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Citrin deficiency as a cause of chronic liver disorder mimicking non-alcoholic fatty liver disease☆

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***Background/Aims*: Citrin deficiency caused by SLC25A13 gene mutations develops into adult-onset type II citrullinemia (CTLN2) and may be accompanied with hepatic steatosis and steatohepatitis. As its clinical features remain unclear, we aimed to explore the characteristics of fatty liver disease associated with citrin deficiency.**

***Methods*: The prevalence of hepatic steatosis in 19 CTLN2 patients was examined, and clinical features were compared with those of non-alcoholic fatty liver disease (NAFLD) patients without known SLC25A13 gene mutations.**

***Results*: Seventeen (89%) CTLN2 patients had steatosis, and 4 (21%) had been diagnosed as having NAFLD before appearance of neuropsychological symptoms. One patient had steatohepatitis. Citrin deficiency-associated fatty livers showed a considerably lower prevalence of accompanying obesity and metabolic syndrome, higher prevalence of history of pancreatitis, and higher serum levels of pancreatic secretory trypsin inhibitor (PSTI) than fatty livers without the mutations. Receiver operating characteristic curve analyses revealed that a body mass index < 20 kg/m^2 and serum PSTI > 29 ng/mL were associated with citrin deficiency.**

***Conclusions*: Patients presenting with non-alcoholic fatty liver unrelated to obesity and metabolic syndrome might have citrin deficiency, and serum PSTI may be a useful indicator for distinguishing this from conventional NAFLD.**

***Keywords*: Adult-onset type II citrullinemia; Body mass index; Non-alcoholic fatty liver disease; Pancreatic secretory trypsin inhibitor**

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Abbreviations: AGC, aspartate–glutamate carrier; NADH, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; ASS, argininosuccinate synthetase; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; CTLN2, adult-onset type II citrullinemia; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; US, ultrasonography; CT, computed tomography; HCV, hepatitis C virus; ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; PSTI, pancreatic secretory trypsin inhibitor; NAS, NAFLD activity score; ROC, receiver-operating characteristic; AUC, area under the ROC curve; γGT, γ-glutamyltransferase; HCC, hepatocellular carcinoma.

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1. Introduction

Citrin is a liver-type mitochondrial aspartate–glutamate carrier (AGC) known to exchange mitochondrial aspartate for cytosolic glutamate and a proton [1–3]. This function is important in translocating cytosolic nicotinamide adenine dinucleotide (NADH) reducing equivalents into the mitochondria as a part of the malate-aspartate shuttle, and NADH produced by malate is oxidized to generate adenosine triphosphate (ATP) in the oxidative phosphorylation pathway. Moreover, liver-specific AGC also plays an important role in supplying aspartate to argininosuccinate synthetase (ASS) in the cytosol to generate argininosuccinate in the urea cycle. Thus, a deficiency of liver-specific AGC, namely citrin, results in dysfunction of the urea cycle and hyperammonemia [2,3].

Citrin deficiency is an autosomal recessive disorder caused by a mutation of the SLC25A13 gene encoding citrin, which is located on chromosome 7q21.3 [2–4]. Some of the mutations identified in Japanese patients are frequently found in the East Asian population, and the prevalence of these mutations has been estimated to be 1/65 in China, 1/112 in Korea, and 1/69 in Japan [3,5,6]. Furthermore, several cases with SLC25A13 gene mutations have been reported in Israel, USA, and elsewhere [7–9], indicating a world-wide incidence of citrin deficiency.

The SLC25A13 gene mutation leads to neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type II citrullinemia (CTLN2) [2,3]. Citrin deficiency is sometimes asymptomatic in adults probably by metabolic adaptation, but CTLN2 is characterized by the sudden onset of various neuropsychological symptoms including disorientation, abnormal behavior, convulsions, and coma due to hyperammonemia, which may result in rapidly progressive and irreversible brain edema and death. Generally speaking, the prognosis of patients with CTLN2 is not favorable after the onset of encephalopathy, and liver transplantation is required for severe CTLN2 which is refractory to various ammonia-lowering and neuroprotective therapies. Furthermore, certain types of conventional treatments for brain edema, such as intravenous administration of hyperosmotic and high sugar solutions, have been reported to aggravate encephalopathy [2,10,11], and the mechanism of toxicity of carbohydrate overload for citrin deficiency has been recently clarified using mouse models [12]. Thus, early diagnosis of citrin deficiency and CTLN2 and appropriate treatment might improve prognosis.

Citrin deficiency has been demonstrated to present with hepatic steatosis and steatohepatitis. Previously, we reported the case of a patient with non-alcoholic fatty liver disease (NAFLD) who was later shown to carry SLC25A13 gene mutation [13]. The patient had elevation of serum aminotransferase levels and fatty liver of unknown causes, and was first diagnosed as having NAFLD by liver biopsy at the age of 23. Ten years afterwards, serious encephalopathy suddenly appeared after accidental consumption of alcohol. The patient was then diagnosed as having CTLN2, but did not respond to any conservative treatments and required urgent liver transplantation [13,14]. Moreover, Takagi et al. have also reported three CTLN2 patients with liver histologies of steatohepatitis [15]. These findings suggest the possibility that citrin deficiency may be present in adults who are diagnosed as having NAFLD or non-alcoholic steatohepatitis (NASH), and elucidating the characteristics of fatty liver related to citrin deficiency may be helpful in the early distinction of this disease from conventional NAFLD. However, the clinical features of this phenomenon have not been fully investigated.

In the present study, we evaluated the clinical characteristics of citrin deficiency-associated fatty liver through analysis of CTLN2 patients presenting with fatty liver, and sought to find key indicators to detect citrin deficiency in NAFLD patients.

2. Methods

2.1. Patients

Nineteen CTLN2 patients (11 men and 8 women, mean age 37 ± 10 yrs), who had been admitted to Shinshu University Hospital between 1979 and 2007, were examined in this study. All patients had experienced neuropsychological symptoms due to hyperammonemia. CTLN2 was suspected from the presence of neurological abnormalities and elevated plasma ammonia and citrulline levels, and diagnosis was confirmed by the presence of SLC25A13 gene mutations. In these patients, the presence of fatty liver was confirmed by liver histology (i.e., lipid accumulation in more than 5% of hepatocytes) or imaging modalities such as ultrasonography (US) and/or computed tomography (CT) scan.

For comparison, 25 NAFLD patients (10 men and 15 women, mean age 61 ± 13 yrs), who had been admitted to Shinshu University Hospital between 2003 and 2004 for the purpose of percutaneous liver biopsy, were analyzed. These patients did not have any of the 16 different mutations frequently found in the SLC25A13 gene of Japanese patients with citrin deficiency. Three patients were diagnosed as having NASH according to the histological criteria proposed by Kleiner et al. [16].

NAFLD patients without SLC25A13 mutations satisfied the following exclusion criteria: no consumption of alcohol or steatosis-inducing drugs such as tamoxifen or anti-retroviral agents, negative results for hepatitis B surface antigen, anti-hepatitis B core and anti-hepatitis C virus (HCV) antibodies, and no presence of other types of chronic liver disease such as autoimmune liver diseases, Wilson's disease, hereditary hemochromatosis or α1-antitrypsin deficiency.

2.2. Analysis of clinical features

At the time of admission, body height, weight, and waist circumference were measured by hospital staff unaware of the patients' medical information, and clinical course and past history were taken for each patient and family. History of alcohol or drug consumption and food preferences were also recorded. The time of diagnosis of CTLN2 was taken as the age of appearance of neuropsychological symptoms and detection of hyperammonemia and/or hypercitrullin-

emia. The time of diagnosis of fatty liver was taken as the age of diagnosis by liver biopsy or imaging. The time of diagnosis of hyperlipidemia, type 2 diabetes, and elevation of alanine aminotransferase (ALT) level (> 35 U/L) was taken as the age of detection of these abnormalities by blood examination. The time of diagnosis of pancreatitis was taken as the age of diagnosis by physical, laboratory, and imaging examinations.

The presence of obesity was defined as having a body mass index (BMI) of more than 25 kg/m^2, based on criteria released by Japan Society for the Study of Obesity [17]. Patients were considered to be hypertensive if their systolic/diastolic blood pressure was greater than 140/90 mmHg, or if they were taking anti-hypertensive drugs. Patients were considered to be diabetic if they had a fasting glucose level equal to or higher than 7.0 mmol/L (126 mg/dL) or if they were taking insulin or oral hypoglycemic drugs. Patients were considered to have hyperlipidemia if their fasting serum levels of cholesterol or triglyceride were equal to or higher than 5.7 mmol/L (220 mg/dL) or 1.7 mmol/L (150 mg/dL), respectively, or if they were taking lipid-lowering drugs [18]. The presence of metabolic syndrome was judged according to the criteria released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome [19].

2.3. Laboratory and biochemical examination

At the time of admission, blood samples were obtained in a fasting state, and complete blood counts and blood chemistries, including lipoprotein, were determined by standard methods. The homeostasis model assessment of insulin resistance (HOMA-IR), a simple and reliable indicator of insulin resistance, was calculated by the following formula: [fasting plasma glucose (mmol/L) × immunoreactive insulin (μU/mL)]/22.5. A HOMA-IR of greater than 2 was defined as the presence of insulin resistance [18]. Serum pancreatic secretory trypsin inhibitor (PSTI) concentration was measured by immunoradiometric assay (Eiken Chemical Co., Ltd, Tokyo, Japan). Hepatic ASS activity was measured as described by Su et al. [20], and values were expressed as percentages of control.

2.4. Imaging examination

Each patient underwent abdominal US or CT scan. The presence of fatty liver was assessed according to positive findings such as hepatorenal contrast, blurring of the vascular wall, and/or profound attenuation of the diaphragm [18,21]. In CT scans, the presence of hepatic steatosis was assessed according to criteria described elsewhere [22].

2.5. Liver histology

Liver samples were obtained from 14 CTLN2 patients with fatty liver by percutaneous US-guided biopsy (6 patients; Nos. 1, 7, 8, 11, 12, and 14 in Table 1) or from explanted livers at living donor liver transplantation (8 patients). Liver samples were fixed in 10% neutral formalin, and sections cut at 4-μm thickness were stained with hematoxylin and eosin or Azan-Mallory methods. Histological diagnosis was performed by two experienced pathologists (KS, EH) in a blinded fashion. The NAFLD activity score (NAS) was assessed according to the criteria proposed by Kleiner et al. [16]. The diagnosis of NASH was confirmed by a NAS of more than 5.

2.6. SLC25A13 gene mutation analysis

DNA was extracted from peripheral blood using standard methods. Sixteen SLC25A13 mutations found relatively frequently in Japan, 13 mutations described previously [4,6,23–25] and three unpublished novel mutations (Tabata et al., manuscript in preparation), were tested by the polymerase chain reaction–restriction fragment length polymorphism

Table 1
Characteristics of 19 CTLN2 patients at time of admission

No.	Age	Sex	Symptom	Prolonged neonatal jaundice	Fondness for beans	Ammonia (ng/mL)	PSTI (ng/mL)	Citrulline (nmol/mL)	SLC25A13 mutation	Hepatic ASS activity (%)	Reference No.
1	42	M	Disorientation	−	+	**96**	**110**	**106**	V/V	**26**	
2	43	F	Coma	−	+	**201**	**66**	**606**	IV/IV	**21**	14
3	21	F	Consciousness disturbance	−	+	**223**	**300**	**310**	I/II	**14**	34
4	52	F	Disorientation	−	−	35	**21**	**1241**	I/II	**1**	34
5	41	F	Coma	−	+	**223**	**32**	**373**	II/II	**10**	10, 39
6	48	M	Disorientation	−	+	59	**29**	**1542**	I/II	**21**	
7	32	M	Consciousness disturbance	−	+	**92**	**77**	**416**	IV/IV	**17**	13, 14
8	38	M	Coma	−	+	**239**	**35**	**590**	I/XXXVII	**8**	
9	51	M	Consciousness disturbance	−	+	67	**64**	**86**	II/II	70	27
10	24	F	Disorientation	−	−	**100**	**110**	**200**	I/II	**4**	34, 37
11	19	F	Consciousness disturbance	+	+	**201**	**29**	**498**	I/II	**6**	34
12	26	F	Consciousness disturbance	−	+	**142**	NE	**155**	II/II	**41**	
13	48	F	Abnormal behavior	−	+	**178**	**29**	**486**	I/I	NE	
14	52	M	Consciousness disturbance	−	+	**83**	**60**	**408**	I/V	NE	
15	25	M	Tremor	−	−	**224**	NE	**474**	I/I	**6**	34
16	45	M	Disorientation	−	−	**172**	NE	**154**	V/VI	**10**	34
17	32	M	Visual impairment	−	−	**91**	**35**	**404**	I/I	**3**	34
18	31	M	Abnormal behavior	−	−	33	14	**117**	I/II	**9**	10, 32, 34,
19	40	M	Consciousness disturbance	−	+	29	**30**	**187**	II/XXII	**4**	10, 32

Nineteen CTLN2 patients have been admitted to our hospital up to 2007.
SLC25A13 gene mutations were indicated as follows. I, 851del4 [4]; II, IVS11+1G > A [4]; IV, S225X[4]; V, IVS13+1G > A [4]; VI, 1800insl [4]; XXII, Ex1-1G > A (Tabata et al, manuscript in preparation); and XXXVII, Ex9-1G > A (Song et al., manuscript in preparation).
Hepatic argininosuccinate synthetase (ASS) activity was determined as described in Methods.
The activities of these patients are expressed as percentages relative to those of normal controls.
M, male; F, female; PSTI, pancreatic secretory trypsin inhibitor; NE, not examined.
Normal ranges are as follows: ammonia, < 75 ng/mL; PSTI, < 20 ng/mL; and citrulline, < 43 nmol/mL.

method and/or multiple methods using Genescan/SNaPshot. A novel mutation identified by PCR/sequencing of all 18 exons and the flanking sequences in an allele of a CTLN2 patient (No. 8 in Table 1) will be described elsewhere (Song et al., manuscript in preparation).

2.7. Ethics

This study was approved by the ethical committees of the Shinshu University School of Medicine and Kagoshima University Faculty of Medicine, and adheres to the principles of the Declaration of Helsinki.

2.8. Statistics

Categorical variables were expressed as percentages, and continuous variables were expressed as median and range (in parentheses). Statistical analyses were performed using SPSS software 11.5J for Windows (SPSS Inc., Chicago, IL). Comparisons were made using Fisher's exact probability test for categorical variables, the χ^2 test for histological scores, and the Mann–Whitney *U*-test for continuous variables. To assess the clinical parameters in differentiating citrin deficiency from NAFLD, receiver-operating characteristic (ROC) curves were constructed by plotting sensitivity against reverse specificity (1 minus specificity) for each value. In this assessment, a larger area under the ROC curve (AUC) corresponded to a more useful marker for differentiating citrin deficiency. The most appropriate cut-off point was the point at which the sum of the sensitivity and specificity was maximized. A probability value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Prevalence of fatty liver in CTLN2 patients at time of admission

Clinical features of the 19 CTLN2 patients are summarized in Table 1. Their median age was 40 years. Only one patient (No. 11) had a past history of prolonged neonatal jaundice, a symptom suggestive of NICCD [26]. Thirteen (68%) patients tended to consume with frequency protein-rich foods such as beans, eggs, or cheese, but the others did not have any peculiar dietary preferences. All patients demonstrated hypercitrullinemia and had SLC25A13 gene mutations (9 homozygotes), and all patients except one (No. 9) demonstrated marked reduction in hepatic ASS activity. Elevation of serum ALT and γ-glutamyltransferase (γGT) levels and fatty liver were observed in 17 (89%), 15 (79%), and 17 (89%) patients, respectively, at the time of admission (Table 2). None of the patients had a history of alcohol consumption. Interestingly, in CTLN2 patients with fatty liver, median BMI values were as low as 18.3 kg/m^2, and none was classified as obese (Table 2). Increases in waist circumference, a reliable anthropometric indicator of visceral obesity, were also not observed in these patients. These results corroborate with the high prevalence of non-obese non-alcoholic fatty liver in CTLN2 patients.

3.2. Time of diagnosis

Next, the times of diagnosis of CTLN2, hyperlipidemia, fatty liver, elevation of serum ALT levels, and pancreatitis were recorded (Table 3). The median age of the initial diagnosis of CTLN2 was 40 years. Hypertriglyceridemia was present in 6 (32%) patients. Interestingly, hypertriglyceridemia, fatty

Table 2
Biochemical and anthropometric parameters of 19 CTLN2 patients at time of admission

No	ALT (U/L)	AST (U/L)	ALP (U/L)	γGT (U/L)	Bilirubin (mg/dL)	Albumin (g/dL)	Triglyceride (mg/dL)	Fatty liver	BMI (kg/m^2)	WC (cm)
1	**102**	**80**	**840**	**376**	0.8	**4.1**	**163**	+	18.6	72
2	**48**	**76**	NE	**225**	0.9	NE	85	+	16.2	68
3	**126**	**205**	**513**	**343**	**1.3**	**3.7**	**374**	+	15.7	58
4	**47**	**54**	**422**	**180**	**1.7**	**3.5**	143	+	21.5	NE
5	**107**	**84**	**399**	**215**	0.5	**3.1**	78	+	14.8	60
6	**69**	30	204	**59**	0.6	**3.7**	111	+	16.1	69
7	**132**	**123**	262	**146**	0.8	**3.7**	136	+	18.0	72
8	**42**	25	208	46	**1.3**	**3.1**	60	+	18.1	74
9	**51**	33	234	**55**	0.8	4.5	53	+	22.1	76
10	**62**	**125**	70	**190**	0.7	**3.5**	**918**	+	14.9	NE
11	**37**	18	164	**235**	0.3	4.3	56	+	23.6	74
12	**37**	28	NE	24	1.1	**3.9**	54	+	20.8	72
13	29	24	**389**	24	0.4	**4.0**	111	+	20.0	71
14	**47**	**56**	331	**235**	**1.4**	**3.9**	**411**	+	22.3	72
15	**140**	**104**	**521**	**205**	1.2	**3.3**	**303**	+	18.4	69
16	**39**	**81**	**633**	**111**	0.5	**3.6**	**684**	+	17.1	68
17*	**161**	**92**	353	**238**	1.2	4.2	58	+	18.8	69
18	**74**	**151**	NE	17	0.9	**3.9**	65	–	24.1	78
19	24	26	296	**57**	0.6	**3.2**	56	–	14.8	62

Patients' numbers correspond to those in Table 1. The presence of fatty liver was evaluated by means of liver histology or imaging.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; γGT, γ-glutamyltransferase; BMI, body mass index; WC, waist circumference; NE, not examined.
*, Patient No. 17 was positive for anti-hepatitis C virus (HCV) antibody and HCV-RNA in serum. Normal ranges are as follows: ALT, 7–35 U/L; AST, 12–37 U/L; ALP, 124–367 U/L; γGT, 8–50 U/L; bilirubin, 0.3–1.2 mg/dL; albumin, 4.2–5.1 g/dL; and triglyceride, 30–150 mg/dL.

Table 3
Age of diagnosis of abnormalities/diseases in CTLN2 patients

No.	CTLN2	Hypertriglyceridemia	Hypercholesterolemia	Fatty liver	ALT elevation	Pancreatitis
1	42	42	NE	41*	41*	ND
2	42	NE	42	42	42	ND
3	21	21	NE	21	16*	ND
4	51	NE	NE	52	52	ND
5	40	NE	NE	41	41	ND
6	48	NE	NE	48	48	ND
7	32	NE	22*	22*	22*	ND
8	37	NE	NE	38	38	ND
9	51	NE	NE	51	20*	ND
10	24	10*	NE	24	18*	10*
11	19	NE	NE	19	0*	ND
12	26	NE	NE	26	26	ND
13	47	NE	NE	38*	38*	ND
14	52	51*	NE	51*	51*	ND
15	25	25	NE	25	25	ND
16	45	20*	NE	44*	45	43*
17	29	NE	NE	29	29	18*
18	25	NE	NE	NE	31	14*
19	40	NE	NE	NE	ND	33*

Patients' numbers correspond with those in Tables 1 and 2.
Time of diagnosis of CTLN2 was taken as the age of appearance of neuropsychological symptoms and detection of hyperammonemia and hypercitrullinemia.
Time of diagnosis of fatty liver was taken as the age of diagnosis by liver biopsy or imaging.
Time of diagnosis of hyperlipidemia and ALT elevation (>35 U/L) was taken as the age of indication of abnormalities in blood examination.
Time of diagnosis of pancreatitis was taken as the age of diagnosis by means of physical, laboratory, and imaging examinations.
*, before the diagnosis of CTLN2; NE, not examined; ND, not detected.

liver, and elevation of serum ALT levels had been pointed out in 3 (16%), 5 (26%), and 8 (42%) patients, respectively, before appearance of neuropsychological symptoms, i.e., diagnosis of CTLN2. Furthermore, 5 (26%) patients had experienced pancreatitis, all of which preceded diagnosis with CTLN2.

3.3. Histological findings of fatty liver with citrin deficiency

In the 17 CTLN2 patients with fatty liver, one patient (No. 17) was excluded because of positive results for anti-HCV antibody and serum HCV-RNA (HCV genotype 1b). The remaining 16 patients (Nos. 1–16) had

Table 4
Histological examination of hepatic steatosis associated with SLC25A13 gene mutations

No.	Steatosis	Microvesicular steatosis	Fibrosis	Lobular inflammation	Portal inflammation	Ballooning	MH	GN	NAS	
1	1 (30%)	1	0	1	0	0	0	0	2	
2	1 (20%)	0	0	0	0	0	0	0	1	
3	2 (60%)	0	3	1	1	0	0	0	3	
4	3 (70%)	0	0	1	0	0	0	0	4	
5	2 (60%)	0	1C	1	1	0	0	0	3	
6	3 (80%)	0	1A	1	0	1	1	1	5	NASH
7	1 (20%)	1	1C	0	0	0	0	0	1	
8	1 (30%)	0	1A	1	0	0	0	0	2	
9	1 (30%)	1	1A	1	1	0	0	0	2	
10	3 (70%)	0	1C	1	1	0	0	0	4	
11	1 (30%)	0	1C	1	0	0	0	0	2	
12	1 (30%)	0	1A	1	0	1	0	0	3	
13	1 (30%)	1	1A	1	1	1	0	0	3	
14	1 (20%)	0	1A	1	1	1	0	0	3	

Patients' numbers correspond with those in Tables 1–3.
Liver samples were obtained from 14 of 16 CTLN2 patients presenting with fatty liver.
Six samples (Nos. 1, 7, 8, 11, 12, and 14) were obtained by percutaneous liver biopsy, and the remainder was obtained from explanted livers at transplantation.
The histological activity and severity were scored according to the criteria proposed by Kleiner et al. [16].
MH, Mallory hyaline; GN, glycogenated nuclei; NAS, NAFLD activity score.

hepatic steatosis, and liver samples were obtained from 14 patients (Nos. 1–14) (Table 4). All samples demonstrated various degrees of macrovesicular steatosis mainly present in zone 3. Apparent microvesicular steatosis was also present in 4 (29%) samples. Perisinusoidal fibrosis was detected in 11 (79%) samples, and one (8%) sample demonstrated bridging fibrosis (Fig. 1A). Mild lobular and portal inflammation consisting of mononuclear cell infiltration was observed in 12 (86%) and 6 (43%) samples, respectively. Hepatocyte ballooning, Mallory hyaline, and glycogenated nuclei were detected in 4 (29%), 1 (7%), and 1 (7%) sample, respectively. Assessment of histological severity of NAFLD revealed that 1 (7%) patient (No. 6) had a NAS of more than 5 (Fig. 1B), but approximately 50% of the patients had a NAS of less than 3 (Table 4).

3.4. Comparison of clinical and biochemical findings between fatty liver with and without SLC25A13 gene mutations

In order to investigate the clinical characteristics of fatty liver associated with citrin deficiency, clinical and biochemical findings of these patients were compared with fatty liver cases without SLC25A13 mutations, i.e., conventional NAFLD. As shown in Table 5, fatty liver patients with the mutations had a lower frequency of obesity (0 vs 72%, $P < 0.001$), metabolic syndrome (0 vs 36%, $P < 0.001$), type 2 diabetes (0 vs 32%, $P < 0.001$), hypertension (0 vs 44%, $P < 0.001$), and a higher frequency of history of pancreatitis (13 vs 0%, $P = 0.002$) than those without the mutations. The median BMI of citrin deficiency patients was significantly lower (18.3 vs 27.3 kg/m^2, $P < 0.001$), as were waist circumference (72 vs 89 cm, $P < 0.001$), serum albumin (3.7 vs 4.4 g/dL, $P = 0.001$), cholesterol (4.8 vs 5.8 mmol/L, $P = 0.003$), low-density-lipoprotein cholesterol (2.1 vs 4.2 mmol/L, $P = 0.002$), and HOMA-IR (1.7 vs 4.8, $P = 0.006$) (Table 5). Serum PSTI concentrations were significantly greater in patients with citrin deficiency (74 vs 10 ng/mL, $P < 0.001$) (Fig. 2).

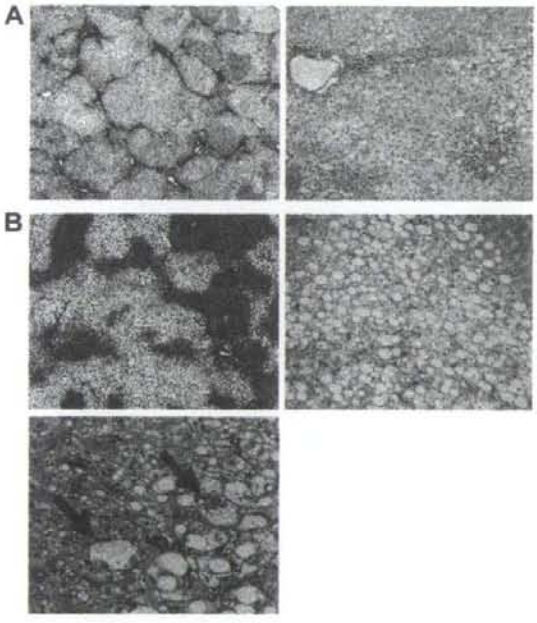

Fig. 1. Liver histology of CTLN2 patients. (A) Liver specimen obtained from patient No. 3. Azan-Mallory staining showed the presence of bridging fibrosis with perisinusoidal fibrosis. Macrovesicular steatosis and mild lobular inflammation were observed (Azan-Mallory staining, ×40; hematoxylin and eosin staining, ×100). (B) Liver specimen obtained from patient No. 6. Diffuse macrovesicular steatosis mainly present in zone 3 was observed. Ballooned hepatocytes containing Mallory hyaline were also found (arrows) (Azan-Mallory staining, ×40; hematoxylin and eosin staining, ×100 and ×600).

3.5. ROC curve analysis

We first tried multivariate logistic regression analysis to identify key clinical parameters for detecting citrin deficiency in NAFLD, but were unable to obtain any meaningful data due to the remarkable differences in BMI and PSTI. Thus, ROC curves were constructed and evaluated for each parameter. The AUC of BMI and PSTI were found to be quite large (0.968 and 0.989, respectively). The odds ratio for BMI was 0.48 (95% confidence interval, 0.924–1.011; $P < 0.001$), and that for PSTI was 1.37 (95% confidence interval, 0.963–1.015; $P < 0.001$). The most appropriate cut-off values for distinguishing citrin deficiency from NAFLD were identified as a BMI < 20 kg/m^2 and serum PSTI > 29 ng/ml, respectively (Fig. 2).

4. Discussion

The present study demonstrates that citrin deficiency can superficially mimic NAFLD before the onset of neurological abnormalities. However, fatty liver patients with SLC25A13 mutations lacked the complications of obesity, metabolic syndrome, and diabetes. Furthermore, BMI and serum PSTI appeared to be clues in distinguishing citrin deficiency from conventional NAFLD. As far as we know, this is the first study to review the clinical features of citrin deficiency-associated fatty liver disease and compare the characteristics with those of NAFLD without SLC25A13 mutations.

As evidenced in Table 3, it is plausible that many patients homozygous for SLC25A13 mutations may be diagnosed as having other diseases, such as NAFLD, hyperlipidemia, and pancreatitis, before the appearance of neurological abnormalities and diagnosis of CTLN2. There is also great variety in the time and mode of onset of neurological episodes in CTLN2; some patients do

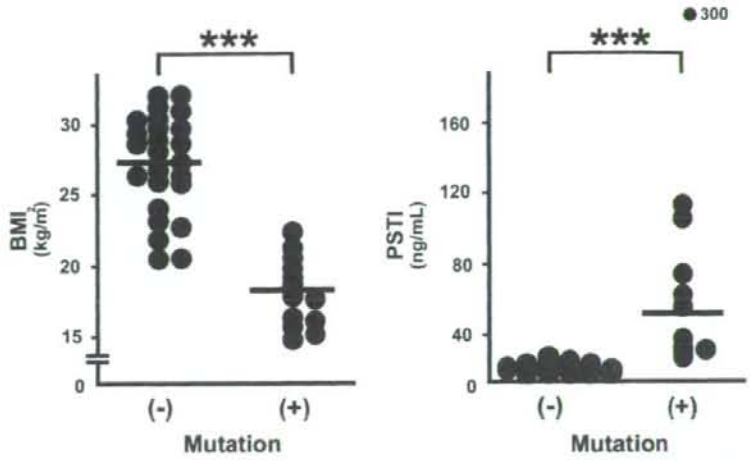

Fig. 2. Comparison of BMI and serum PSTI between fatty liver patients without (−) and with (+) SLC25A13 gene mutations. Each value was plotted, and median values were indicated in the lines. The cut-off values of BMI and serum PSTI were calculated as 20 kg/m² and 29 ng/mL, respectively. ***, $P < 0.001$.

not experience neurological symptoms until middle or old age. For example, in one patient, the first neurological episode occurred at 79 years of age [23]. Indeed, the patient (No. 9), who had been diagnosed as having hepatocellular carcinoma (HCC) of unknown etiology experienced consciousness disturbance for the first time after hepatic resection at 51 years of age, leading to the diagnosis of CTLN2 [27]. This diversity may be partially explained by varying degrees of reduction in hepatic ASS activity. The frequency of homozygotes in Japan (1/19,000) [3] calculated from carrier rate is quite different from the actually reported incidence of CTLN2 (1/100,000) [28], suggesting that many people with citrin deficiency devoid of neurological symptoms may exist in the general population.

The mechanism of steatogenesis by citrin deficiency may be explained as follows: NADH reducing equivalents are supplied to the mitochondria mainly via the malate-aspartate shuttle in human hepatocytes (Fig. 3A). However, citrin deficiency loses this shuttle, resulting in the compensatory up-regulation of the malate–citrate shuttle, which increases citrate in the cytosol (Fig. 3B). A large amount of citrate is converted to acetyl-CoA by ATP-citrate lyase, further leading to overproduction of fatty acids in hepatocytes (Fig. 3C). In fact, elevated mRNA levels of ATP-citrate lyase have been confirmed in livers of citrin/mitochondrial glycerol-3-phosphate dehydrogenase double knockout mouse, a suitable model of human citrin deficiency [12]. The mRNA levels of CD36 (fatty acid translocase) were also elevated in these mouse livers [12], suggesting increased fatty acid uptake capacity into hepatocytes. Based on these findings, enhancement of *de novo* lipogenesis and fatty acid uptake into hepatocytes are considered to be key events in the development of hepatic steatosis in patients with citrin deficiency. Additionally, the observation that plasma ketone body levels are relatively low in CTLN2 patients [29] implies that mitochondrial fatty acid-degrading capacity might be reduced to some degree [30,31].

A striking finding in this study is that some citrin deficiency patients presenting with fatty liver had a history of pancreatitis, indicating a possible susceptibility to citrin deficiency in NAFLD/NASH patients who have experienced this disease. Pancreatitis unrelated to alcohol consumption, which is sometimes chronic and recurrent, is one of the hallmark features of citrin deficiency [32], but its etiology remains unclear. Recently, we reported the case of a non-obese patient with NASH caused by pancreatic exocrine insufficiency [33]. This case was characterized by the absence of obesity and insulin resistance and decrease in serum levels of albumin and cholesterol, showing several resemblances to fatty liver with citrin deficiency. A significant reduction in plasma methionine concentrations was also observed in this case, and both hepatic steatosis and decreased plasma methionine levels were significantly improved by pancreatic enzyme supplementation [33]. Therefore, impaired pancreatic exocrine function due to chronic pancreatitis, latent malabsorption of essential amino acids, and the resultant deficiency of methionine might at least in part contribute to the pathogenesis of fatty liver with citrin deficiency.

The most remarkable characteristics of fatty liver disease due to citrin deficiency were low BMI and high serum PSTI. All CTLN2 patients with fatty liver were lean, which is consistent with other previous reports [13,14,34,35]. This phenomenon may derive from a unique food habit of disliking high calorie and high car-

Table 5
Comparison of clinical, anthropometric, and biochemical characteristics of fatty liver with SLC25A13 gene mutations and NAFLD without such mutations

Parameter	Mutation (+) ($n = 16$)	Mutation (−) ($n = 25$)	P
Age	38 (19–52)	61 (26–77)	**0.001**
Female (%)	50	60	0.313
Body height (m)	1.63 (1.41–1.75)	1.57 (1.34–1.83)	**0.048**
Body weight (kg)	48 (34–68)	68 (45–106)	**<0.001**
WC (cm)	72 (58–76)	89 (70–102)	**<0.001**
BMI (kg/m^2)	18.3 (14.8–23.6)	27.3 (22.2–32.9)	**<0.001**
Obesity (%)	0	72	**<0.001**
Metabolic syndrome (%)	0	36	**<0.001**
Type 2 diabetes (%)	0	32	**<0.001**
Hypertension (%)	0	44	**<0.001**
Hypercholesterolemia (%)	19	52	0.265
Hypertriglyceridemia (%)	38	40	0.874
History of pancreatitis (%)	13	0	**0.002**
Platelet (×10^3/mm^3)	194 (68–257)	187 (78–297)	0.514
PT (%)	73 (32–109)	108 (59–121)	**0.002**
Albumin (g/dL)	3.7 (3.1–4.5)	4.4 (3.5–5.1)	**0.001**
AST (U/L)	56 (18–205)	57 (24–141)	0.063
ALT (U/L)	54 (29–140)	76 (25–298)	0.829
γGT (U/L)	185 (24–376)	82 (36–278)	**0.004**
Cholesterol (mmol/L)	4.8 (2.9–6.7)	5.8 (3.6–9.6)	**0.003**
(mg/dL)	186 (112–259)	221 (140–370)	
Triglyceride (mmol/L)	1.4 (0.6–10.4)	1.8 (0.7–4.6)	0.188
(mg/dL)	124 (53-918)	158 (65–408)	
HDL-C (mmol/L)	1.1 (0.4–2.3)	1.1 (0.8–1.5)	0.893
(mg/dL)	49.5 (19–101)	49.5 (36–68)	
LDL-C (mmol/L)	2.1 (1.0–5.1)	4.2 (1.3–8.8)	**0.002**
(mg/dL)	66 (32–163)	136 (42–283)	
Glucose (mmol/L)	5.6 (4.3–8.1)	6.8 (4.9–10.1)	**0.003**
(mg/dL)	100 (77–145)	122 (88–181)	
Glycohemoglobin (%)	4.8 (4.4–5.1)	6.4 (5.1–9.9)	**0.018**
IRI (μU/mL)	5.4 (3.0–22.8)	14.1 (5.4–50.3)	**0.002**
HOMA-IR	1.7 (0.6–7.0)	4.8 (1.5–14.4)	**0.006**
HOMA-IR > 2 (%)	19	92	**0.006**
PSTI (ng/mL)	74 (21–300)*	10 (6–29)	**<0.001**

These data were compared between CTLN2 patients presenting with fatty liver (Nos. 1–16 in Tables 1–3) and NAFLD patients without the mutations.
Comparisons were made using Fisher's exact probability test for categorical variables and the Mann–Whitney U-test for continuous variables.
Data are expressed as percentages and median (ranges in parenthesis).
*, Data obtained from 13 patients (except patient Nos. 12, 15, and 16).
WC, waist circumference; BMI, body mass index; PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; IRI, immunoreactive insulin; HOMA-IR, homeostasis model assessment for insulin resistance; PSTI, pancreatic secretory trypsin inhibitor.
A HOMA-IR > 2 was considered indicative of the presence of insulin resistance.

bohydrate foods such as rice and sweets [2,3]. An increase in serum PSTI is known to sometimes precede neurological episodes [36,37]. Indeed, in a patient we reported [13], a retrospective measurement revealed a marked elevation of serum PSTI (170 ng/mL) at the time of liver biopsy in which he appeared conscious. Therefore, we may postulate that measurement of BMI and serum PSTI may be useful as an initial screening method to distinguish citrin deficiency from NAFLD. We are prospectively examining serum PSTI in non-obese NAFLD patients, but all patients analyzed thus far have had normal PSTI and no SLC25A13 gene mutations. Verifying this finding will require further large-scale examinations.

It is quite noteworthy that some CTLN2 patients with steatosis developed severe fibrosis (Fig. 1A) or cirrhosis, suggesting that citrin deficiency may progress to cirrhosis. Since all liver samples analyzed were obtained shortly after the onset of neurological abnormalities and the initiation of ammonia-reducing therapy, the possibility that the development of hepatic fibrosis can be attributed to inadequate therapeutic interventions (e.g., strict protein restriction, long-term intravenous hyperalimentation) seems very low. More importantly, citrin deficiency can induce HCC [2,27,38–40], meaning clinicians should also consider citrin deficiency as one of the etiologies of cryptogenic cirrhosis or HCC.

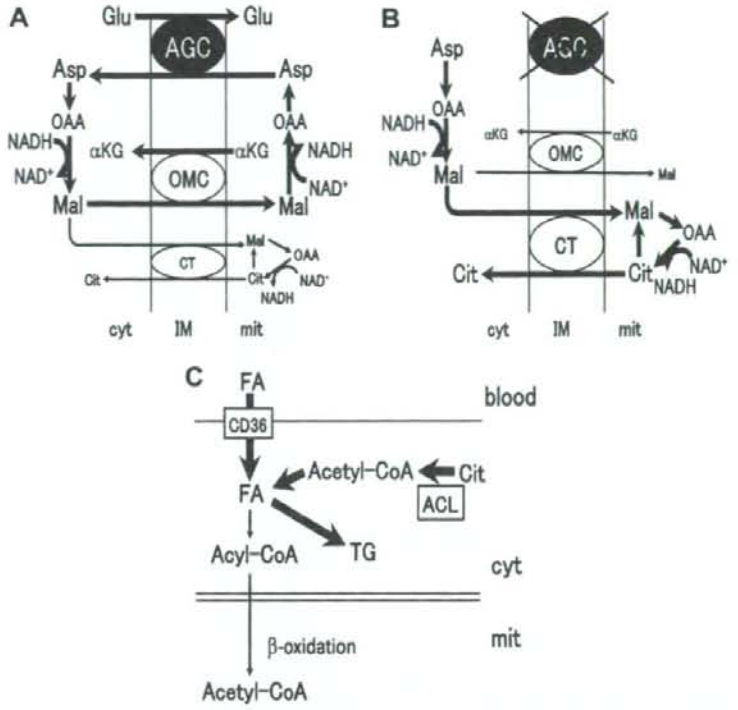

Fig. 3. A proposed mechanism of steatogenesis in patients with citrin deficiency. (A) NADH-supplying systems into mitochondria in human hepatocytes. The malate–aspartate shuttle plays a central role in translocating NADH from the cytosol to mitochondria, and citrin (aspartate–glutamate carrier, AGC) is one of the essential components of this pathway. Asp, aspartate; Cit, citrate; CT, citrate transporter; cyt, cytosol; Glu, glutamate; IM, intermembrane space; αKG, α-ketoglutarate; Mal, malate; mit, mitochondrial matrix; NADH, nicotinamide adenine dinucleotide; OAA, oxaloacetate; OMC, oxoglutarate malate transporter. (B) Changes in NADH-supplying systems caused by citrin deficiency in human hepatocytes. Citrin deficiency causes loss of the malate–aspartate shuttle and compensatory up-regulation of the malate–citrate shuttle, resulting in increased citrate in the cytosol. Abbreviations are identical to those in panel A. (C) Changes in fatty acid metabolism in patients with citrin deficiency. Overproduction of fatty acids due to increases in cytosolic citrate and enhancement of fatty acid uptake into hepatocytes are proposed to be associated with steatogenesis. ACL, ATP-citrate lyase; CD36; fatty acid translocase; Cit, citrate; FA, fatty acid; TG, triglyceride; cyt, cytosol; mit, mitochondrial matrix.

The results found in the current study lead to several points that need further study. Firstly, the prevalence of citrin deficiency in patients with cryptogenic chronic liver diseases should be clarified. Secondly, simple, non-invasive, and useful indicators to find apparently healthy homozygotes need to be established. For instance, the usefulness of immunoblot analysis of citrin by means of lymphocytes has recently been documented [41]. Finally, possible pharmacological interventions against asymptomatic citrin deficiency patients should be discussed as therapeutic interventions to decrease cytosolic citrate might attenuate hepatic steatosis. A recent study using citrin-deficient mice demonstrated that pyruvate replenishment may be effective to alleviate metabolic disturbances [42].

In conclusion, patients who present with fatty liver, especially non-obese individuals, might have citrin deficiency mimicking NAFLD. Medical history, dietary preference, and anthropometric and laboratory data such as BMI and serum PSTI may provide clues to discriminate citrin deficiency from NAFLD.

Acknowledgements

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ARTICLE TYPE

Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C

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Key words
hepatocellular carcinoma, liver cirrhosis, non-alcoholic steatohepatitis.

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Abstract

Background and Aim: Ethnic differences in non-alcoholic steatohepatitis (NASH) are well-documented, but there has been no study on the prognosis of Japanese NASH patients with cirrhosis. Accordingly, we compared cirrhotic NASH with liver cirrhosis caused by chronic hepatitis C (LC-C) to clarify its clinical features and define the risk factors for death.

Methods: A prospective evaluation of the outcomes of NASH patients with severe fibrosis was started in 1990. Data on age- and sex-matched patients with biopsy-proven LC-C were collected retrospectively and used as the control.

Results: There were 68 patients with cirrhotic NASH and 69 with LC-C. The Child–Turcotte–Pugh (CTP) class was similar in these two groups. Although the outcome of the NASH group was better than that of the LC-C group, cirrhotic NASH followed a similar course to that of LC-C; that is, complications of cirrhosis developed, including hepatocellular carcinoma (HCC; the 5-year HCC rate was 11.3% for NASH and 30.5% for HCV) and death (the 5-year survival rates were 75.2% and 73.8%, respectively). HCC was the leading cause of death in both groups (NASH, 47%; HCV, 68%). The occurrence of HCC and the CTP class were significant risk factors for mortality in NASH patients according to a multivariate analysis (HCC: hazard ratio [HR] 7.96, 95% confidence interval [CI] 2.45–25.88, CTP class A: HR 0.17, 95% CI 0.06–0.50).

Conclusion: In conclusion, the present study confirmed that cirrhotic NASH has a similar course to LC-C. The occurrence of HCC was the strongest predictor of mortality in the NASH groups. These findings may be helpful when deciding on therapeutic interventions for NASH and also for the daily management of these patients.

Introduction

Recently, lifestyle-related diseases and metabolic syndromes (diabetes, hypertension, and hyperlipidemia) have become leading public health problems because of the dramatic increase of these diseases in both Western countries and Asia. Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the metabolic syndrome, so NAFLD and non-alcoholic steatohepatitis (NASH) have now become major liver diseases in these countries.[1–3]

Annual health checks have resulted in 20–35% of Japanese adults being diagnosed with NAFLD by ultrasonography (US).[4,5]

Based on this high prevalence of NAFLD, the prevalence of NASH is estimated to be 1–2% among Japanese adults. It has been reported that NASH progresses to cirrhosis in up to 20% of patients.[6,7] In NASH patients, old age, obesity, insulin resistance, hypertension, and diabetes mellitus are all associated with a higher risk of cirrhosis.[8–11] We previously reported a case series of NASH patients with hepatocellular carcinoma (HCC) development,[12] but there have not been many studies about NASH and HCC.[12–18] NASH shows a wide range of severity, from minimal fibrosis to cirrhosis, so it is important to clarify the natural history of each stage (especially cirrhotic NASH) in order to determine how to manage these patients. A prospective study of the natural history of NASH patients with severe fibrosis was started at Tokyo Women's Medical University Hospital (Tokyo, Japan) in 1990. We have reported on the natural history of NASH, showing liver failure and HCC as the major causes of death in NASH patients with advanced fibrosis.[15] Although several studies have provided data about the natural history of NASH, there have been few reports on cirrhotic NASH.[11,20] Accordingly, the present study was performed to clarify the clinical features of cirrhotic NASH, by comparing the clinical features of liver cirrhosis due to NASH with those of liver cirrhosis caused by hepatitis C virus (HCV) infection, as well as to

define the risk factors for the development of HCC and mortality in cirrhotic NASH patients.

Methods

Patients

From 1990 to December 2006, 412 Japanese patients were diagnosed with biopsy-proven NAFLD at Tokyo Women's Medical University. Among them, 70 patients had cirrhosis. An evaluation of the natural history of NASH patients with severe fibrosis was started in 1990. All patients gave informed consent to participate in a study examining the natural history of their disease, and their clinical data were collected prospectively. Data on age- and sex-matched patients with biopsy-proven cirrhosis due to HCV who were concurrently managed at our hospital were also collected retrospectively and used as the control.

We excluded two patients because of a lack of informed consent, so 68 cirrhotic NASH patients (39 women and 29 men) were included in this study. A total of 69 age- and sex-matched patients with liver cirrhosis due to chronic hepatitis C (LC-C) formed the control group.

Definitions

Follow up was started from the time of liver biopsy, which was performed in all of the patients. Although some patients had ascites at baseline, it was resolved after treatment with diuretics, and a liver biopsy was performed following the disappearance of ascites. A diagnosis of NASH was based on the following criteria: (i) detection of steatohepatitis by liver biopsy; (ii) intake of less than 100 g ethanol per week; and (iii) appropriate exclusion of other liver diseases.[21–23] Cirrhosis was diagnosed by histological examination. The HCV group had histologically-proven cirrhosis and the patients were positive for HCV viral RNA by a quantitative polymerase chain reaction assay. They had either not been treated with interferon or were virological non-responders to interferon therapy.

Obesity was defined as a body mass index (BMI) of more than 25 according to the Japanese Obesity Association criteria. The diagnosis of type II diabetes mellitus was based on the Japanese criteria. Hyperlipidemia was diagnosed if the patient was being treated with lipid-lowering medications or had elevated levels of total cholesterol (> 220 mg/dL) and/or triglycerides (> 150 mg/dL) on at least three occasions. Hypertension was diagnosed if the patient was on antihypertensive therapy or had blood pressure greater than 140/90 mmHg on at least three occasions.

All liver biopsy specimens of the cirrhotic NASH patients were examined for fibrosis, and the NAFLD activity score was calculated.[21–25] Liver biopsy specimens from the HCV patients with cirrhosis were evaluated according to the criteria of Desmet *et al.*[26]

HCC was diagnosed histologically or by the detection of consistent findings using at least two imaging techniques from among US, computed tomography (CT), magnetic resonance imaging (MRI), and selective hepatic arteriography.[27]

Patient management

A complete history was obtained and a physical examination was performed in all of the patients. The following laboratory parameters were measured: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase, γ-glutamyltranspeptidase (γGTP), albumin, platelet count, prothrombin time, hepaplastin test, and Child–Turcotte–Pugh (CTP) class. Screening for HCC was performed in the cirrhotic NASH and HCV patients at least three times a year by measuring serum α-fetoprotein and/or prothrombin induced by vitamin K absence-II and by US. Other imaging studies (CT, MRI, and/or selective hepatic angiography) were performed in patients with suspected HCC.

In obese patients, weight was mainly controlled by diet and exercise. Thirty-five patients received drug treatment for NASH (ursodeoxycholic acid in 24 and/or vitamin E plus vitamin C in 22 patients). None of the patients underwent bariatric surgery, and liver transplantation was only performed in one patient. None of the patients were on hormone replacement therapy for menopause.

Statistical analysis

Analysis was performed with SPSS software (SPSS, Chicago, IL, USA). The Mann–Whitney test or the χ^2-test was used to compare baseline variables between the NASH and HCV groups. The starting date for the analysis was the date of liver biopsy in all patients. Patients in both groups were followed up until they died and were censored at the time of their last clinic visit. The patient who underwent liver transplantation was followed up until the day of transplantation, which was handled in the same way as the day of death. The primary outcomes were the occurrence of complications of cirrhosis (gastrointestinal varices and variceal hemorrhage, ascites, and encephalopathy), the development of HCC, overall survival, and liver-related mortality. The time frame for a particular outcome was defined as the interval from liver biopsy until the occurrence of the relevant event. The time-to-failure analysis (Kaplan–Meier) was performed, and the log–rank test was used for comparisons between the NASH and HCV groups. To clarify the risk factors for the development of HCC and mortality among cirrhotic NASH patients, the Cox proportional hazards analysis was used. Age, BMI, diabetes, hyperlipidemia, hypertension, bilirubin, albumin, AST, ALT, γGTP, platelet count, prothrombin time, gastrointestinal varices, ascites, encephalopathy, HCC, and CTP class A were included in the model. All parameters that had a *P*-value of less than 0.1 according to univariate analysis were selected for the multivariate analysis with the Cox proportional hazards model. A *P*-value of 0.05 or less was considered statistically significant.

Results

Baseline data

Baseline demographic, clinical, and laboratory data from the patients with cirrhotic NASH or cirrhotic HCV are shown in Table 1. The mean age of the patients with cirrhosis due to NASH was 62.7 years, with a range of 16 to 89 years (two pediatric patients). The mean age of the patients with cirrhosis due to HCV was 61.3 years (range: 53 to 75 years).

The NASH patients had a higher prevalence of obesity and lifestyle-related diseases, and the between-group differences in the prevalence of obesity, diabetes mellitus, and hyperlipidemia were

Table 1 Baseline demographic, clinical features, and laboratory findings

	NASH $n=68$ mean ± SD or %	HCV $n=69$ n/mean% or ± SD	*P*-value
Follow-up period (months)	41.1 ± 39.3	74.5 ± 52.4	0.001
Age (years)	62.7 ± 13.2	61.3 ± 5.8	N.S.
Sex (female)	57%	57%	N.S.
BMI (kg/m^2)	27.8 ± 5.4	24.0 ± 3.0	> 0.001
Obesity (BMI ≥ 25 kg/m^2)	66%	32% (/65)	> 0.001
Severe obesity (BMI ≥ 30 kg/m^2)	24%	3% (/65)	0.002
Diabetes	68%	33% (/67)	> 0.001
Hyperlipidemia	34%	13%	0.004
Hypertension	47%	43%	N.S.
Alb (g/dL)	3.6 ± 0.7	3.6 ± 0.6	N.S.
T-bil (g/dL)	1.1 ± 0.9	0.9 ± 0.5	N.S.
AST (IU/L)	60.9 ± 42.2	92.2 ± 45.4	> 0.001
ALT (IU/L)	54.9 ± 41.3	81.9 ± 42.1	> 0.001
ALP (IU/L)	332.3 ± 202.2	287.6 ± 132.0	N.S.
γGTP (IU/L)	116.7 ± 90.1	58.0 ± 36.5	> 0.001
Plt (× 10^4/μL)	11.8 ± 4.3	9.5 ± 3.4	0.001
Prothrombin time (%)	77.6 ± 15.6	69.0 ± 18.1	0.006
HPT (%)	75.9 ± 21.1	68.6 ± 18.3	0.034
Child–Turcotte–Pugh score	6.1 ± 1.4	6.1 ± 1.4	N.S.

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; HCV, hepatitis C virus; HPT, hepaplastin test; NASH, non-alcoholic steatohepatitis; NS, not significant; Plt, platelet count; T-bil, total bilirubin.

Table 2 Complications of cirrhosis

	NASH ($n=68$)			HCV ($n=69$)		
	At baseline (*n*)	During the follow-up period (*n*)	At the end of the follow-up period (*n*)	At baseline (*n*)	During the follow-up period (*n*)	At the end of the follow-up period (*n*)
Ascites	9% (6)	10% (7)	19% (13)	6% (4)	36% (25)	42% (29)
GI varices*	44% (27)	15% (9)	58% (36)	42% (27)	26% (17)	68% (44)
Encephalopathy	3% (2)	10% (7)	13% (9)	1% (1)	12% (8)	13% (9)
HCC	21% (14)	10% (7)	31% (21)	13% (9)	35% (24)	48% (33)

*Sixty-two patients underwent endoscopy in the non-alcoholic steatohepatitis (NASH) group, and 65 patients in the hepatitis C virus (HCV) group. GI, gastrointestinal; HCC, hepatocellular carcinoma.

significant. Transaminases were significantly higher in the HCV group, while the γGTP level was significantly higher in the NASH group. The platelet count was significantly higher, and the prothrombin time was significantly shorter in the cirrhotic NASH group. However, 67% of NASH patients had a CTP score of less than 7 at the time of entry, as did 62% of HCV patients. Histologically, steatosis and ballooning degeneration were detected in all of the NASH patients, while necroinflammatory changes were variable.

Complications of cirrhosis

Ascites was present in six NASH patients and four HCV patients at the time of the initial evaluation. Of the remaining patients, seven with NASH and 25 with HCV developed ascites during follow up (Table 2). The 5-year occurrence rate of ascites was 19.1% in the NASH group versus 29.6% in the HCV group. The Kaplan–Meier analysis showed that HCV patients developed ascites more frequently after 100 months of follow up, but there was no significant difference between the two groups ($P = 0.175$).

At entry into the study, 44% of cirrhotic NASH patients (62 patients underwent endoscopy) and 42% of cirrhotic HCV patients (65 patients underwent endoscopy) had evidence of esophageal varices. During the follow-up period, nine additional patients with NASH and 17 with HCV were diagnosed as having esophageal varices by endoscopy. During follow up, 15 patients with NASH versus nine with HCV suffered from variceal hemorrhage, and one and five patients died of bleeding, respectively (Table 2). The 5-year occurrence rate of varices was 28.2% in the NASH group versus 34.5% in the HCV group, and the Kaplan–Meier analysis showed a similar incidence in both groups ($P = 0.789$).

At enrollment, two patients with NASH and one patient with HCV had a history of encephalopathy. Over time, seven other patients with NASH and eight patients with HCV developed encephalopathy (Table 2). The 5-year occurrence rate of ascites was 16.1% in the NASH group versus 9.1% in the HCV group.

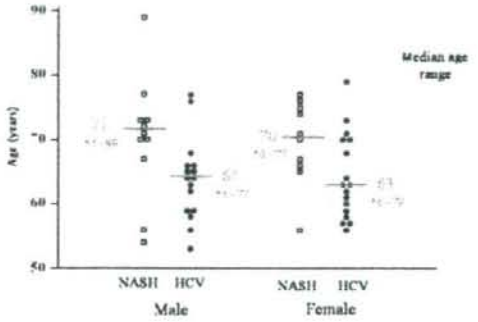


Figure 1 Age distribution at diagnosis of hepatocellular carcinoma. HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis.

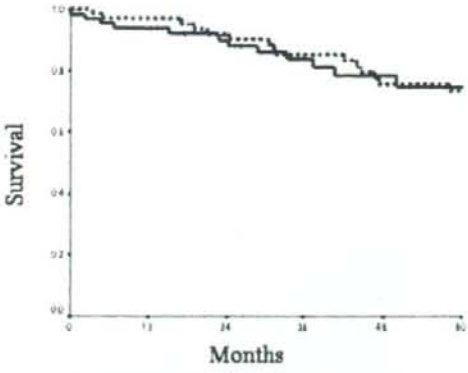


Figure 2 Survival curves. (a) Non-alcoholic steatohepatitis (NASH; $n = 68$); (b) hepatitis C virus ($n = 69$). $P = 0.393$. —, NASH; ....., hepatitis C.

Although NASH patients showed a higher incidence of encephalopathy, according to the Kaplan–Meier analysis there was no significant difference between the groups ($P = 0.253$).

Fourteen patients with NASH and nine patients with HCV had HCC at enrollment. During the follow-up period, seven patients with NASH developed HCC, as did 24 patients with HCV (Table 2). There were 11 males and 10 females with HCC in the cirrhotic NASH group and the median age was 71 for males and 70 for females. There were 16 males and 17 females with HCC in the cirrhotic HCV group and the median age was 64 for males and 63 for females. The median ages of the males and females in each group are shown in Figure 1. The median ages of the male and female NASH patients were significantly higher than those of the HCV patients. The 5-year occurrence rate of HCC was 11.3% in the NASH group versus 30.5% in the HCV group. The development of HCC showed a higher rate in the latter group, but the difference was not significant ($P = 0.185$).

A total of 19 patients with NASH (including one patient who underwent liver transplantation) and 28 patients with HCV died. Survival curves are shown in Figure 2. Survival was slightly better for cirrhotic NASH patients (the 5-year survival rate was 75.2% in the NASH group and 73.8% in the HCV group), but the difference was not significant. The causes of death are shown in Figure 3. HCC was the leading cause of death in both groups (nine deaths in the NASH group and 19 in the HCV group), followed by liver failure (six and seven deaths, respectively). Among the patients with liver failure, one NASH patient and five HCV patients died of variceal bleeding. There were more non-liver-related deaths in the NASH group, but the difference was not significant. The causes of non-liver related death in the NASH group were endometrial cancer in one patient, cholangitis in one patient, interstitial pneumonia in one patient, and cerebral infarction in one patient. Only two patients suffered from non-liver-related death in the HCV group (interstitial pneumonia and cerebral hemorrhage).

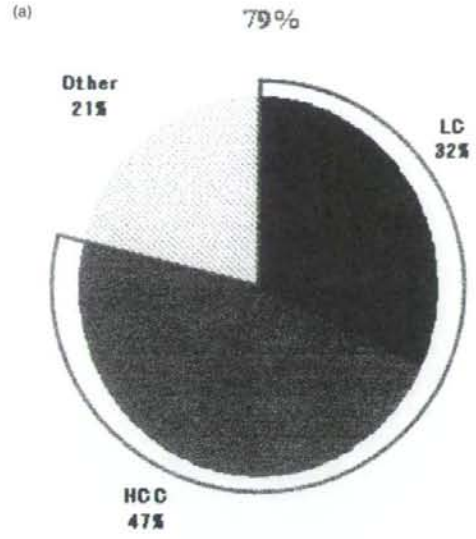
There were no differences in the baseline characteristics between the HCV patients with and without prior anti-HCV therapy, and the long-term outcome was similar for these two subgroups.

The Cox proportional hazards analysis did not identify any risk factors for the development of HCC, although we also investigated the metabolic factors. The univariate analysis showed that HCC, ascites, albumin, bilirubin, and CTP class A were significant risk factors for mortality, but diabetes was not. According to the multivariate analysis, HCC and CTP class A were significant risk factors for mortality (Table 3). Figure 4 shows the survival of cirrhotic NASH patients with or without HCC, which was a significant risk factor that led to the death of these patients.

Figure 5 shows the prevalence of lifestyle-related diseases in all of the NASH patients, cirrhotic NASH patients, and NASH patients with HCC. The prevalence of diabetes was significantly higher among the NASH patients with HCC, but the prevalence of obesity and hyperlipidemia was lower in the NASH patients with cirrhosis or HCC.

Discussion

Ethnic differences with respect to the prevalence and features of NAFLD are well documented,[28] but there has been no information available on the prognosis of Japanese NASH patients with cirrhosis. The present report provides data from the first Japanese prospective study on the natural history of NASH patients with cirrhosis who underwent follow up using a predefined screening protocol for HCC at a single tertiary care hospital. All of the patients were Japanese, and follow up was started at the time of liver biopsy. Several therapeutic approaches have been tested for the treatment of NASH, but there is no pharmacological therapy that has conclusively proven to be effective.[29,30] In the present series, none of the patients underwent bariatric surgery, and liver transplantation was only performed in one patient. Furthermore, only two patients were lost to follow up. Therefore, our patients were suitable for assessing the natural history of Japanese NASH patients with cirrhosis, despite the existence of referral hospital bias. The main limitation of this study is that the control group was investigated retrospectively, but a predefined screening protocol for HCC was also employed in the HCV group. Furthermore, there



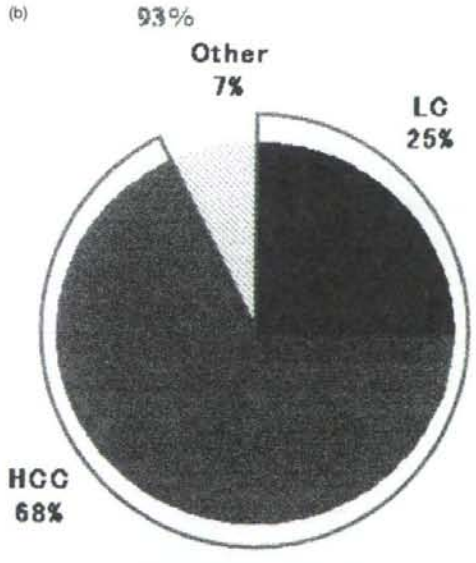


Figure 3 Causes of death (%). In cirrhotic non-alcoholic steatohepatitis patients, non-liver-related death accounted for 21% of all deaths, while in the case of cirrhotic hepatitis C patients, it only accounted for 7%. LC, liver cirrhosis.

Table 3 Cox proportional hazards analysis of factors related to mortality in non-alcoholic steatohepatitis patients with cirrhosis

	P-value	HR	95% CI
Hepatocellular carcinoma	0.001	7.957	2.447–25.877
Child–Turcotte–Pugh score grade A	0.001	0.170	0.058–0.502

CI, confidence interval; HR, **.

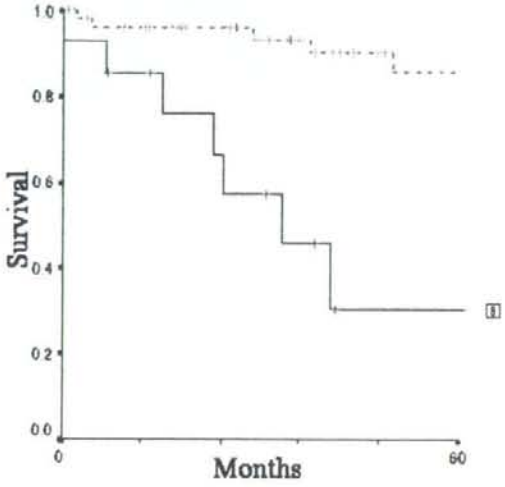


Figure 4 Survival curves of cirrhotic non-alcoholic steatohepatitis (NASH) patients with or without hepatocellular carcinoma (HCC). $P < 0.001$. —, NASH with HCC; ....., NASH without HCC.

were no differences in the baseline characteristics between patients from the HCV group with and without prior anti-HCV therapy. The long-term outcome was similar for these two subgroups, as was found in a recent large-scale nationwide study.[31]

When we compared the outcome of cirrhotic NASH with that of cirrhosis due to HCV, our study confirmed that the morbidity and mortality rates of the NASH group were better than those of the HCV group. However, cirrhosis due to NASH still followed a similar course to cirrhosis caused by LC-C; that is, the development of complications, occurrence of HCC, and then death.

Interestingly, baseline data showed significant differences between the NASH and HCV groups. As expected, there was a significantly higher prevalence of obesity, diabetes, and hyperlipidemia in the NASH group. In addition, the NASH patients with

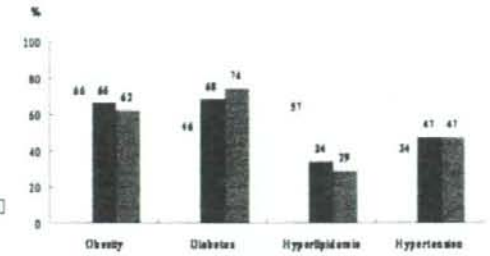


Figure 5 Prevalence of lifestyle-related diseases in all non-alcoholic steatohepatitis (NASH), cirrhotic NASH patients, and NASH patients with hepatocellular carcinoma. , NASH All; ■, Liver cirrhosis; ▩, Hepatocellular carcinoma.

cirrhosis had significantly lower transaminase levels and higher γGTP levels than the HCV patients with cirrhosis. Although there was no significant difference of the CTP class between the two groups, the NASH patients had a significantly higher platelet count and prothrombin time than the HCV patients. It is unlikely that this was because of the earlier detection of cirrhotic NASH, because NASH is still not well known in Japan, while HCV tends to be diagnosed early because of screening for this disease by government heath services. The reasons for these differences in the laboratory data are unknown. It has been reported that a low normal ALT value does not guarantee the absence of underlying steatohepatitis with advanced fibrosis,[32] so the differences that we detected might be related to the characteristics of cirrhotic NASH.

Hui *et al.* reported on the long-term morbidity and mortality (median follow up: 60 months) of 23 patients with NASH-associated cirrhosis.[18] The outcomes were compared with those of 46 age- and sex-matched patients who had cirrhosis related to LC-C. Liver failure was the main cause of morbidity and mortality in patients with NASH-associated cirrhosis, and the prognosis was either similar or less severe than that of HCV-related cirrhosis. These findings were confirmed by Sanyal *et al.*,[20] who reported on the largest prospective study of patients with cirrhosis due to NASH or HCV. Their study compared 152 patients with cirrhosis due to NASH and 150 matched patients with cirrhosis caused by LC-C who were followed up for 10 years. They reported that the outcome for NASH patients with CTP class A cirrhosis was significantly better than that for patients with HCV-related cirrhosis, while there were no significant differences of mortality for patients with CTP class B or C cirrhosis. They also found that death was most commonly due to sepsis and multiple organ failure associated with ascites, while the risk of developing ascites was significantly lower in NASH patients than in patients with HCV. Our series was small, but we found that CTP class A was a significant negative risk factor for mortality. The reasons for such a difference in the complication of cirrhosis need to be studied further.

Another important finding was that no patient died of heart disease among the cirrhotic NASH and HCV groups, although several reports have indicated that NAFLD or NASH is a risk factor for heart disease.[20,33] As seen in Figure 4, the lipid levels, platelet count, and prothrombin time were not high in NASH patients with advanced fibrosis, which might be part of the reason for the lack of cardiac-related death.[10] It is also possible that patients with symptomatic heart disease were not referred to our tertiary liver center.

Evidence of progression from NASH to HCC in prospective studies is minimal. However, with the exception of Hui *et al.*'s report, previous studies have shown that some patients develop HCC within several years after the diagnosis of cirrhotic NASH. Hui *et al.* did not find any NASH patients who developed HCC during follow up. The most important reason for this difference was probably the ages of the patients (a mean of 52.6 ± 13.6 years in Hui *et al.*'s study *vs* a median of 64 years for cirrhotic NASH and 70 years for NASH with HCC in our study). Therefore, Hui *et al.*'s patients were relatively young and unlikely to develop HCC. In NASH, as in other liver diseases, the severity of fibrosis and age are important risk factors for HCC.

The highest rate of HCC was reported by Ratziu *et al.*[34] They assessed survival, occurrence of HCC, and complications of hepatic insufficiency in patients with obesity-related cryptogenic cirrhosis, which was suggested to be burnt-out NASH, and compared the results with those for cirrhosis due to other causes. HCC was detected in eight of 27 (27%) cryptogenic cirrhosis patients versus 21% of the matched HCV-infected controls of similar age, suggesting a comparable carcinogenic potential. This is reasonable because burnt-out NASH is the most advanced stage of cirrhotic NASH.[13,34] In the present study, we found that HCC was a significant independent risk factor for mortality, as was the CTP class (Table 3).

In conclusion, the present study confirmed that, although the morbidity and mortality of NASH cirrhosis were lower than those of cirrhosis due to LC-C, both followed a similar course with respect to the onset of complications, development of HCC, and eventual death. The occurrence of HCC was the strongest predictor of mortality in both groups. These findings are likely to be helpful when devising therapeutic interventions for NASH and also for the daily management of these patients.

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A novel amplification target, *ARHGAP5*, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells

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ABSTRACT

RhoA, a member of the Rho family of small GTPases, directs the organization of the actin cytoskeleton and is involved in regulating cell shape and movement. Its activity is negatively regulated by p190-B RhoGAP (GTPase-activating protein). We investigated DNA copy number aberrations in human hepatocellular carcinoma and esophageal squamous cell carcinoma cell lines using a high-density oligonucleotide microarray and found a novel amplification at chromosomal region 14q12. We identified *ARHGAP5* (the gene encoding p190-B RhoGAP) as a probable target for the amplification at 14q12, and our results showed that p190-B RhoGAP promotes cells spreading and migration by negatively regulating RhoA activity in Huh-7 hepatocellular carcinoma cells.

1. Introduction

Members of the Rho family of small GTPases act as molecular switches. In response to extracellular signals, they direct the organization of the actin cytoskeleton and alter gene expression [1]. Rho proteins, which include the much-studied Cdc42, Rac1 and RhoA, are involved in regulating cell shape, polarity and movement and establishing cell-cell junctional complexes. Accordingly, their activity is tightly controlled by regulatory proteins that determine whether GTP or GDP is bound. Rho proteins are activated by guanine nucleotide exchange factors, which catalyze the release of GDP and thus allow GTP to bind the proteins. Rho proteins in turn are inactivated by Rho GTPase-activating proteins (GAPs), which bind to the Rho proteins and induce them to hydrolyze their bound GTP to GDP. p190-B RhoGAP, a member of the RhoGAP family, negatively regulates RhoA activity [2,3].

Amplification of DNA in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies, specifically when proto-oncogenic target genes within those amplicons are overexpressed. Oncogenes that are often amplified in cancers include *MYC*, *ERBB2* and *CCND1*.

In the present study, we investigated DNA copy number aberrations in human hepatocellular carcinoma (HCC) and

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esophageal squamous cell carcinoma (ESCC) cell lines and found a novel amplification at chromosomal region 14q12. Because the region may harbor one or more proto-oncogenes whose overexpression following amplification contributes to the initiation or progression of HCC and ESCC, we carried out molecular definition of the amplicon. We show here that the p190-B RhoGAP gene (*ARHGAP5*) within the 14q12 amplicon is amplified and overexpressed, and that p190-B RhoGAP promotes cell spreading and migration in Huh-7 hepatocellular carcinoma cells.

2. Materials and methods

2.1. Cell lines

A total of 10 HCC cell lines (JHH-6, JHH-7, SNU354, SNU398, SNU423, SNU475, Huh-1, Huh-7, HLE and PLC/PRF/5) and 10 ESCC cell lines (T.T, EC-GI-10, KYSE140, KYSE220, TE-4, TE-5, TE-6, TE-10, TE-14 and TE-15) were examined. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Genomic DNA was isolated from each cell line using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA).

2.2. Array analysis

Array analyses were performed using the GeneChip Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. In brief, 250 ng of genomic DNA was digested with a restriction enzyme (StyI), ligated to an adaptor and amplified by PCR. Amplified products were fragmented, labeled by biotinylation and hybridized to the microarrays. Hybridization was detected by incubation with streptavidin–phycoerythrin conjugate, and the array was scanned. Analysis was performed as previously described [4]. Copy number changes were calculated using the Copy Number Analyzer for Affymetrix GeneChip Mapping Arrays (CNAG; http://www.genome.umin.jp) [5].

2.3. Fluorescence in situ hybridization (FISH)

We performed FISH using three bacterial artificial chromosomes (BACs), RP11-113E19, RP11-431H16 and RP11-54H22 as probes (Invitrogen, Carlsbad, CA, USA), as described previously [6]. The BACs were selected based on homology with locations in the human genome according to the database provided by the UCSC (http://genome.ucsc.edu/).

2.4. Real-time quantitative PCR

We quantified genomic DNA and mRNA using a real-time fluorescence detection method, as described previously [6]. The primers used were as follows: *ARHGAP5* mRNA (forward, 5′-CATCTGTTTTTGGCCAACCT-3′; reverse, 5′-gtggaggagccacaatgttt-3′); *HEATR5A* mRNA (forward, 5′-TGTGCCTCCTACTCATGCTG-3′; reverse, 5′-gagatggcctgagct tgaac-3′); *c14orf126* mRNA (forward, 5′-gtgctttttcaaggga gctg-3′; reverse, 5′-ttcctccaagggtagcttga-3′); *NUBPL* mRNA (forward, 5′-cttggccttgtccaaaacat-3′; reverse, 5′-acaattggc tggcctgtatc-3′). These primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *GAPDH* and long interspersed nuclear element 1 (LINE-1) were used as endogenous controls for mRNA and genomic DNA levels, respectively.

2.5. RNA interference (RNAi)

For RNAi, small interfering RNA (siRNA) duplex oligoribonucleotides targeting *ARHGAP5* (5′-CAAGATCATAATATCAATCTA-3′) and control (non-silencing) siRNA duplexes were synthesized by QIAGEN (Valencia, CA, USA). The siRNAs were delivered into Huh-7 cells using HiPerfect Transfection Reagent (QIAGEN), according to the manufacturer's protocol.

2.6. Immunoblotting

Immunoblots were prepared according to previously reported methods [7]. Cell lysates (20 μg protein per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels. Anti-p190-B RhoGAP monoclonal antibody was obtained from BD Transduction Laboratories (Lexington, KY, USA); anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-β-actin monoclonal antibody was from Sigma–Aldrich (Tokyo, Japan). For immunoblotting, we used anti-p190-B RhoGAP, anti-RhoA and anti-β-actin at dilutions of 1:250, 1:100 and 1:5000, respectively. For secondary immunodetection, we used anti-mouse IgG (Amersham, Tokyo, Japan) diluted 1:5000. Protein binding was detected using the ECL system (Amersham).

2.7. RhoA activity assay

Active RhoA levels were measured using the enzyme-linked immunosorbent assay (ELISA)-based G-LISA RhoA activation assay Biochem Kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. In brief, Huh-7 cells were transfected with siRNA targeting *ARHGAP5* or negative control siRNA, or were left untreated. Cells were then cultured under the standard conditions in DMEM containing 10% FCS. After 48 h, cells were harvested for the RhoA activity assay or trypsinized and held in suspension for 1 h in DMEM containing 1% FCS. The suspended cells were then plated on 6-well plates coated with 5 μg/ml fibronectin (BD Transduction Laboratories) and harvested for the RhoA activity assay at the indicated time points. For the RhoA activity assay, cells were lysed in 70 μl of G-LISA lysis buffer, scraped into tubes and snap frozen in liquid nitrogen. Cell lysates were subsequently thawed, clarified for 2 min at 10,000*g*, and protein concentrations were normalized between the various time points. Equal amounts of total protein were added to a 96-well plate coated with the Rho-binding domain of Rho effector pro-