	Class C
NASH ($n = 686$)	40.8% (280/686)*	31.8% (138/434)**	56.1% (111/198)	57.4% (31/54)
ALD ($n = 2365$)	54.5% (1289/2365)***	44.2% (486/1099)†	59.0% (447/757)	69.9% (356/509)
FLD ($n = 81$)	40.7% (33/81)	36.5% (23/63)	50.0% (7/14)	75.0% (3/4)
PBC ($n = 331$)	61.9% (205/331)††	53.5% (100/187)††	70.6% (72/102)	78.6% (33/42)
AIH ($n = 278$)	48.2% (134/278)	39.7% (52/131)	53.5% (53/99)	60.4% (29/48)
Unknown etiology ($n = 401$)	45.9% (184/401)	42.9% (94/219)	47.2% (60/127)	54.5% (30/55)

P-values were analyzed by Fisher's exact test or χ^2-test.
*$P < 0.05$, vs ALD, PBC and AIH; **$P < 0.01$, vs ALD, PBC and unknown etiology; ***$P < 0.05$, vs NASH, FLD and unknown etiology; †$P < 0.0001$ vs NASH; ††$P < 0.05$ vs NASH, ALD, FLD, AIH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.

($P < 0.0001$). BMI in the NASH, ALD and FLD patients was 26.8, 24.0 and 25.8 kg/m^2, respectively, and the differences among them were statistically significant. (Table 9).

Table 10 shows the analysis of the risk factors associated with HCC in patients with ALD LC. Obesity and complication of DM were the risk factors of hepatic carcinogenesis in ALD LC patients as well as male sex and being older. Conversely, portal hypertension and anemia of ALD LC patients without HCC were worse than those with HCC. Accordingly, we investigated the comparison of the clinical features between the two ALD LC groups divided based on BMI (Table 11). Although the mean age was similar in these two groups, the prevalence of HCC in the ALD LC patients with obesity (BMI, ≥25 kg/m^2) was significantly higher compared with that in those without obesity (BMI, <25 kg/m^2) (48.3% vs 35.7%, $P < 0.001$) and similar to that in the NASH LC patients (48.3% vs 50.9%, not significant).

Of the NBNC LC patients, 31.3% were anti-HBc positive. Anti-HBc positivity was 30.7%, 30.8%, 34.7% and 43% in the patients with NASH, ALD, FLD and unknown etiology, respectively. The positivity was significantly higher in the patients with unknown etiology compared with the NASH, ALD and FLD patients ($P < 0.001$). Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC (41.1% vs 24.8%, $P < 0.001$).

DISCUSSION

THIS NATIONWIDE SURVEY revealed the following clinical features in the NBNC LC patients:

1 Compared with the previous nationwide survey,[1] the percentage of ALD among the NBNC LC patients remained unchanged, whereas that of NASH increased.
2 The NASH LC patients were significantly older, predominantly female, heavier, hypertensive and more likely to have DM and HCC.
3 The ALD LC patients were significantly younger, predominantly male, had low hepatic reserve and were more likely to have portal hypertension than NASH LC.
4 The FLD LC patients were observed at an age between that of the NASH and ALD patients, were predominantly male (similar to the ALD patients) and were more likely to have DM and HCC similar to the NASH patients.
5 Approximately 10% of the NBNC LC patients still had an unknown etiology, and these patients were more likely to have HCC similar to both the NASH and FLD patients.
6 Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC.

Although the natural history of NASH is not completely understood, Matteoni *et al.* reported that 23% of NASH patients progressed to cirrhosis within 10–15 years.[6] In addition, Starley *et al.* recently stated that approximately 26–37% of NASH patients demonstrate the progression of fibrosis over time periods up to 5.6 years, with up to 9% patients progressing to cirrhosis.[7] BMI and DM have been found to be independent risk factors associated with the progression of fibrosis in NASH patients.[8] Therefore, it is thought that the NASH LC patients in the present study had significantly more severe disease and were more likely to have DM. Conversely, the prevalence of NAFLD in Japan appears to be twice as high in males than in females;[9] however, the NASH LC patients in the present study were

Table 9 Clinical features of patients with HCC

	Percentage (%)	Prevalence of HCC (%)	Age of onset of HCC (years)	M : F ratio	Child–Pugh classification (A/B/C, %)	BMI	Platelet ($10^3/mm^3$)
Total ($n = 2438$)	100	35.9	67.2 ± 10.1	3.06	62.6/28.8/8.6	24.6 ± 4.0	127 ± 66
NASH ($n = 485$)	19.9	50.9*	70.8 ± 9.0**	1.06	66.0/28.9/5.1	26.8 ± 4.3‡‡	128 ± 61
ALD ($n = 1302$)	53.4	34.3	64.8 ± 9.4†	19.05††	60.3/29.8/9.9	24.0 ± 3.8	126 ± 66
FLD ($n = 91$)	3.7	54.5*	68.4 ± 8.8***	17.20††	82.5/15.9/1.6	25.8 ± 4.0‡‡‡	120 ± 61
PBC ($n = 79$)	3.2	14.4	68.0 ± 10.4***	0.32†††	53.2/35.9/10.9	22.3 ± 3.0	110 ± 54
Other biliary cirrhosis ($n = 4$)	0.2	6.8	–	–	–	–	–
AIH ($n = 119$)	4.9	26.0	68.8 ± 8.7**	0.23†††	42.5/42.5/15.0‡	24.3 ± 4.1	107 ± 60§
Metabolic disease ($n = 2$)	0.1	5.1	–	–	–	–	–
Congestive disease ($n = 16$)	0.7	32.0	52.0 ± 16.6	1.67	57.2/21.4/21.4	23.6 ± 3.2	127 ± 72
Parasites ($n = 3$)	0.1	30.0	–	–	–	–	–
Unknown etiology ($n = 337$)	13.8	47.5*	70.9 ± 10.9**	1.57	70.8/22.4/6.8	23.6 ± 3.7	143 ± 76

Results of age are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test and χ^2-test as appropriate.
*$P < 0.0001$, vs ALD, PBC and AIH; **$P < 0.0001$, vs ALD and congestive disease; ***$P < 0.001$, vs ALD and congestive disease; †$P < 0.001$, vs congestive disease; ††$P < 0.0001$, vs NASH, PBC, AIH and unknown etiology; †††$P < 0.0001$, vs NASH, ALD, FLD and unknown etiology; ‡$P < 0.0001$, vs NASH, FLD and unknown etiology; ‡‡$P < 0.0001$, vs ALD, PBC, AIH and unknown etiology; ‡‡‡$P < 0.0001$, vs ALD, PBC and unknown etiology; §$P < 0.001$, vs NASH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; BMI, body mass index; FLD, fatty liver disease; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

predominantly female. Yasui *et al.* reported that NASH HCC patients were predominantly male, although the prevalence of cirrhosis among these patients was significantly lower in male patients compared with that in female patients.[10] These studies suggest that sex is implicated in the progression of fibrosis in NASH patients in Japan. In addition, the prevalence of HCC in the NASH LC patients in the present study was significantly higher compared with that in the previous nationwide survey (50.9% vs 31.5%, $P < 0.001$).[1] The incidence of NASH and NASH HCC has been gradually increasing in Japan, contrary to the decreased incidence of virus-related HCC.[4] Starley *et al.* found that as many as 4–27% of cases of NASH transform to HCC after the development of cirrhosis, and that the prevalence of HCC in NAFLD is 0–0.5%, whereas that of HCC in NASH is 0–2.8% over time periods of up to 19.5 years.[7] Yatsuji *et al.* reported the prospective evaluation of NASH LC and HCV-related LC (LC-C). They reported that NASH LC followed a course similar to that of LC-C, namely, complications of cirrhosis developed, including HCC (the 5-year cumulative rate of HCC development was 11.3% for NASH LC and 30.5% for LC-C).[11] Therefore, NASH LC patients need to be followed up carefully with respect to the occurrence of HCC, similar to LC-C patients.

Alcoholic liver disease remains the most prevalent cause of NBNC LC in Japan, accounting for approximately 55% of all NBNC LC cases. In the present study, the prevalence of HCC was significantly lower in the ALD LC patients than in the NASH LC patients, whereas the ALD LC patients were significantly younger and had a lower hepatic reserve. Regarding the comparison of outcomes with LC-C, Toshikuni *et al.* reported that the risk of HCC was lower in ALD LC than in LC-C, whereas the risk of hepatic decompensation and mortality was the same.[12] It is estimated that there are approximately 2.4 million heavy drinkers in Japan, and the number of ALD patients has been increasing because of increased alcohol consumption.[13] Therefore, ALD LC patients need to be followed up carefully with respect to the occurrence of hepatic decompensation, similar to LC-C patients. Obesity appears to be involved in the progression of ALD LC.[13] Accordingly, we investigated the risk factors associated with HCC and clarified that obesity and complication of DM could be the risk for hepatic carcinogenesis in ALD LC patients. The comparison of the clinical features between the two ALD LC groups divided based on BMI revealed that the prevalence of HCC in the ALD LC patients with obesity was significantly higher compared with that in those without obesity. Horie *et al.* also reported similar results.[14] Thus,

Table 10 Factors associated with HCC in patients with ALD

Factors	HCC (–), ($n = 2494$)	HCC (+), ($n = 1303$)	Univariate analysis, *P*-value	Multivariate analysis, *P*-value
Sex (M : F)	83.7%:16.3%	95.0%:5.0%	<0.0001	<0.0001
Age (years)	57.9 ± 11.0	64.8 ± 9.4	<0.0001	<0.0001
Body mass index (kg/m^2)	22.8 ± 3.8	24.0 ± 3.8	<0.0001	<0.0001
Hypertension (– : +)	77.4%:22.6%	61.9%:38.1%	<0.0001	0.068
Dyslipidemia (– : +)	87.0%:13.0%	81.6%:18.4%	<0.0001	0.482
Diabetes mellitus (– : +)	67.2%:32.8%	50.2%:49.8%	<0.0001	<0.0001
Child–Pugh classification (A : B + C)	38.5%:61.5%	60.3%:39.7%	<0.0001	0.188
Esophageal varices (– : +)	42.3%:57.7%	57.9%:42.1%	<0.0001	<0.0001
Ascites (– : +)	57.1%:42.9%	76.5%:23.5%	<0.0001	<0.0001
WBC (/mm^3)	6014 ± 3465	5532 ± 3484	0.001	0.547
Hemoglobin (g/dL)	11.3 ± 2.6	12.7 ± 2.2	<0.0001	<0.0001
Platelet (×10^3/mm^3)	114.6 ± 67.1	126.1 ± 65.5	<0.0001	0.104
AST (IU/L)	93 ± 209	65 ± 71	<0.0001	0.974
ALT (IU/L)	51 ± 118	45 ± 43	0.159	0.786
Bilirubin (mg/dL)	2.8 ± 3.9	1.6 ± 2.4	<0.0001	0.006
Albumin (g/dL)	3.3 ± 1.0	3.5 ± 0.7	<0.0001	0.281
PT%	69 ± 22	79 ± 19	<0.0001	0.628

Results of age are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test, χ^2-test and multivariate Cox's proportional hazard model as appropriate.
ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; PT, prothrombin time; WBC, white blood cells.

obesity appears to be involved in the progression of HCC in ALD LC. Therefore, ALD LC patients with obesity need to be followed up carefully with respect to the occurrence of HCC, similar to NASH LC and LC-C patients. Not only abstinence from alcohol but also improvement in lifestyle is important to decrease the progression of ALD LC.

In the present study, we established a new clinical etiologic criterion: FLD. According to previous clinical etiologic criteria in Japan, mild drinkers (intake of >20 g and <70 g of ethanol/day) with steatohepatitis were not diagnosed with both NASH and ALD. The prevalence of minor homozygote or heterozygote type of the aldehyde dehydrogenase-2 gene (*ALDH2*), which oxidizes acetaldehyde to acetate and is a key enzyme in alcohol metabolism, is very high in Asian countries. The enzyme activity of a minor homozygote of *ALDH2* is completely defective. Moreover, the enzyme activity of a heterozygote is only 1/16th. Our survey is the first to reveal that these FLD LC patients were observed in 2.5% of NBNC LC patients. Considering the frequencies of mild drinkers and obese people in Japan, it is thought that the frequency of FLD LC is lower than that of LC with unknown etiology. This is because there were many patients whose amounts of daily alcohol intake were unknown; therefore, some were diagnosed as having an

Table 11 Clinical features of patients with ALD LC

	BMI <25 ($n = 1915$)	BMI ≥25 ($n = 749$)	*P*-value
Sex (M : F)	1644:317 (83.4%:16.6%)	692:57 (92.4%:7.6%)	$P < 0.001$
Age	60.2 ± 11.1	61.0 ± 10.2	N.S.
Diabetes mellitus	35.1%	43.9%	$P < 0.001$
HCC	35.7%	48.3%	$P < 0.001$

Results of age are expressed as mean ± standard deviation, *P*-values were analyzed by by Mann–Whitney *U*-test and χ^2-test as appropriate.
ALD, alcoholic liver disease; BMI, body mass index; HCC, hepatocellular carcinoma; N.S., not significant.

unknown etiology. Interestingly, the clinical features of the FLD LC patients overlapped with those of the NASH LC and ALD LC patients. Because the mean age of the FLD LC patients was between that of the NASH and ALD patients, the FLD LC patients were predominantly male, similar to the ALD LC patients, and they were more likely to have DM and HCC similar to the NASH LC patients. Horie *et al.* described a category such as FLD as overlap steatohepatitis.[13,14] The most important clinical feature in FLD LC patients was that the prevalence of HCC was high, similar to that in the NASH LC patients. This finding suggests that steatohepatitis per se is a potent risk factor of HCC, irrespective of alcohol consumption.

The LC patients with unknown etiology (or cryptogenic LC) were approximately 10% of the NBNC LC patients and were more likely to have HCC similar to the NASH and FLD patients. Some FLD LC patients whose daily alcohol intake was unknown may have been included in this group, and some "burnt-out" NASH LC patients whose liver showed complete disappearance of steatosis[15] may have also been included in this group. In addition, some patients who had been HBV carriers but had become HBsAg negative or those with occult HBV may have also been included in this group. Anti-HBc positivity was significantly higher in this group than in the NASH, ALD and FLD LC groups. Several studies have suggested a high prevalence of occult HBV among cryptogenic LC and NBNC HCC patients and also the participation of occult HBV in the progression to cirrhosis and occurrence of HCC.[16,17] In the present study, anti-HBc positivity was significantly higher in the NBNC LC patients with HCC than in those without HCC; however, the role of occult HBV in the progression to cirrhosis and carcinogenesis remains unclear. Occult HBV is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in serum) for patients testing HBsAg negative.[18] Because of the lack of a HBV DNA assay in the present study, the impact of occult HBV on carcinogenesis could not be evaluated. Thus, a HBV DNA assay in the liver is needed for the evaluation of occult HBV on carcinogenesis. Although NBNC LC seemed to include varied etiology, occult HBV should be taken into account in the prediction of future HCC development in NBNC LC.

Our nationwide survey determined the etiology of NBNC LC in Japan. Future changes in etiology must be considered for the establishment of precise diagnostic strategies. We hope that these results contribute new ideas toward understanding NBNC LC and NBNC HCC.

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APPENDIX I

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Special Report

Guidelines on nutritional management in Japanese patients with liver cirrhosis from the perspective of preventing hepatocellular carcinoma

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Aim: The Japanese Nutritional Study Group for Liver Cirrhosis (JNUS) was assembled in 2008 with the support of a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan. The goal of the study group was to propose new nutritional guidelines for Japanese patients with liver cirrhosis (LC), with the aim of preventing hepatocellular carcinoma.

Methods: Between 2008 and 2010, the member investigators of JNUS conducted various clinical and experimental studies on nutrition on LC. These included anthropometric studies, a questionnaire study on daily nutrient intake, clinical trials, experimental studies using animal models, re-evaluation of previous publications and patient education. Over this 3-year period, the group members regularly discussed the nutritional issues related to LC, and a proposal was finally produced.

Results: Based on the results of JNUS projects and discussions among the members, general recommendations were made on how Japanese patients with LC should be managed nutritionally. These recommendations were proposed with a specific regard to the prevention of hepatocarcinogenesis.

Conclusion: The new JNUS guidelines on nutritional management for Japanese patients with LC will be useful for the actual nutritional management of patients with LC. The JNUS members hope that these guidelines will form the basis for future discussions and provide some direction in nutritional studies in the field of hepatology.

Key words: hepatocellular carcinoma, liver cirrhosis, malnutrition, nutrition, protein-energy malnutrition

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INTRODUCTION

THE LIVER IS a major organ in nutritional metabolism. Therefore, metabolic abnormalities in nutritional elements are generally observed in the progression of chronic liver disease (CLD). Malnutrition, which is characterized by protein-energy malnutrition (PEM), is known as an essential complication in patients with liver cirrhosis (LC) and is closely associated with LC prognosis.[1–7] On the other hand, the major cause of CLD in Japan is infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) and approximately 34 000 patients with CLD die annually due to hepatocellular carcinoma (HCC).[8–11] Importantly, 90% of HCC cases are associated with LC.[12,13] Therefore, standard therapeutic guidelines on the use of antiviral agents for CLD patients with HBV and HCV infection have been established to prevent the occurrence of HCC.[14,15] However, because the number of elderly CLD patients is increasing, patients are often unable to tolerate full antiviral therapy. CLD caused by non-alcoholic steatohepatitis (NASH), which is associated with overweight status, has also been increasing.[16–18] These findings indicate that total nutritional management, including both diet and nutritional supplements, is required in order to prevent the progression of CLD and onset of HCC.[19–22]

Japanese dietitians were consulted in preparing the guidelines of both the Japan Society of Metabolism and Clinical Nutrition (2003)[23] and the European Society of Parenteral and Enteral Nutrition (2006 and 2009).[24,25] In addition, nutritional recommendations for the treatment of LC in Japan were incorporated into guidelines in Japan in 2010.[26] However, specific and detailed guidelines for the nutritional management of patients with LC in Japan have been lacking.

From these perspectives, the Japanese Nutritional Study Group for Liver Cirrhosis (JNUS) was assembled between 2008 and 2010 in order to establish new nutritional guidelines for LC. The study group was supported by a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan (H20-Hepatitis-General-005). Here, we describe the guidelines on nutritional management of Japanese LC patients, with the aim of preventing HCC.

METHODS

THE JNUS GROUP performed the following projects: (i) investigation of clinical and anthropometric characteristics in Japanese patients with LC; (ii) evaluation of daily nutrient intake (total calories, and individual intake of protein, fat, carbohydrate, trace elements such as iron and zinc, and sodium) using a 3-day questionnaire in CLD patients; (iii) development of new biomarkers representing non-protein respiratory quotients (npRQ) measured by indirect calorimetry; (iv) development of a new analytical system to estimate iron status in the blood; (v) a prospective controlled trial to examine whether branched-chain amino acid (BCAA) granule supplementation prevents recurrence of HCC after primary HCC treatment; (vi) a prospective double-blind controlled study evaluating the effects of zinc supplementation on the ammonia metabolism in LC patients with hyperammonemia; (vii) a pilot study to evaluate the effects of late-evening snacks (LES) and a new treatment (α-glucosidase inhibitor) in LC patients with impaired glucose tolerance; (viii) an experimental study to estimate the effects of supplementation of BCAA granules on the development of HCC in a mouse model of NASH; (ix) education programs for nutritional management in both LC patients and the general Japanese population; and (x) re-evaluation of previous publications concerning nutritional therapies in LC patients. After repeated discussion of the results, we then proposed the new guidelines for nutritional management of Japanese LC patients with the aim of preventing HCC.

RESULTS

THE FOLLOWING FINDINGS were obtained: (i) approximately 30% of patients with LC are overweight (body mass index >25), with the incidence being higher in male LC patients due to NASH and alcohol; (ii) only 30% of LC patients have adequate dietary intake for both energy and protein; (iii) iron intake (mean value, 6.7 mg/day) does not differ among CLD patients; (iv) percent arm circumference, percent arm muscle circumference, and serum concentrations of free fatty acid, tumor necrosis factor (TNF)-α and soluble TNF receptors are significantly correlated with npRQ;[27–29] (v) serum non-transferrin-bound iron (NTBI) determined by a newly developed high-performance liquid chromatography system is elevated in LC patients,[30,31] although further study is necessary to clarify whether serum NTBI levels are associated with the development of HCC; (vi) plasma amino acid imbalance is closely associated with the numbers and functions of peripheral dendritic cells;[32] (vii) long-term zinc supplementation therapy in LC patients tends to decrease HCC occurrence; (viii) LES and administration of α-glucosidase inhibitor improve impaired glucose

Table 1 Recommendations for nutritional management of liver cirrhosis: part 1

I. Assessment before nutrition and diet therapy
  (1) Evaluate clinical stage (compensated or decompensated liver cirrhosis) and the severity of liver damage (i.e. Child–Pugh classification) as well as presence of portal-systemic shunt.
  (2) Perform SGA† and anthropometry.‡
  (3) Evaluate impaired glucose tolerance, insulin resistance§ and postprandial hyperglycemia.
  (4) Evaluate oxidative stress conditions.¶
  (5) Examine dietary intake using a questionnaire.
  (6) Perform indirect calorimetry†† and trace element measurement.

†Subjective global assessment (SGA) is an effective method in the screening of malnourished patients. It examines age, sex, height, bodyweight, changes in bodyweight, changes in food intake, the presence of gastrointestinal symptoms, intensity of activities of daily living (ADL), the condition of loss of subcutaneous fat and muscles, the presence of edema/ascites, hair condition, among other factors.
‡In addition to height, bodyweight and body mass index (BMI: bodyweight [kg]/height [m]^2), arm circumference (AC) and triceps skinfold thickness (TSF) are measured using an insert tape and adipometer. Moreover, arm muscle circumference (AMC) is calculated by AC – 3.14 × TSF. Data are evaluated using standard values for the physical measurements of a Japanese individual (Japanese Anthropometric Reference Data: JARD 2001).[39] This allows the calculation of basal energy expenditure, resting energy expenditure and protein (amino acid) requirements according to age, sex difference and physical measurements. More detailed body composition analysis methods have recently become available, and these are based on bioelectrical impedance analysis.
§Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR = blood fasting insulin [μU/mL] × fasting blood glucose level [mg/dL] / 405) is used as an index for insulin resistance, with HOMA-IR ≥2.5 considered to indicate insulin resistance. However, this equation assumes that the fasting blood glucose levels are <140 mg/dL.
¶Although there are numerous biomarkers for evaluating oxidative stress, the measurement of serum ferritin levels should be used for the purpose of preventing hepatocellular carcinoma. In addition, the presence of anemia is examined using hemoglobin concentrations.
††Where indirect calorimeters are available, measurement of resting energy expenditure, non-protein respiratory quotient (npRQ) and oxidation rates for various nutrients (carbohydrate, fat, protein) after overnight fasting is useful in evaluating protein-energy malnutrition. Anthropometric values (%AC, %AMC) and the serum free fatty acid levels are useful indexes for npRQ during routine care; serum levels of tumor necrosis factor (TNF)-α and soluble TNF receptors and plasma ghrelin levels may also be used as references.

tolerance;[33–35] (ix) supplementation of BCAA granules and BCAA-enriched nutrients improve liver function and energy metabolism;[36,37] and (x) supplementation of BCAA granules inhibits carcinogenesis in a mouse model of NASH, possibly via improvement of insulin resistance.[38]

Based on these data and discussions among the members of JNUS, guidelines for nutritional management of Japanese LC patients were prepared and are shown in Tables 1 and 2. The guidelines consist of two parts. The first part (Table 1) describes essential nutritional assessments that should be performed before instituting nutritional and diet therapy. The second part (Table 2) describes the recommended dietary management for each nutrient, including energy, protein, fat, sodium chloride, iron and other nutrient requirements. Restriction of sodium chloride was decided based on the therapeutic guidelines for hypertension by the Japanese Society of Hypertension.[40] We also included supplemental descriptions in the tables in order to ensure that dietitians are able to perform nutritional assessment and therapy in accordance with these guidelines.

At this point, it is not clear whether supplementation with BCAA granules has any preventive effects on HCC recurrence after primary treatment for HCC, as the number of enrolled patients is small. A double-blind controlled study for zinc supplementation in LC patients with hyperammonemia is also still on-going. The final results of this study are expected to be available by the end of 2012.

DISCUSSION

IN ORDER TO establish new guidelines on nutritional management in LC patients, it is important to consider hepatocarcinogenesis. In this article, based on the results of JNUS projects between 2008 and 2010 and re-evaluation of previous publications concerning nutritional therapies in LC patients with or without HCC, we proposed new guidelines for nutritional management of Japanese LC patients, with the aim of preventing HCC.

We hope these guidelines will form a basis for future discussions on nutritional management of LC by specialists such as hepatologists and dietitians.

Table 2 Recommendations for the nutritional management of liver cirrhosis: part 2

II. Nutrition and diet therapy
(1) Energy requirements[a]
25–35 kcal/kg (ideal bodyweight) per day, based on *Standards for Dietary Intake* (2010 Edition, Recommended Dietary Allowance According to Intensity of Daily Activity).
If any abnormalities are seen in glucose tolerance, intake should be 25 kcal/kg (ideal bodyweight) per day.
(2) Required protein intake[b]
If there is no protein intolerance: 1.0–1.5 g/kg/day (including oral BCAA granules).[c]
If there is protein intolerance: 0.5–0.7 g/kg per day + BCAA-enriched enteral nutrient mixture.[d]
(3) Required fat intake:[e] lipid energy ratio 20–25%.
(4) Sodium chloride:[f] ≤6 g/day and <5 g/day if there are ascites and/or edema, respectively
(5) Iron:[g] ≤7 mg/day if serum ferritin levels are above the upper limit of the reference interval.
(6) Others: zinc supplementation,[h] adequate intake of vitamins and dietary fiber (e.g. vegetables, fruits).
(7) LES as a divided meal (4 times/day) (amounts to 200 kcal).[i]

[a]Resting energy expenditure is often accelerated in liver cirrhosis patients and protein-energy malnutrition (PEM) is observed in approximately 80–90% of patients. However, approximately 30% of patients are obese, with a body mass index (BMI) of ≥25. Moreover, in cases of hepatitis C, there is a high frequency of insulin resistance exhibited. It is important to determine the required amount of energy by taking into account such nutritional conditions.
[b]Required protein intake includes the protein content of branched-chain amino acid (BCAA) formulation (BCAA granules or BCAA-enriched nutrient mixture for chronic liver failure). The majority of patients with decompensated liver cirrhosis (LC) often have protein intolerance, which is determined by referring to the blood ammonia levels.
[c]Patients in the decompensated state, including cases with hyperammonemia, are judged as having protein intolerance. The administration of BCAA granules (e.g. Livact Granules) is essential for the patient with serum albumin <3.5 g/dL, Fischer's ratio <1.8 and/or BTR < 3.5, and is usually administrated by dividing the dosage of 3 packs/day (12 g) into 3 administrations, but there is also a method whereby 2 packs are administrated (before sleep). Prevention of hepatocellular carcinoma (HCC) is expected in male hepatitis C patients with BMI >25 due to long-term administration of this formula. Improvement of the amino acid imbalance is also useful in recovering decreased dendritic cell functions.
[d]When administrating BCAA-enriched enteral mixtures (e.g. Aminoleban EN and Hepan ED), the amount of energy and protein present in this nutrient should be included in the total intake of energy and protein for the day. BCAA-enriched enteral mixtures should be the first choice in patients with PEM, regardless of the presence of protein intolerance.
[e]Ideal ratio of fatty acid composition for the inhibition of HCC has not been clarified, but a decline in n-6 and n-3 polyunsaturated fatty acids has been observed in patients with LC.
[f]Even patients who are not physically observed to have edema/ascites have a tendency for water retention, so fundamentally salt should be restricted.
[g]Excess deposition of iron in the liver causes oxidative stress and promotes hepatocarcinogenesis; thus, unless severe anemia is observed, an iron-restricted diet should be standard. Moreover, although the standard value of serum ferritin level differs with sex, phlebotomy in small amounts should be considered for patients with values ≥150 ng/mL.
[h]Zinc supplementation improves hyperammonemia and may suppress the occurrence of HCC in patients with LC over long-term administration.
[i]Lifestyle and eating habits of patients should examine. Late-evening snack (LES) is also useful for managing the blood glucose level in patients with impaired glucose tolerance, and combined use with α-glucosidase inhibitor enhances this effect. Usually, snacks such as rice balls (*onigiri*) are provided, but with the recommendation of using enteral nutrients, food products rich in BCAA are also used.
Fischer's ratio: BCAA/tyrosine + phenylalanine.
BTR: molar ratio of BCAA and tyrosine.

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Original Article

Analysis of vanin-1 upregulation and lipid accumulation in hepatocytes in response to a high-fat diet and free fatty acids

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High-fat diet is one of the causes of nonalcoholic fatty liver disease. We have previously demonstrated that high-fat diet induces upregulation of adipose differentiation-related protein mRNA expression accompanied by lipid droplet formation in mouse liver. Vanin-1 is a ubiquitous epithelial ectoenzyme that has pantetheinase activity and produces cysteamine, a potent endogenous antioxidant. In the present study, we analyzed the expression of hepatic vanin-1 mRNA following the administration of a high-fat diet in mice as well as free fatty acids in hepatocyte cultures and speculated its possible mechanism. Vanin-1 mRNA levels in the livers of mice were upregulated within a day of the high-fat diet, even before the expression of adipose differentiation-related protein mRNA and lipid accumulation. An *in vitro* analysis using HuH-7 cells revealed a significant upregulation of vanin-1 mRNA by as low as 0.01 mM oleic acid; however, lipid accumulation in hepatocytes was not affected at this concentration. Furthermore, vanin-1 mRNA was differentially upregulated by various free fatty acids irrespective of the grade of lipid accumulation. These findings indicate that the upregulation of vanin-1 precedes lipid accumulation and is differentially mediated by various types of free fatty acids in the model, presenting vanin-1 as a novel player in the pathogenesis of nonalcoholic fatty liver disease.

Key Words: **fatty acids, lipid droplet, nonalcoholic fatty liver disease, vanin-1**

Hepatic cells are important stores of neutral lipids that act as a physiological buffer. Although lipid storage is necessary for energy preservation and synthesis of lipoproteins and steroid hormones, excess accumulation often leads to disorders such as fatty liver, obesity, and atherosclerosis.[1,2] Nonalcoholic fatty liver disease (NAFLD) covers a wide array of pathological conditions, ranging from benign fatty liver to different forms of nonalcoholic steatohepatitis (NASH) including steatosis with inflammation, steatosis with inflammation and mild-to-advanced fibrosis, steatosis with fibrosis alone, cirrhosis, and end-stage liver disease.[3,4] NASH is a more severe histological form of NAFLD, and has gained clinical importance in developing countries due to its progression to cirrhosis.[3,4] A "two-hit" hypothesis has been suggested for the pathogenesis of NAFLD.[5] The "first hit" is caused by fat deposition in the liver that is closely associated with insulin resistance, leading to NAFLD. The "second hit" is a result of oxidative stress, leading to lipid peroxidation and increased cytokine production and inflammation in the hepatocytes, ultimately resulting in NASH.[6]

Currently, the mechanism for the development of hepatic steatosis is being elucidated through the identification of genes involved in lipid metabolism using mouse models of fatty liver disease. In these studies, vanin-1 has been recognized as a candidate for lipid metabolism-related genes. Vanin-1 gene expression was upregulated 17.1-fold in peroxisome proliferator-activated receptor alpha-deficient (PPARα$^{-/-}$) mice who overexpressed PPARγ when compared with fatty livers induced by fasting or choline deficiency.[7] Similarly, DNA microarray analysis of phosphatase and tensin homolog-deficient (Pten$^{-/-}$) mice exhibiting hepatic steatohepatitis revealed a 3-fold upregulation of the vanin-1 gene when compared with their non-fatty liver counterparts.[8] Furthermore, vanin-1 mRNA expression in apolipoprotein E-deficient (apoE$^{-/-}$) mice, in whom fatty liver was accelerated by administering *trans*-10, *cis*-12-conjugated linoleic acid, was demonstrated to be higher than that in *trans*-11, *cis*-9 linoleic acid-administered mice exhibiting amelioration of fatty liver. In this report, the vanin-1 gene was considered to be associated with fatty liver based on its high steatotic index.[9] These studies clearly raise the possibility of an association between vanin-1 and the pathologic course of fatty liver disease.

Vanin-1 is an ectoenzyme anchored to the surface of epithelial cells by a glycosylphosphatidyl inositol moiety.[10] Its pantetheinase activity is involved in the metabolic pathway of pantothenate (vitamin B5) and is a main provider of cysteamine to tissues.[11] Interestingly, both the presence and absence of vanin-1 has been demonstrated to have a cytoprotective role in different cell types. Vanin-1 deficient mice show elevated glutathione (GSH) levels that are associated with better resistance to oxidative injury and with reduced apoptosis, suggesting that vanin-1 may be a negative regulator of cellular GSH storage.[12] Further, vanin-1-deficient mice show a remarkably increased resistance to stress and decreased intestinal inflammation.[13,14] Epithelial vanin-1 was also found to regulate inflammation-driven cancer development in a colitis-associated colon cancer model.[15] The presence of mouse vanin-1 has been shown to play a cytoprotective role for islet beta cells possibly through the antioxidant property of cysteamine, and shown to regulate the development of type 1 diabetes. It has been suggested that the impact of cysteamine on a disease state may directly depend upon local tissue concentrations, although the mechanisms of control of inflammation vs survival by cysteamine are poorly characterized.[16] In the light of these studies, we hypo-

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thesized that the regulation of vanin-1 may determine the progression of fatty liver disease.

Although microarray studies in PPARα$^{-/-}$, Pten$^{-/-}$, and apoE$^{-/-}$ mice have demonstrated the upregulation of vanin-1 in the lipid metabolic pathway in the liver,[7–9] the contribution of vanin-1 to the development of hepatic steatosis is not known. Therefore, the aim of this study was to evaluate the induction of the vanin-1 gene in obese mice as well as in human hepatocyte cells treated with various fatty acids.

Materials and Methods

Animals, biochemical parameters, and tissue analyses. Nine-week-old male C57Bl/6N and C57Bl/6J-*ob/ob* (*ob/ob*) mice, purchased from Sankyo Labo Service (Tokyo, Japan), were housed at 22°C under a 12-h light-dark cycle (lights on at 0700 h) and were allowed ad libitum access to food and water. Blood glucose was measured using Glucocard (Arkray, Kyoto, Japan). Serum parameters were measured using a Clinical Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan). For histology and immunohistochemistry, the mice were anesthetized with 2.5% avertin and perfused through the left ventricle with 20 mL of ice-cold PBS and then with 4% paraformaldehyde in PBS. Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and the sections stained with hematoxylin and eosin (HE) for histopathologic evaluation. All the experiments were conducted in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

***In situ* hybridization.** *In situ* hybridization was performed using a digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany)-labeled copy RNA (cRNA) probe for vanin-1 mRNA as described previously.[17]

Cell culture and histopathological lipid-droplet evaluation. Human hepatocellular carcinoma cell line HuH-7 was obtained from RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Free fatty acids (FFAs) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 M and stored at −20°C before use. In some cultures, fatty acids (as a sodium salt, conjugated with 2% bovine serum albumin (BSA) and/or other reagents were added to the medium at the indicated concentrations. HuH-7 cells were plated in glass-bottom culture dishes (Matsunami Glass, Osaka, Japan) and cultured in DMEM containing 10% FBS. The medium was changed to DMEM containing fatty acids conjugated with BSA and 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Invitrogen) as a lipid probe. The cells were fixed with 4% paraformaldehyde, permeabilized, and then incubated with TO-PRO-3 (Invitrogen) for nuclear staining. Fluorescent images were observed with a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Real-time RT-PCR analyses were performed as described previously.[18,19] Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using a RETROscript kit (Applied Biosystems/Ambion, Austin, TX). Real-time PCR analysis was performed using an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA) according to the manufacturer's specifications. TaqMan probes for mouse vanin-1 (Mm00495965_m1), human vanin-1 (Hs00190982_m1), human PPARα (Hs00947539_m1), and human adipose differentiation-related protein (ADRP; Hs00605340_m1) were purchased from Applied Biosystems. To normalize the relative expression of the genes of interest, eukaryotic 18S rRNA (Hs99999901_s1, X03205.1) was used as an endogenous control.

Statistical analysis. Statistical analysis was performed either by 1-way analysis of variance along with the Tukey multiple comparison test or by 2-way analysis of variance with subsequent Bonferroni post-test. All tests were performed using GraphPad Prism ver. 5 (GraphPad Software, La Jolla, CA). $p<0.05$ was considered significant.

Results

Vanin-1 mRNA expression following the high-fat diet. Fig. 1A shows the relative vanin-1 mRNA expression in the livers of mice fed with the high-fat diet for 2, 4, 6, and 12 weeks. Vanin-1 mRNA was upregulated in the high-fat diet groups compared with the normal diet groups starting from the early disease stage, although there was no significant difference between the 2-week and the 12-week groups, indicating that vanin-1 mRNA expression was not regulated by the duration for which the high-fat diet was administered. In a previous study, we observed that normal mice fed with a high-fat diet for 2 to 6 weeks did not develop diabetes; however, liver steatosis was confirmed as early as 2 weeks through the detection of triacylglycerol (TG) accumulation by Oil Red O staining.[20] To determine a possible association between the onset of high-fat diet-induced steatosis and vanin-1 mRNA expression, we focused on the vanin-1 mRNA expression in the first week of high-fat dietary administration. Although liver steatosis did not occur within the first week (H&E staining; data not shown) in mice on a high-fat diet, vanin-1 mRNA expression was increased from Day 1 when compared to that in Day 0 control mice (Fig. 1B). Vanin-1 mRNA expression level had returned to control level when the mouse fed the normal diet for 4 days following the high-fat diet for 3 days (Day 3 + 4).

Effects of the high-fat diet condition on physiological and biochemical parameters. Total cholesterol (T-Cho), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and FFA levels were significantly elevated in the high-fat diet groups accompanied by an increase in body weight, liver weight, and white adipose tissue (WAT) (Table 1). There were no significant differences between Day 3 + 4 and Day 0 control mice, except glutamic-pyruvic transaminase (GPT) level. These results indicated that FFA uptake to the liver was higher in the high-fat diet group, which possibly affected lipid metabolism and vanin-1 mRNA expression in the hepatocytes of these mice.

Steatosis in *ob/ob* mice and vanin-1 mRNA expression in *ob/ob* mouse livers. Spontaneously obese (*ob/ob*) mice have been used as an effective model to understand the mechanisms leading to the development of hepatic steatosis.[21] The grade of liver steatosis gradually advanced in the 14- and 32-week-old mice (Fig. 2A). The relative expression of vanin-1 mRNA in the livers of 14- and 32-week *ob/ob* mice was approximately 6–7 times higher than in the livers of wild type mice (Fig. 2B). The expression of vanin-1 mRNA between the 2 age groups was not significantly different, although the grade of liver steatosis gradually advanced (Fig. 2A).

Hepatic distribution of vanin-1 mRNA by *in situ* hybridization. In 34-week-old *ob/ob* mice, vanin-1 mRNA was expressed strongly and particularly in hepatocytes near large lipid droplets in the central vein area (Fig. 3 A and B). These results suggest that the expression of vanin-1 mRNA is more prominent in the areas of lipid droplet formation in the liver.

Effect of FFAs on lipid accumulation and *in vitro* expression of hepatic vanin-1 mRNA. The high-fat diet fed to the experimental mice comprised of 58% lard obtained from pigs, and oleic acid constitutes about 44–47% of the FFAs in the lard.[22] So we used the hepatoma cell line HuH-7 as an *in vitro* lipid-accumulation model to investigate the direct effect of oleic acid in hepatocytes. Oleic acid concentrations higher than 1 mM induced lipid droplet formation, whereas 0.1 and 0.01 mM were ineffective

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