Table 2 Prevalence of esophagogastric varices in NASH patients with severe fibrosis

	All ($n = 72$)	Bridging fibrosis ($n = 25$)	Cirrhosis ($n = 47$)
Esophageal varices	25 (34.7%)	4 (16.0%)	21 (44.7%)
Variceal form			
F1	12	1	11
F2	7 (2)†	1 (1)†	6 (1)†
F3	6 (2)†	2 (1)†	4 (1)†
Red color sign (-/+/++/+++)	9/5/8/3	1/1/2/0	8/4/6/3
Gastric varices	9 (12.5%)	0 (0.0%)	9 (19.1%)
Variceal form			
F1	1	0	1
F2	3 (1)†	0	3 (1)†
F3	5 (1)†	0	5 (1)†
Red color sign (-/+)	6/3	0/0	6/3
No varices	38 (52.8%)	21 (84.0%)	17 (36.2%)

†Number of bleeding cases. Data are number of patients (percentage).
NASH, non-alcoholic steatohepatitis.

61.5 years (P = NS). Among 38 patients without varices, 34 (89.5%) were classified as Child–Pugh class A. Among 34 patients with varices, 18 patients (52.9%) were Child–Pugh class A and 15 patients (44.1%) Child–Pugh class B. Thus, patients with varices were more likely to be class B. Transaminases tended to be lower in patients with varices than in patients without varices. The median platelet count of the patients with varices was $9.6 \times 10^4/mm^3$, while that of patients without varices was $17.9 \times 10^4/mm^3$. Patients with varices had a significantly lower platelet count than those without varices ($P < 0.0001$) (Table 3).

Results of treatment of esophageal varices in NASH patients

The cumulative recurrence-free probability at 24 months after endoscopic treatment of esophageal varices was 63.6% in the NASH group, 45.7% in the alcoholic group and 73.3% in the HCV group (Fig. 1). The cumulative bleeding-free probability at 24 months was 90.9% in the NASH group, 78.8% in the alcoholic group and 89.7% in the HCV group (Fig. 2). The 5-year survival probability was 100% in the NASH group, 82.8% in the alcoholic group and 75.9% in the HCV group (Fig. 3). There were no significant differences in these rates between the NASH group and the alcoholic group or between the NASH group and the HCV group. In the NASH group, only one patient died of liver failure at 70 months after treatment of the varices. In the alcoholic group, three patients each died of liver failure and variceal bleeding. In the HCV group, 23 patients died of liver failure and 13 patients died of HCC that developed during the course of follow-up (Table 4).

DISCUSSION

IN THE PRESENT study, we determined the incidence and morphological features of esophagogastric varices in NASH patients with bridging fibrosis (F3) or cirrhosis (F4). Of 72 NASH patients who were diagnosed as having severe fibrosis by liver biopsy, esophagogastric varices were detected in 34 (47.2%) patients, esophageal varices in 25 (34.7%) patients and gastric varices in nine (12.5%) patients. Six of these patients presented with variceal bleeding. Esophagogastric

Table 3 Comparison of subjects with and without varices

	Varices present ($n = 34$)	Varices absent ($n = 38$)	P-value
Male/Female	17/17	18/20	NS
Age (years)	64.5 (39–82)	61.5 (16–89)	NS
Child–Pugh classification (A/B/C)	18/15/1	34/2/2	<0.05
Concomitant HCC	2	5	NS
Liver histology (bridging fibrosis/cirrhosis)	4/30	21/17	<0.05
AST (IU/L)	44.5 (12–132)	52.0 (9–392)	NS
ALT (IU/L)	36.0 (9–212)	66.0 (5–740)	<0.05
Platelet count ($\times 10^4/mm^3$)	9.6 (3.7–21.9)	17.9 (5.2–45.1)	<0.0001

Data are number of patients or median (range). Mann–Whitney U-test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; NS, not significant.

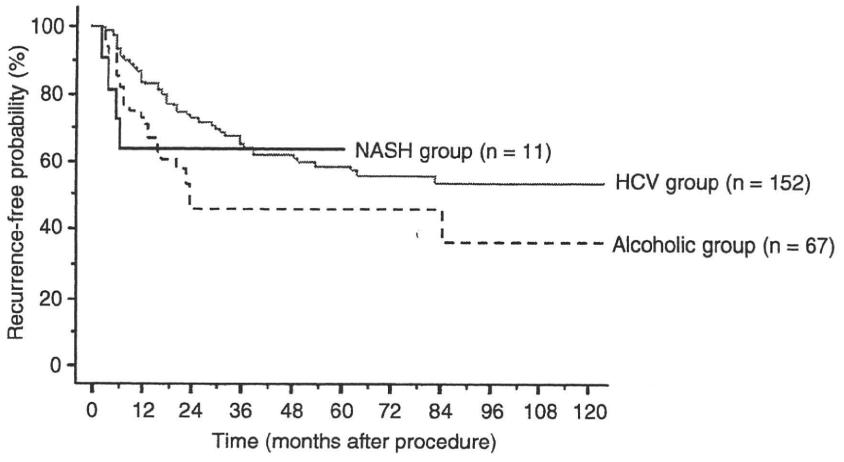

Figure 1 Kaplan–Meier analysis of cumulative recurrence-free curves. The cumulative recurrence-free probability at 24 months after endoscopic procedure for the non-alcoholic steatohepatitis (NASH) group (63.6%) was not significantly different from that of the alcoholic group (45.7%) or the hepatitis C virus (HCV) group (73.3%).

varices were found in four out of 25 (16.0%) patients who had bridging fibrosis versus 30 out of 47 (63.8%) patients who had cirrhosis. Thus, the rate of esophagogastric varices is higher in patients with cirrhosis than in those with fibrosis. Development of varices did not correlate with age and gender; no differences were detected in these variables between 34 patients with varices and 38 patients without varices. A high percentage of

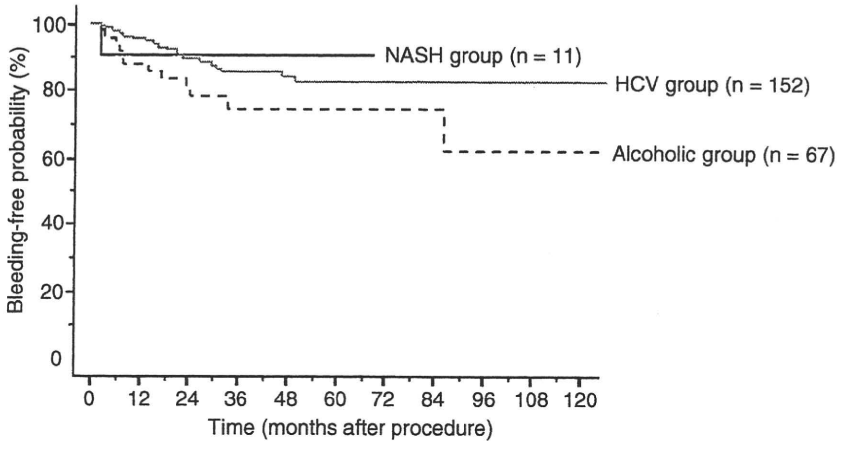

Figure 2 Kaplan–Meier analysis of cumulative bleeding-free curves. The cumulative bleeding-free probability at 24 months after endoscopic procedure for the non-alcoholic steatohepatitis (NASH) group (90.9%) was not significantly different from that of the alcoholic group (78.8%) or the hepatitis C virus (HCV) group (89.7%).

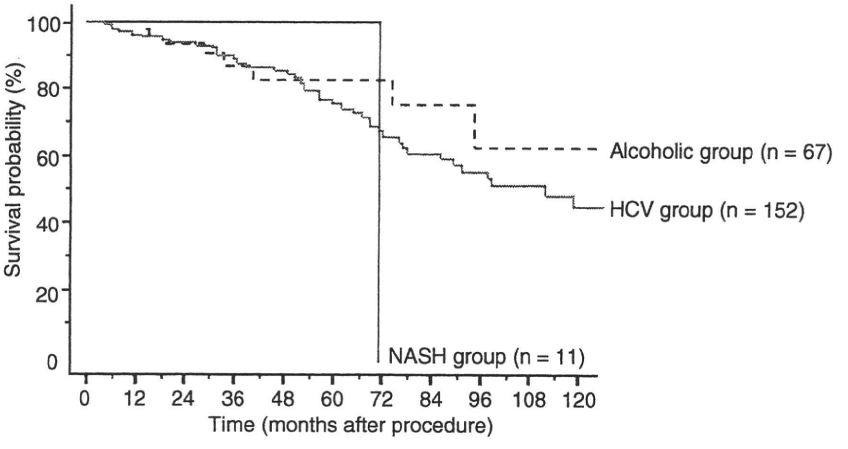

Figure 3 Kaplan–Meier analysis of survival curves. The survival probability at 5 years after endoscopic procedure for the non-alcoholic steatohepatitis (NASH) group (100%) was not significantly different from that of the alcoholic group (82.8%) or the hepatitis C virus (HCV) group (75.9%).

Table 4 Characteristics and prognosis of NASH, alcoholic and HCV patients with esophageal varices treated by endoscopic procedures

	NASH group ($n = 11$)	Alcoholic group ($n = 67$)	HCV group ($n = 152$)
Male/Female	4/7	64/3	82/70
Age (years)	68.0 (45–82)	56.0 (22–72)	62.5 (29–83)
Child–Pugh classification (A/B)	3/8	25/42	44/108
Variceal form (F1/F2/F3)	1/4/6	4/40/23	16/88/48
Red color sign (−/+/++/+++)	0/1/7/3	0/14/35/18	2/32/78/40
Endoscopic treatment (ligation/sclerotherapy)	8/3	45/22	91/61
Indication for treatment (prophylaxis/bleeding)	8/3	38/29	125/27
Follow-up period (months)	21.0 (8–70)	28.0 (3–157)	45.5 (3–159)
Mortality	1 (9.1%)	8 (11.9%)	46 (30.3%)
Causes of death			
Liver failure	1	3	23
HCC	0	1	13
Variceal bleeding	0	3	1
Others, unknown	0	1	9

Data are number of patients (percentage) or median (range).
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis.

patients with varices had cirrhosis, which meant that they had advanced liver disease on histopathological examination. The median platelet count of patients with varices ($9.6 \times 10^4/mm^3$) was significantly lower than that of patients without varices ($17.9 \times 10^4/mm^3$).

Sanyal *et al.*[28] evaluated 1016 patients with HCV infection who had advanced fibrosis. They found that 16% of patients with bridging fibrosis and 39% of those with cirrhosis had esophageal varices ($P < 0.0001$), while 2% of patients with bridging fibrosis and 11% of those with cirrhosis had medium to large esophageal varices. They reported that the risk of esophageal varices in such patients increased along with a decrease of the platelet count, an increase of bilirubin and a higher international normalized ratio (INR), and that the risk of medium to large varices was negligible in the patients with platelet counts >150 000/mm^3. We compared the risk of esophagogastric varices in our NASH patients and those with HCV infection reported by Sanyal *et al.*,[28] since the risk of development of esophagogastric varices (approximately 16%) was similar in the two groups with bridging fibrosis. Esophagogastric varices occurred more frequently in our NASH patients who had cirrhosis and more than half of these patients had varices classified as F2 or more severe. More NASH patients had medium to large varices compared with patients suffering from HCV-associated cirrhosis. This is because NASH is characterized by fibrosis around the central veins that tends to cause portal hypertension. Zaman *et al.*[12] performed endoscopy in 98 patients to evaluate their eligibility for liver transplantation, and they found esophageal varices in 68% of the patients and gastric varices in 15%. They also reported that a platelet count of ≤88 000/mm^3 was a predictor for the presence of esophagogastric varices. Levy *et al.*[29] detected esophageal varices in 56 out of 113 patients (49.6%) with primary biliary cirrhosis and reported that the frequency of detection by screening endoscopy was 37% when 22 patients with variceal bleeding were excluded. A platelet count of ≤140 000/mm^3 was regarded as an independent predictor of esophageal varices.

Considered together, our results indicate that the incidence of esophagogastric varices in NASH patients with severe fibrosis is equivalent to or higher than that in patients with advanced fibrosis and cirrhosis caused by other liver diseases. In addition, NASH patients with severe fibrosis often developed bleeding. Kaneda *et al.*[30] reported that high serum hyaluronic acid and low platelet count are useful indicators of fibrosis in NASH and that the platelet count of patients with cirrhosis is ≤160 000/mm^3, with a median count of 130 000/mm^3. We suggest that platelet count can be a predictor of esophagogastric varices in NASH patients and that a count of <96 000/mm^3 could be a marker of cirrhosis in such patients, warranting endoscopy.

In general, the serum alanine aminotransferase (ALT) level tends to decrease with the progression of liver fibrosis (in cirrhosis). In our patients, the ALT level

varied widely during follow-up within a wide range (5–740 IU/L). Although the statistical analysis found ALT level to be a predictor of varices, we consider that further investigation including a larger sample is needed to analyze the reason for this finding.

The therapeutic outcome of endoscopically treated NASH patients with esophageal varices was compared with that of patients with alcohol- and HCV-associated cirrhosis. The NASH group included 11 patients, after excluding one patient with HCC from the 12 patients who underwent endoscopy. The control groups were the alcoholic group (67 patients) and the HCV group (152 patients). All patients were Child–Pugh A and B without HCC at the start of endoscopic treatment. The cumulative recurrence-free probability at 24 months after endoscopic treatment of esophageal varices was 63.6% for the NASH group, 45.7% for the alcoholic group and 73.3% for the HCV group. The cumulative bleeding-free probability after 24 months was 90.9% for the NASH group, 78.8% for the alcoholic group and 89.7% for the HCV group. Thus, the NASH group had better bleeding-free and recurrence-free probabilities than the alcoholic group, although the differences were not significant. Comparable results were noted for the NASH group and the HCV group. The 5-year survival probability was 100% for the NASH group, 82.8% for the alcoholic group and 75.9% for the HCV group. The NASH group showed better survival, although the difference was not significant. In the alcoholic group, three patients died of variceal bleeding associated with liver failure. Many patients with alcoholic cirrhosis cannot stop drinking, which leads to hepatopathy and gastrointestinal mucosal damage. These events tend to be recurrent and can readily cause bleeding, resulting in death from variceal hemorrhage in some cases. The largest number of deaths from liver failure was noted in the HCV group, and there were also many deaths due to HCC that occurred during the course of follow-up. In patients with HCV-associated cirrhosis, liver failure and HCC are important prognostic factors. In the NASH group, only one patient died of liver failure and the prognosis was better than that of alcoholic cirrhosis or HCV-associated cirrhosis. A number of reports described NASH patients who developed HCC, but the risk is considered to be lower than that for patients with HCV-associated cirrhosis. Thus, the main cause of death in NASH patients seems to be liver failure. In fact, Hui *et al.*[9] reported that liver failure was the most common cause of morbidity and mortality in patients with NASH-associated cirrhosis. HCC appears to be less common in these patients than in those with cirrhosis due to hepatitis C. Accordingly, the prognosis of NASH patients who develop cirrhosis appears to be similar to or better than that of patients with HCV-associated cirrhosis. Sanyal *et al.*[10] have also reported that compensated cirrhosis due to NASH is associated with a lower mortality rate than that due to HCV and that there is also a lower risk of ascites, hyperbilirubinemia and HCC. HCC can occur in patients with NASH-related cirrhosis, but the risk is lower than that reported for patients with cirrhosis due to hepatitis C. However, cardiovascular mortality is higher in NASH patients. Although our study included only a small group of patients and should thus be considered preliminary in nature, we can recommend that patients with cirrhosis should be followed up to monitor the progression of liver disease (function), development of HCC and other complications. Lifestyle modification including diet, physical activity and pharmacological treatment should be the first line and mainstay of management. NASH should be recognized as a part of the metabolic syndrome and managed in a multidisciplinary approach that addresses liver disease in the context of risk factors for diabetes and premature cardiovascular disease.

CONCLUSION

IN CONCLUSION, 47.2% of our NASH patients who were diagnosed with severe liver fibrosis had esophagogastric varices. In particular, 63.8% of the patients with cirrhosis (F4) had esophagogastric varices. More than half of NASH patients had varices classified as F2 or more severe. The survival probability of NASH was better than that associated with alcoholic cirrhosis or HCV-associated cirrhosis. However, the cumulative recurrence-free probability and bleeding probability of NASH were similar to those of alcoholic cirrhosis and HCV-associated cirrhosis. The clinical status and course of esophagogastric varices in NASH patients do not necessarily improve after endoscopic treatment, compared with alcoholic cirrhosis or HCV-associated cirrhosis. Therefore, NASH with esophagogastric varices needs to be followed up carefully, like other chronic liver diseases. We consider that NASH patients with treated varices will increase in the future and their natural history need to be defined by long-term follow-up.

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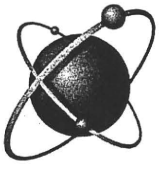
REFERENCES

1 Harrison SA, Torgerson S, Hayashi PH. The natural history of non-alcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol* 2003; **98**: 2042–7.
2 Ono M, Saibara T. Clinical features of non-alcoholic steatohepatitis in Japan: evidence from literature. *J Gastroenterol* 2006; **41**: 725–32.
3 Chitturi S, Farrell GC, Hashimoto E, Saibara T, Lau GKK, Sollano JD, The Asia-Pacific Working Party on NAFLD. Non-alcoholic fatty liver disease in the Asia-Pacific region: definitions and overview of proposed guidelines. *J Gastroenterol Hepatol* 2007; **22**: 778–87.
4 Shimada M, Hashimoto E, Taniai M *et al.* Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J Hepatol* 2002; **37**: 154–60.
5 Ratziu V, Bonyhay L, Di Martino V *et al.* Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. *Hepatology* 2002; **35**: 1485–93.
6 Hashimoto E, Yatsuji S, Kaneda H *et al.* The characteristics and natural history of Japanese patients with non-alcoholic fatty liver disease. *Hepatol Res* 2005; **33**: 72–6.
7 Ludwig J, Viggiano TR, McGill DB, Oh BJ. Non-alcoholic steatohepatitis: Mayo Cinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434–8.
8 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Non-alcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467–74.
9 Hui JM, Kench JG, Chitturi S *et al.* Long-term outcomes of cirrhosis in non-alcoholic steatohepatitis compared with hepatitis C. *Hepatology* 2003; **38**: 420–7.
10 Sanyal AJ, Banas C, Sargeant C *et al.* Similarities and differences in outcomes of cirrhosis due to non-alcoholic steatohepatitis and hepatitis C. *Hepatology* 2006; **43**: 682–9.
11 Cales P, Desmorat H, Vinel JP *et al.* Incidence of large oesophageal varices in patients with cirrhosis: application to prophylaxis of first bleeding. *Gut* 1990; **31**: 1298–302.
12 Zaman A, Hapke R, Flora K, Rosen HR, Benner K. Factors predicting the presence of esophageal or gastric varices in patients with advanced liver disease. *Am J Gastroenterol* 1999; **94**: 3292–6.
13 Jensen DM. Endoscopic screening for varices in cirrhosis: findings, implications, and outcomes. *Gastroenterology* 2002; **122**: 1620–30.
14 Madhotra R, Mulcahy HE, Willner I *et al.* Prediction of esophageal varices in patients with cirrhosis. *J Clin Gastroenterol* 2002; **34**: 81–5.
15 Sanyal AJ. AGA technical review on non-alcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 1705–25.
16 Brunt EM. Non-alcoholic steatohepatitis. *Semin Liver Dis* 2004; **24**: 3–19.
17 Neuschwander-Tetri BA, Caldwell SH. Non-alcoholic steatohepatitis: summary of an AASLD single topic conference. *Hepatology* 2003; **37**: 1202–19.
18 Adams LA, Sanderson S, Lindor KD *et al.* The histological course of non-alcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**: 132–8.
19 Kleiner DE, Brunt EM, Van Natta M *et al.* Design and validation of a histological scoring system for non-alcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313–21.
20 The Japan Society for Portal Hypertension. *The General Rules for Study of Portal Hypertension*, 2nd edn. Tokyo: Kanehara Co., 2004; 37–50.
21 Stiegmann GV, Goff JS, Michaletz-Onody PA *et al.* Endoscopic sclerotherapy as compared with endoscopic ligation for bleeding esophageal varices. *N Engl J Med* 1992; **326**: 1527–32.
22 Saeed ZA, Stiegmann GV, Ramirez FC *et al.* Endoscopic variceal ligation is superior to combined ligation and sclerotherapy for esophageal varices: a multicenter prospective randomized trial. *Hepatology* 1997; **25**: 71–4.
23 Kitano S, Koyanagi N, Iso Y, Higashi H, Sugimachi K. Prevention of recurrence of esophageal varices after endoscopic injection sclerotherapy with ethanolamine oleate. *Hepatology* 1987; **7**: 810–15.
24 Nakamura S, Mitsunaga A, Murata Y, Suzuki S, Hayashi N. Endoscopic induction of mucosal fibrosis by argon plasma coagulation (APC) for esophageal varices: a prospective randomized trial of ligation plus APC vs. ligation alone. *Endoscopy* 2001; **33**: 210–15.
25 Cipolletta L, Bianco MA, Rotondano G, Marmo R, Meucci C, Piscopo R. Argon plasma coagulation prevents variceal recurrence after band ligation of esophageal varices: preliminary results of a prospective randomized trial. *Gastrointest Endosc* 2002; **56**: 467–71.
26 Kanagawa H, Mima H, Kouyama H, Gotoh K, Uchida T, Okuda K. Treatment of gastric fundal varices by balloon-occluded retrograde transvenous obliteration. *J Gastroenterol Hepatol* 1996; **11**: 51–8.
27 Lo GH, Lai KH, Cheng JS, Chen MH, Chiang HT. A prospective, randomized trial of butyl cyanoacrylate injection versus band ligation in the management of bleeding gastric varices. *Hepatology* 2001; **33**: 1060–4.
28 Sanyal AJ, Fontana RJ, Di Bisceglie AM *et al.* The prevalence and risk factors associated with esophageal varices in subjects with hepatitis C and advanced fibrosis. *Gastrointest Endosc* 2006; **64**: 855–64.
29 Levy C, Zein CO, Gomez J *et al.* Prevalence and predictors of esophageal varices in patients with primary biliary cirrhosis. *Clin Gastroenterol Hepatol* 2007; **5**: 803–8.
30 Kaneda H, Hashimoto E, Yatsuji S, Tokushige K, Shiratori K. Hyaluronic acid levels can predict severe fibrosis and platelet counts predict cirrhosis in patients with non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2006; **21**: 1459–65.

Familial Aggregation in Patients with Non-Alcoholic Steatohepatitis

Katsutoshi Tokushige, Satoru Yatsuji, Etsuko Hashimoto, Ayae Kabutake, Maki Tobari, Makiko Taniai and Keiko Shiratori



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INTERNAL MEDICINE

□ CASE REPORT □

Familial Aggregation in Patients with Non-Alcoholic Steatohepatitis

Katsutoshi Tokushige, Satoru Yatsuji, Etsuko Hashimoto, Ayae Kabutake, Maki Tobari, Makiko Taniai and Keiko Shiratori

Abstract

We encountered three families that showed NASH accumulation. In family #1, a 21-year-old son and 10-year-old daughter were diagnosed with nonalcoholic steatohepatitis (NASH). They shared two adiponectin-gene single nucleotide polymorphisms (SNP). In family #2, a 51-year-old mother and 27-year-old son were diagnosed with NASH and shared the SNPs of other genes. In family #3, a 66-year-old mother and 34-year-old son were diagnosed with NASH and shared the SNPs of other genes. SNP sites differed among the three families, suggesting that the genes associated with the occurrence of NASH might be different in each patient.

Key words: NASH, SNP, familial aggregation

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Introduction

In lifestyle-related diseases such as type 2 diabetes mellitus (DM/) hypertension, accumulation in a family is often observed (1, 2). Hara et al reported that two single nucleotide polymorphism (SNP) sites, Intron 2 and Exon 2 of the adiponectin gene, are associated with DM onset and insulin resistance (3). In addition, SNPs of the PPARγ2 gene, calpains 10 (intron polymorphism) gene and adrenergic receptor (Trp64Ayg polymorphism) gene have been reported to be associated with the onset of DM, suggesting that genetic background might be associated with DM (4-6). In hypertension, a polymorphism of the angiotensinogen gene (M235 T) is reported to be correlated with serum renin levels and to related to hypertension (7). It is reported that insertion/deficiency (I/D) polymorphisms of the angiotensin-converting enzyme (ACE) gene are correlated with the serum ACE level, which is related to cardiovascular disease and essential hypertension (8). These results suggested that the occurrence and progression of lifestyle-related diseases are associated with genetic background as well.

Non-alcoholic fatty liver disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function tests. Its spectrum ranges from simple fatty liver, which is usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis (9, 10). Patients with NASH usually have insulin resistance syndrome as well. In addition, NASH is increasingly being recognized as a major cause of cryptogenic cirrhosis and as an indication for liver transplantation. Its etiology remains unclear, but most investigators agree that development of NASH requires underlying steatosis followed by a "second hit" that induces inflammation, fibrosis, or necrosis (10). As NASH is included in metabolic syndrome, familial aggregation is suggested (11-13).

Genetic SNPs of the β3 adrenergic receptor gene, microsomal triglyceride transfer protein (MTP) gene, MnSOD gene, and interleukin 1β gene are reported to be associated with NASH (14, 15). We also demonstrated that SNPs of the TNF promoter region might be associated with the "second hit" of NASH (16). Here, we report three families in which NASH accumulation was observed, and analyze the disease course and gene polymorphisms of these patients.

Case Report

Between January 1991 and December 2005, 249 patients

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Table 1. Laboratory Data

	Case1	Case2	Case3	Case4	Case5	Case 6	Unit
T-bil	0.7	1.5	0.3	0.8	0.6	0.8	(mg/dl)
AST	280	61	43	134	66	30	(IU/l)
ALT	504	154	98	292	69	65	(IU/l)
γ-GTP	54	23	41	184	74	36	(IU/l)
T-chol	205	139	256	211	249	186	(mg/dl)
TG	85	79	83	368	246	224	(mg/dl)
Plt	33.7	24.5	27.2	19.4	20.3	20.9	(10^4/mm^3)
PT%	85.3	82.5	100	89.4	93.8	100	(%)
FBS	99	95	115	91	140	137	(mg/dl)
HbA1c	4.9	4.9	6.2	5.6	8.0	7.3	(%)
IRI	10.5	3.3	9.1	16.2	16.6	11.2	(μU/ml)
HOMA-R	2.57	0.77	2.58	3.64	5.74	3.79	
HBsAg	(-)	(-)	(-)	(-)	(-)	(-)	
HCV Ab	(-)	(-)	(-)	(-)	(-)	(-)	
DM	(-)	(-)	(+)	(-)	(+)	(+)	
HT	(-)	(-)	(-)	(-)	(-)	(+)	
Hyperlipidemia	(-)	(-)	(+)	(+)	(-)	(+)	

were diagnosed as having biopsy-proven NASH at Tokyo Women's Medical University Hospital. Three families were found to have NASH between siblings or between mother and child. The diagnosis of NASH was based on the following criteria: 1) the presence of steatosis (>10%), lobular inflammation, and ballooning, with or without Mallory bodies and perivenular or pericellular fibrosis; 2) intake of less than 40 g of ethanol per week, as confirmed by physicians and family members in close contact with the patient; and 3) appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, biliary obstruction, and metabolic liver diseases.

Family #1 (Case 1, 10-year-old girl; Case 2, 21-year-old man)

In the summer of 1999, Case 1, a 10-year-old girl had eaten ice cream every day and had gained 3 kg of body weight in one month. Because her mother had type 2 DM, a parent-and-child medical examination for DM was performed, revealing hepatic dysfunction. Laboratory and physical data show BMI 21.8 kg/m^2, AST 280, ALT 504 (Table 1). Liver biopsy showed steatohepatitis (Stage 1, Grade 2-3) according to Brunt's NASH classification (17). Thus, she was diagnosed with NASH. Diet and exercise therapy was started, and her serum AST/ALT levels normalized. Her older brother (Case 2) had had mild liver dysfunction since 1999. His body weight had increased by 5 kg from 2002 to 2003, and his serum AST/ALT levels were markedly increased. Laboratory and physical data showed BMI 23.3 kg/m^2, AST 61, and ALT 154 (Table 1). Liver biopsy showed steatohepatitis (Stage 1, Grade 2), and he was diagnosed with NASH. After diet and exercise therapy, his serum AST/ALT levels improved to the normal range. We investigated the lifestyle and food preference by questionnaires. In cases 1 and 2, both liked to eat snacks and ice cream. Figure 1 shows the family tree of these cases. Their mother had DM. Both her children (cases 1 and 2) were diagnosed with NASH at relatively young ages. In addition, we investigated 10 SNP sites reported to be associated with NASH or DM (3, 14-16), and found that both cases shared two adiponectin-gene SNP sites that have been suggested to be related to DM (Table 2).

Family #2 (Case 3, 51-year-old woman; Case 4, 27-year-old man)

Case 3 had been treated for DM from 1990. Around 2000, liver dysfunction was discovered, and she was referred to our hospital in 2001. Laboratory and physical data showed BMI 26.4 kg/m^2, AST 43, ALT 98 (Table 1). Liver biopsy showed steatohepatitis (Stage 0-1, Grade 2), and she was diagnosed with NASH. Figure 2 shows her clinical course. We recommended diet and exercise therapy, but her body weight did not decrease. Serum AST/ALT levels increased and decreased in direct proportion to the increase and decrease in her body weight. In 2004, her serum AST/ALT levels were markedly increased. A second biopsy was performed, showing steatohepatitis (Stage 2, Grade 2-3), and the fibrosis was observed to have progressed during the four years since diagnosis. Her son (Case 4) had had hepatic dysfunction since his late teens. Serum AST/ALT levels, which at one time were normalized, had increased again in 2004, prompting a medical examination at that time. In 2005, his serum AST/ALT levels were increased to 134/292 when his body weight increased from 78 kg to 82 kg, as shown in Fig. 2. Liver biopsy showed steatohepatitis (Stage 1, Grade 3). With his weight loss, liver function improved. In cases 3

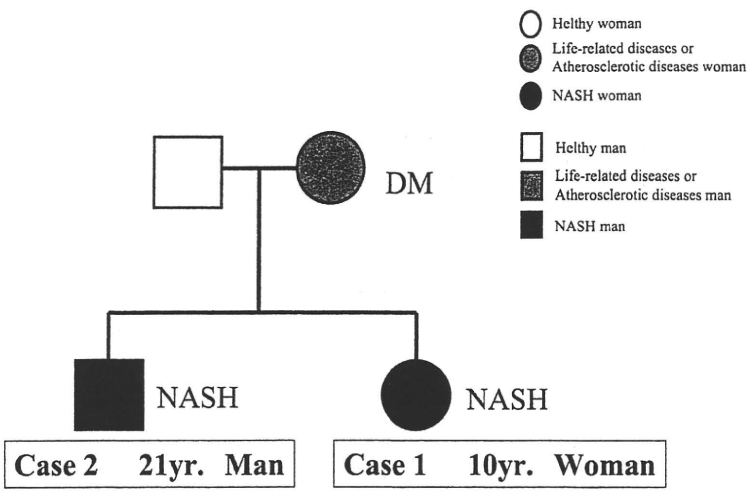

Figure 1. The family tree of family #1. Cases 1 and 2 were diagnosed with NASH. Their mother had type 2 diabetes mellitus (DM).

Table 2. SNP Analysis

	TNF-α -238	TNF-α -308	TNF-α -863	TNF-α -1031	Adipo +45	Adipo +276	IL-1β +511	β3-Ad	MTP	MnSOD
Case1	G/G	G/G	C/C	T/T	G/T	G/G	T/T	Trp/Arg	T/G	T/T
Case2	G/G	G/G	C/A	C/T	G/T	G/G	C/T	Trp/Trp	G/G	C/T
Case3	G/G	G/G	C/A	C/T	T/T	G/G	T/T	Trp/Arg	G/G	C/T
Case4	G/G	G/G	A/A	C/C	G/T	G/G	C/T	Arg/Arg	G/G	T/T
Case5	G/G	G/G	C/C	T/T	G/T	T/G	C/T	Trg/Trg	G/G	T/T
Case6	G/G	G/G	C/C	T/T	T/T	T/T	T/T	Trp/Arg	G/G	T/T

Shaded genotypes indicate those thought to contribute to the occurrence or progression of NASH or DM.

TNF, tumor necrosis factor

Adipo, adiponectin

IL-1, interleukin-1

β3-Ad, β3-adrenergic receptor

MTP, microsomal triglyceride transfer protein

and 4, there was no common tendency of lifestyle or food preference. Figure 3 shows the family tree of Cases 3 and 4. DM and hypertension were found in 3 other relatives. In the SNP analysis, both cases shared the SNPs of TNF-α promoter regions -1,031 and -863, which have been suggested to be related to TNF-α production, and the SNPs of adiponectin gene, the β3-adrenergic receptor and MTP genes, reportedly related to NASH (Table 2).

Family #3 (Case 5, 34-year-old man; Case 6, 66-year-old woman)

Case 5 had been obese since primary school age. At age 24, his serum AST/ALT levels were slightly increased and his body weight had increased to 90 kg (BMI 28.7 kg/m^2). He was diagnosed with DM at age 29. As his control of DM was poor, medical treatment was started. In 2004, he was referred for hepatic dysfunction to our hospital. Laboratory and physical data showed BMI 33.5 kg/m^2, AST 66, ALT 69 (Table 1). Liver biopsy showed steatohepatitis (Stage 3, Grade 3). We recommended diet and exercise therapy, after which his serum AST/ALT levels decreased. His mother (Case 6) had had hypertension from about age 50. At about age 60, hyperlipemia, hyperuricemia, and fatty liver were discovered. At age 63, therapy against DM was started. She was referred to our hospital for continuous hepatic dysfunction in 2005. Laboratory and physical data showed BMI 26.6 kg/m^2, AST 30, ALT 65 (Table 1). Liver biopsy showed steatohepatitis (Stage 3, Grade 3). In cases 5 and 6, there was no common tendency of lifestyle or food preference. Figure 4 shows the family tree of Cases 5 and 6. DM and arteriosclerotic disease were found in 3 other relatives. In SNP analysis, both cases shared the SNPs of the MTP and MnSOD genes, which are reportedly related to NASH (Table 2).

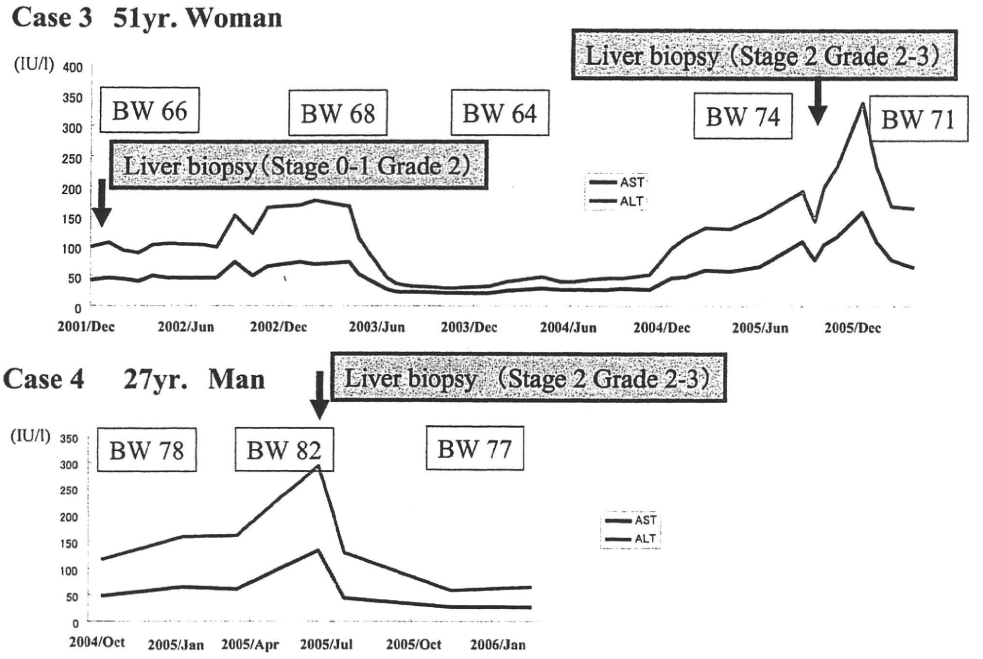

Figure 2. Clinical courses of cases 3 and 4 as well as the serum AST/ALT levels and body weight are shown. In case 3, the fibrosis stage progressed for 4 years. BW: body weight (kg).

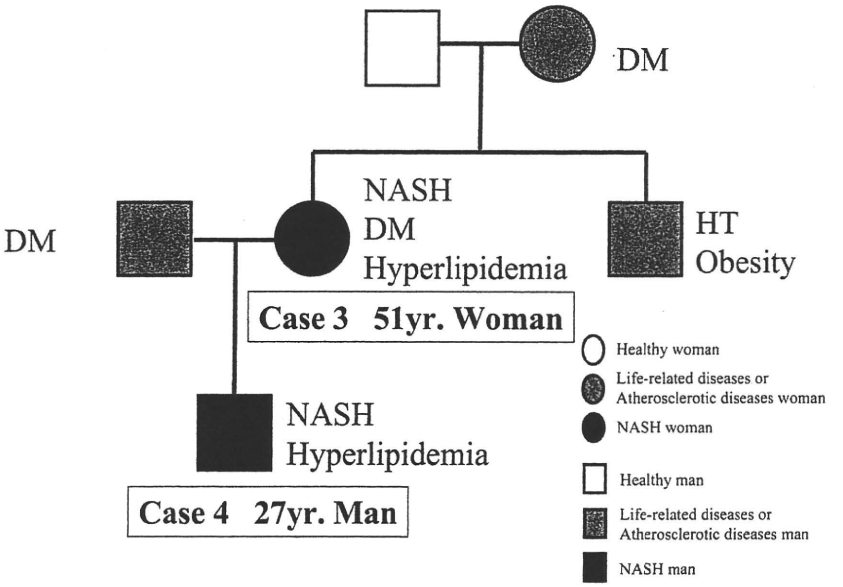

Figure 3. The family tree of family #2. Cases 3 and 4 were diagnosed with NASH. The mother of case 3 and the father of case 4 had type 2 diabetes mellitus (DM).

Discussion

We encountered three families in which NASH occurred in two siblings, or in both parent and child, similar to other lifestyle-related diseases. In these families, DM and other lifestyle-related diseases were frequently observed. The NASH-associated SNP sites that were found in each family were different, but all patients had some SNPs that are reportedly related to NASH or DM. For example, in family # 1, two SNP sites of adiponectin were shared between siblings, and each patient had other genetic SNPs. In family # 2, both patients shared an adiponectin gene SNP associated with DM, an economizing genotype of the β3-adrenergic receptor gene, a genotype of MTP that decreases the release capacity of very low-density lipoprotein (VLDL), and SNPs related to high TNF-α production. In family #3, both patients shared SNPs of the MnSOD gene and MTP gene, which are associated with oxidative stress. It was not confirmed whether these SNPs actually contributed to the occurrence and progression of NASH in our patients. For example, case 2 did not show insulin resistance, raising suspicion as to whether the adiponectin gene contributed to the pathogenesis of NASH in this case. The biological function

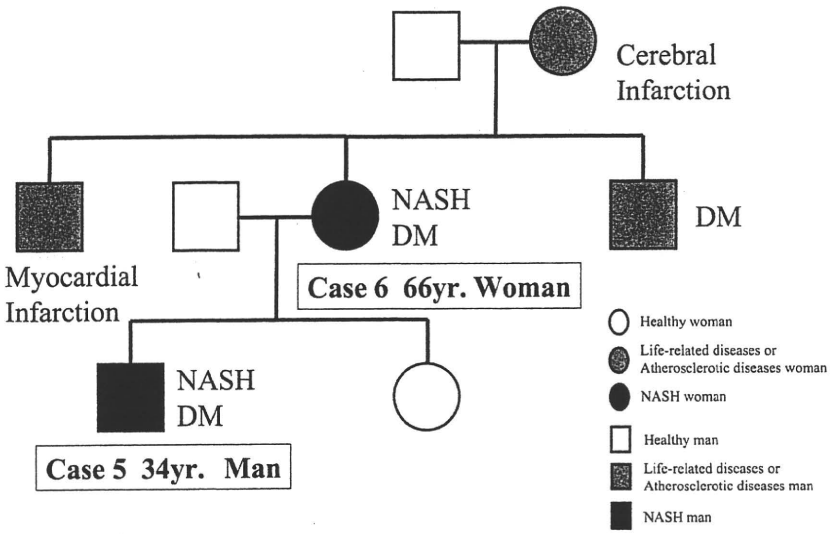

Figure 4. The family tree of family #3. Cases 5 and 6 were diagnosed with NASH. The mother and brothers of case 6 had type 2 diabetes mellitus (DM) or atherosclerotic diseases.

of adiponectin has been associated with not only insulin resistance, but also anti-inflammatory and anti-liver fibrosis, and it has been demonstrated that adiponectin is associated with the occurrence and progression of NASH (18, 19). In addition, Hara et al reported that adiponectin SNP might influence the production of adiponectin (3). These data suggest that adiponectin and these SNPs are associated with NASH. Among the SNPs which are reported to be related to the pathogenesis and progression of NASH, only adiponectin SNPs were shared in Cases 1 and 2. Therefore, we suggest that adiponectin SNPs are associated with NASH. In the future, SNP analysis and investigation of lifestyles in other family members who have not developed NASH will clarify which factors are more important.

Abdelmalek et al reported that NAFLD patients were more likely to have DM and that insulin resistance and DM occurred frequently in their first-degree relatives. In addition, a trend toward insulin resistance was noted in the mothers, but not the fathers, of patients with NAFLD (11). In our three families, all mothers had DM and/or NASH. In addition, in families #2 and #3, the grandmothers had DM or atherosclerotic diseases. Yatsuji et al reported that DM was more prevalent in older female NASH patients and suggested the possibility that female NASH patients might have stronger genetic factors (20). At any rate, it is necessary to pay close attention to children of mothers with DM or NASH.

Familial aggregation has been reported for DM, insulin resistance, and arteriosclerotic diseases (1, 2, 11). Here, in three family members, DM, HT, and arteriosclerotic diseases were frequently observed. These data support the hypothesis that NASH is associated with genetic background of these diseases and is part of the metabolic syndrome (12).

Case 5 was at stage 3 in young adulthood. In addition, the fibrosis of Case 3 progressed in the four years after diagnosis. Genetic background and similar eating habits or lifestyles might accelerate the progress of NASH. Considering the rapid progression of NASH in these patients, in addition to their family histories, amelioration of living habits and pharmacotherapy should be started from a young age.

Even when NASH patients had a genetic background for the disease, their liver function improved when body weight was controlled. These results suggested that adiposis based on living habits is the origin of NASH. We investigated the lifestyle and food preference by questionnaires. In cases 1 and 2, both liked to eat snacks and ice cream. In cases 3 and 4, there was no common tendency of lifestyle or food preference. Cases 5 and 6 also showed no common tendency. More detailed investigations will be necessary, because the possibility could not be denied that a similar lifestyle might be the cause of NASH among members of the same family. As overeating is encouraged in contemporary society, it is believed that the incidence of NASH will increase in the future. It is also recommended for the entire family of a NASH patient to undergo medical examinations.

References

1. Altinli S, Elevli M, Ozkul AA, Kara PG, Karsidag K, Dogru M. Insulin resistance and metabolic syndrome in children of parents with diabetes mellitus. J Pediatr Endocrinol Metab **20**: 431-436, 2007.
2. Bensen JT, Li R, Hutchinson RG, Province MA, Tyroler HA. Family history of coronary heart disease and pre-clinical carotid artery atherosclerosis in African-Americans and whites: the ARIC Study: Atherosclerosis Risk in Communities. Genet Epidemiol **16**: 165-178, 1999.
3. Hara K, Boutin P, Mori Y, Tobe K, et al. Genetic variation in the

gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes **51**: 536-540, 2002.

4. Turner MD, Cassell PG, Hitman GA. Calpain-10: from genome search to function. Diabetes Metab Res Rev **21**: 505-514, 2005.
5. Zeggini E, Parkinson JR, Halford S, et al. Examining the relationships between the Pro 12 Ala variant in PPARG and type 2 diabetes-related traits in UK samples. Diabet Med **22**: 1696-1700, 2005.
6. Kawamura T, Egusa G, Okubo M, Imazu M, Yamakido M. Association of beta3-adrenergic receptor gene polymorphism with insulin resistance in Japanese-American men. Metabolism **48**: 1367-1370, 1999.
7. Kuriyama S, Tomonari H, Tokudome G, et al. Association of angiotensinogen gene polymorphism with erythropoietin-induced hypertension: a preliminary report. Hypertens Res **24**: 501-505, 2001.
8. Prasad A, Narayanan S, Waclawiw MA, Epstein N, Quyyumi AA. The insertion/deletion polymorphism of the angiotensin-converting enzyme gene determines coronary vascular tone and nitric oxide activity. J Am Coll Cardiol **36**: 1579-1586, 2000.
9. Teli MR, James OFW, Burt AD, Bennet MK, Day CP. The natural history of nonalcoholic fatty liver: follow-up study. Hepatology **22**: 1714-1719, 1995.
10. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis; summary of an AASLD single topic conference. Hepatology **37**: 1202-1219, 2003.
11. Abdelmalek MF, Liu C, Shuster J, Nelson DR, Asal NR. Familial aggregation of insulin resistance in first-degree relatives of patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol **4**: 1162-1169, 2006.
12. Willner IR, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. Am J Gastroenterol **96**: 2957-2961, 2001.
13. Struben VM, Hespenheide EE, Caldwell SH. Nonalcoholic steatohepatits and crptogenic cirrhosis within kindreds. Am J Med **108**: 9-13, 2000.
14. Namikawa C, Shu-Ping Z, Vyselaar JR, et al. Polymorphisms of microsomal triglyceride transfer protein gene and manganese superoxide dismutase gene in non-alcoholic steatohepatitis. J Hepatology **40**: 781-786, 2004.
15. Nozaki Y, Saibara T, Nemoto Y, et al. Polymorphisms of interleukin-1β and β3-adrenergic receptor in Japanese patients with nonalcoholic steatohepatitis. Alcohol Clin Exp Res **28**: 106S-110S, 2004.
16. Tokushige K, Takakura M, Tsuchiya-Matsushita N, Taniai M, Hashimoto E, Shiratori K. Influence of TNF gene polymorphisms in Japanese patients with NASH and simple steatosis. J Hepatology **46**: 1104-1110, 2007.
17. Brunt EM, Janney CG, Di Biscrglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staining the histological lesions. Am J Gastroenterol **94**: 2467-2474, 1999.
18. Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology **40**: 46-54, 2007.
19. Marra F, Aleffi S, Bertolani C, Petrai I, Vizzutti F. Adipokines and liver fibrosis. Eur Rev Med Pharmacol Sci **9**: 279-284, 2005.
20. Yatsuji S, Hashimoto E, Tobari M, Tokushige K, Shiratori K. Influence of age and gender in Japanese patients with nonalcoholic steatohepatitis. Hepatol Res **37**: 1034-1043, 2007.

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Nonalcoholic steatohepatitis and increased risk of chronic kidney disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD) share common features. Both are associated with visceral obesity, type 2 diabetes mellitus, metabolic syndrome, and insulin resistance. However, the relationship between NAFLD and CKD is poorly understood. We examined the prevalence of and risk factors for CKD in patients with NAFLD. We analyzed 174 Japanese patients with liver biopsy–proven NAFLD using a cross-sectional design. Chronic kidney disease was defined as estimated glomerular filtration rate less than 60 mL/min per 1.73 m^2 and/or overt proteinuria. Of 174 NAFLD patients, 92 (53%) exhibited histologic characteristics of nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD; and 82 (47%) had non-NASH NAFLD. Chronic kidney disease was present in 24 (14%) of 174 NAFLD patients. The prevalence of CKD was significantly higher in NASH patients (19 of 92; 21%) than non-NASH patients (5 of 82; 6%). The presence of CKD was associated with a higher body mass index and the presence of hypertension and NASH. Our results demonstrated a high prevalence of CKD among patients with NASH.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease. The incidence of NAFLD continues to increase, and the prevalence of NAFLD ranges from 17% to 33% in the general population of Western countries [1]. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive nonalcoholic steatohepatitis (NASH) associated with fibrosis, necrosis, and inflammation [2-4]. Nonalcoholic steatohepatitis can progress to cirrhosis and hepatocellular carcinoma.

Chronic kidney disease (CKD) encompasses a spectrum of different processes associated with abnormal kidney function and a progressive decline in glomerular filtration rate (GFR). The prevalence of CKD in American adults was estimated to be 11% (19.2 million) [5]. Chronic kidney disease is increasingly recognized as a major risk factor for not only end-stage renal failure but also cardiovascular disease [6,7].

Nonalcoholic fatty liver disease and CKD share some common features, including visceral obesity, type 2 diabetes mellitus, hypertension, and metabolic syndrome [8-11]. Both diseases are also linked to an increased risk of cardiovascular disease [6,7,11]. Common factors underlying the pathogenesis of NAFLD and CKD include insulin resistance, oxidative stress, activation of the renin-angiotensin system, and inappropriate secretion of inflammatory cytokines [12,13]. However, the relationship between NAFLD and CKD is poorly understood; and the prevalence of and risk factors for CKD in patients with NAFLD remain unknown.

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Whereas laboratory test abnormalities and ultrasound or radiographic findings may be suggestive of NAFLD, histologic evaluation remains the only means of accurately assessing the degree of steatosis and the distinct necroinflammatory lesions and fibrosis of NASH; and it remains the only means of distinguishing NASH from simple steatosis [14]. In the present study, we therefore examined the association between liver biopsy–proven NAFLD and CKD using a cross-sectional design; and we investigated the risk factors associated with CKD in patients with NAFLD.

2. Methods

2.1. Patients

The study included a total of 174 Japanese patients with NAFLD who underwent liver biopsy between 2001 and 2009 at the Hospital of Kyoto Prefectural University of Medicine (Kyoto, Japan) and Nara City Hospital (Nara, Japan). The diagnosis of NAFLD was based on a liver biopsy showing steatosis in more than 5% of hepatocytes, along with exclusion of liver diseases of other etiology. Patients had to be older than 18 years. Exclusion criteria were as follows: patients consuming more than 20 g of alcohol per day; positive for hepatitis B virus surface antigen; positive for anti–hepatitis C virus antibody; other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, Wilson disease, or hemochromatosis; treated with drugs known to produce hepatic steatosis, including corticosteroids, high-dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; and a history of gastrointestinal bypass surgery.

The Ethics Committees of the Kyoto Prefectural University of Medicine and Nara City Hospital approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

2.2. Clinical assessment and laboratory tests

Body mass index (BMI) was calculated using the following formula: weight in kilograms/(height in meters)^2. *Obesity* was defined as a BMI of at least 25 according to the criteria of the Japan Society for the Study of Obesity [15]. Diabetes was defined as a fasting plasma glucose concentration of at least 126 mg/dL or a 2-hour plasma glucose concentration of at least 200 mg/dL during an oral glucose (75 g) tolerance test or the use of insulin or oral hypoglycemic agents to control blood glucose [16,17]. Hypertension was defined as a systolic blood pressure of at least 130 mm Hg, a diastolic blood pressure of at least 85 mm Hg, or the use of antihypertensive agents [18]. Dyslipidemia was defined as serum concentrations of triglycerides of at least 150 mg/dL or high-density lipoprotein (HDL) cholesterol less than 40 mg/dL and less than 50 mg/dL for men and women, respectively, or the use of specific medication [18].

Venous blood samples were taken in the morning after a 12-hour overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), albumin, creatinine, total cholesterol, HDL cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using standard clinical chemistry techniques. Proteinuria was detected by dipstick examination. These clinical and laboratory data were collected at the time of liver biopsy.

Kidney function was estimated using the Japanese equation, which defines the estimated glomerular filtration rate (eGFR) as follows: eGFR = 194 × (serum creatinine$^{-1.094}$) × (age$^{-0.287}$) × 0.739 (if female) [19]. Chronic kidney disease was defined as eGFR less than 60 mL/min per 1.73 m^2 and/or overt proteinuria [20]. Both of these outcome measures had to be confirmed in a least 2 consecutive tests. Stage of CKD was defined according to the criteria proposed by the National Kidney Foundation [21].

2.3. Histopathologic examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes. The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, with Masson trichrome, and by silver impregnation. The sections were analyzed by experienced hepatopathologists (TO and YS) who were blinded to the laboratory parameters and clinical data. Patients with biopsy-established NAFLD were categorized as NASH or non-NASH [2,22]. Nonalcoholic steatohepatitis was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory hyaline (Mallory body) or fibrosis [2,14,23,24]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with nonspecific inflammation were identified as having non-NASH NAFLD. The degree of fibrosis in NASH was evaluated and scored according to the criteria proposed by Brunt et al [24].

2.4. Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a χ^2 test for qualitative factors or a Mann-Whitney U test on ranks for quantitative factors with nonequal variance. Logistic regression analysis was used for multivariate analysis. P values < .05 from 2-sided tests were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. All statistical analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL).

Table 1
Patient characteristics

Characteristic	Total (n = 174)	NASH (n = 92)	Non-NASH (n = 82)	P
Age (y)	54 (18-78)	62 (24-78)	49 (18-78)	<.001
Sex				.009
Male	102 (59%)	45 (49%)	57 (70%)	
Female	72 (41%)	47 (51%)	25 (30%)	
BMI (kg/m^2)	26.2 (18.6-43.4)	26.5 (19.1-39.4)	25.2 (18.6-43.4)	.02
Obesity	106 (61%)	61 (66%)	45 (55%)	.16
Diabetes	53 (31%)	33 (36%)	20 (24%)	.14
Dyslipidemia	84 (48%)	44 (48%)	40 (49%)	1.00
Hypertension	59 (34%)	39 (42%)	20 (24%)	.02
Platelet count (×10^4/μL)	21.8 (4.6-37.3)	18.9 (4.6-35.1)	24.2 (12.3-37.3)	<.001
AST (IU/L)	49 (10-447)	61 (10-447)	39 (16-151)	<.001
ALT (IU/L)	77 (12-358)	79 (16-316)	75 (12-358)	.14
γ-GTP (IU/L)	73 (19-1681)	76 (19-1681)	69 (19-568)	.59
Albumin (g/dL)	4.6 (2.9-5.5)	4.5 (2.9-5.2)	4.8 (4.0-5.5)	<.001
Fasting glucose (mg/dL)	102 (65-452)	103 (65-452)	99 (76-333)	.17
Total cholesterol (mg/dL)	202 (52-344)	192 (52-288)	217 (99-344)	.003
HDL cholesterol (mg/dL)	47 (25-79)	46 (25-79)	49 (35-77)	.12
Triglyceride (mg/dL)	136 (35-1454)	131 (42-1454)	139 (35-410)	.56
eGFR (mL/[min 1.73 m^2])	82.3 (46.5-161.8)	82.1 (46.5-161.8)	82.7 (53.8-137.9)	.18
Proteinuria	17 (10%)	12 (13%)	5 (6%)	.14
CKD	24 (14%)	19 (21%)	5 (6%)	.007
Stage[a]				.07
1	7 (4%)	6 (7%)	1 (1%)	
2	9 (5%)	5 (5%)	4 (5%)	
3	8 (5%)	8 (9%)	0 (0%)	
4	0 (0%)	0 (0%)	0 (0%)	
5	0 (0%)	0 (0%)	0 (0%)	

Values are median (range) or number (percentage). Where no other unit is specified, values refer to number of patients. All patients were of Japanese ethnicity.
[a] According to Levey et al [20].

3. Results

The characteristics of the 174 NAFLD patients included in the study are summarized in Table 1. Of these 174 NAFLD patients, 92 (53%) exhibited histologic characteristics of NASH; and 82 (47%) had non-NASH NAFLD. Five patients had liver cirrhosis (fibrosis stage 4). Patients with NASH, as compared with patients with non-NASH NAFLD, were significantly older, were more often female, had a higher BMI, more often had hypertension, had a higher AST, and had lower platelet count, albumin, and total cholesterol (Table 1).

Chronic kidney disease was present in 24 patients (14%), including 7 (4%) with stage 1, 9 (5%) with stage 2, and 8 (5%) with stage 3. The prevalence of CKD was significantly higher in patients with NASH (19 of 92; 21%) than in those with non-NASH NAFLD (5 of 82; 6%) (Table 1). Patients with NASH tended to have a more advanced stage of CKD than patients with non-NASH NAFLD, although the difference was not statistically significant (Table 1).

We evaluated the relationship between eGFR values and the histologic severity of NASH (fibrosis stage). The median (range) of eGFR values in NASH patients with fibrosis stage 1 (n = 37), 2 (n = 24), 3 (n = 26), and 4 (n = 5) was 79.4 (46.5-122.8), 82.2 (57.0-161.8), 83.8 (48.4-144.1), and 85.0 (66.5-97.6) mL/min per 1.73 m^2, respectively. The correlation between eGFR values and the fibrosis stage was not significant (P = .47).

Univariate correlations between variables and CKD are shown in Table 2. The presence of CKD was associated with a higher BMI and the presence of hypertension and NASH; but it was not associated with age, sex, the presence of diabetes or dyslipidemia, or levels of AST, ALT, or γ-GTP. Multivariate analysis revealed that the presence of hypertension correlated independently with the presence of CKD (Table 3).

Table 2
Univariate analysis of factors associated with CKD in NAFLD patients

Factor	No CKD (n = 150)	CKD (n = 24)	P
Age (y)	55 (18-78)	54 (31-78)	.19
Male	89 (59%)	13 (54%)	.66
BMI (kg/m^2)	25.6 (18.6-43.4)	28.3 (21.1-35.1)	.003
Diabetes	42 (28%)	11 (46%)	.10
Dyslipidemia	72 (48%)	12 (50%)	1.00
Hypertension	43 (29%)	16 (67%)	.001
AST (IU/L)	50 (10-210)	45 (21-447)	.82
ALT (IU/L)	78 (12-358)	65 (18-254)	.45
γ-GTP (IU/L)	77 (19-568)	72 (29-1681)	.87
NASH	73 (49%)	19 (79%)	.007

Values are median (range) or number (percentage). Where no other unit is specified, values refer to number of patients.

Table 3
Multivariate analysis of factors independently associated with CKD in NAFLD patients

Factor	Odds ratio	95% Confidence interval	P
BMI (kg/m^2)	1.09	0.98-1.21	.11
Hypertension	3.90	1.42-10.71	.008
NASH	2.46	0.82-7.42	.11

Data are from a total of 174 patients.

4. Discussion

Our results demonstrated a high prevalence (21%) of CKD among patients with NASH. The prevalence of CKD was significantly higher in NASH than in non-NASH NAFLD.

It is important to identify factors that increase the risk for CKD, even in individuals with normal GFR. In general, known risk factors include hypertension, diabetes, autoimmune disease, older age, African ancestry, a family history of renal disease, a previous episode of acute renal failure, or structural abnormalities of the urinary tract [25]. Our results showed that the risk factors associated with CKD in NAFLD patients include obesity (higher BMI), hypertension, and NASH. In particular, hypertension was an independent risk factor for CKD.

Nonalcoholic fatty liver disease is closely associated with obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus, which are all features of the metabolic syndrome. This strongly supports the idea that NAFLD is the hepatic manifestation of the metabolic syndrome [8]. The presence of insulin resistance is recognized as the pathophysiologic hallmark of NAFLD. A recent study show that NAFLD is more prevalent in nondipper hypertensive patients than dipper hypertensive patients, and a high prevalence of NAFLD is associated with insulin resistance and low adiponectin in the nondippers [26]. Similarly, growing evidence suggests that the metabolic syndrome is an important factor in the pathogenesis of CKD [27]; and there is a positive relationship between insulin resistance and CKD [10].

Relatively few studies have evaluated NAFLD and the risk of CKD. Recent studies found that NAFLD is associated with an increasing incidence of CKD in type 2 diabetes mellitus patients [28] and in nonhypertensive and nondiabetic Korean men [29]. Although these findings are important, their interpretation is limited by the fact that the diagnosis of NAFLD was based on liver ultrasound imaging. Whereas ultrasound is the commonly used for diagnosing NAFLD in clinical practice, it cannot distinguish NASH from simple steatosis. Histologic evaluation remains the only means of diagnosing NASH. To our knowledge, our current study is the first to assess the association between NASH and CKD.

A recent report showed a positive relationship between microalbuminuria and liver fibrosis in nondiabetic patients with NAFLD [30]. In this study, however, we did not find an association between eGFR values and the degree of liver fibrosis in NASH patients. The association of liver fibrosis with low eGFR or proteinuria remains to be verified in future prospective studies using a larger number of samples.

The underlying mechanisms by which NASH increases the risk for CKD remain to be elucidated. It may simply reflect the coexistence of underlying known risk factors. Alternatively, NASH may be a stimulus for further increases in whole-body insulin resistance, leading to the development of CKD. Another possible underlying mechanism is increased oxidative stress and chronic subclinical inflammation. The possible mediators linking NASH and CKD include reactive oxygen species, tumor necrosis factor–α, and other proinflammatory cytokines [11,31].

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding causality between NASH and CKD. Second, an eGFR was used rather than more precise measures to identify and classify kidney disease. However, equations that estimate GFR for the evaluation of renal function are recommended for epidemiological studies and for clinical practice [20]. Third, the dipstick urinalysis has a lower sensitivity and specificity in the diagnosis of proteinuria than 24-hour urine collection or measurement of the albumin-to-creatinine ratio in a random spot collection. Nevertheless, in most cases, screening with urine dipsticks is considered acceptable for detecting proteinuria [21]. Fourth, this was a hospital-based study and therefore may be influenced by selection bias. Finally, the study did not include a control group of nonsteatotic subjects.

In summary, this study shows that it is important to assess the risk of CKD in NASH/NAFLD patients. In addition, our findings suggest that preventing and treating obesity, hypertension, and NASH may help prevent NAFLD patients from developing CKD. Moreover, our results suggest that a higher BMI and the presence of hypertension and NASH are associated with an increasing prevalence of CKD in NALFD patients. Further prospective studies are warranted to establish the causal relationship between NASH and CKD.

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References

[1] Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology 2006;43:S99-S112.
[2] Matteoni CA, Younossi ZM, Gramlich T, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 1999;116:1413-9.
[3] Bacon BR, Farahvash MJ, Janney CG, et al. Nonalcoholic steatohepatitis: an expanded clinical entity. Gastroenterology 1994;107:1103-9.

[4] Teli MR, James OF, Burt AD, et al. The natural history of nonalcoholic fatty liver: a follow-up study. Hepatology 1995;22:1714-9.
[5] Coresh J, Astor BC, Greene T, et al. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. Am J Kidney Dis 2003;41:1-12.
[6] Sarnak MJ, Levey AS, Schoolwerth AC, et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 2003;108:2154-69.
[7] Go AS, Chertow GM, Fan D, et al. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 2004;351:1296-305.
[8] Marchesini G, Bugianesi E, Forlani G, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003;37:917-23.
[9] Agrawal V, Shah A, Rice C, et al. Impact of treating the metabolic syndrome on chronic kidney disease. Nat Rev Nephrol 2009;5:520-8.
[10] Chen J, Muntner P, Hamm LL, et al. Insulin resistance and risk of chronic kidney disease in nondiabetic US adults. J Am Soc Nephrol 2003;14:469-77.
[11] Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia 2008;51:1947-53.
[12] Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002;346:1221-31.
[13] Lastra G, Manrique C, McFarlane SI, et al. Cardiometabolic syndrome and chronic kidney disease. Curr Diab Rep 2006;6:207-12.
[14] Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-21.
[15] Japan Society for the Study of Obesity. New criteria of obesity (in Japanese). J Jpn Soc Study Obes 2000;6:18-28.
[16] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539-53.
[17] Kuzuya T, Nakagawa S, Satoh J, et al. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. Diabetes Res Clin Pract 2002;55:65-85.
[18] Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001;285:2486-97.
[19] Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 2009;53: 982-92.
[20] Levey AS, Coresh J, Balk E, et al. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003;139:137-47.
[21] National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 2002;39:S1-S266.
[22] Rafiq N, Bai C, Fang Y, et al. Long-term follow-up of patients with nonalcoholic fatty liver. Clin Gastroenterol Hepatol 2009;7:234-8.
[23] Brunt EM. Non-alcoholic fatty liver disease. In: Burt AD, Portmann BC, Ferrell LD, editors. MacSween's pathology of the liver. London: Churchill Livingstone; 2006. p. 367-97.
[24] Brunt EM, Janney CG, Di Bisceglie AM, et al. Non-alcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467-74.
[25] Bargman JM, Skorecki K. Chronic kidney disease. In: Fauci AS, Braunwald E, Kasper DL, Hauser SL, Long DL, Jameson JL, editors. Harrison's principles of internal medicine. New York: McGraw-Hill; 2008. p. 1761-71.
[26] Fallo F, Dalla Pozza A, Sonino N, et al. Nonalcoholic fatty liver disease, adiponectin and insulin resistance in dipper and nondipper essential hypertensive patients. J Hypertens 2008;26:2191-7.
[27] Chen J, Muntner P, Hamm LL, et al. The metabolic syndrome and chronic kidney disease in U.S. adults. Ann Intern Med 2004;140: 167-74.
[28] Targher G, Chonchol M, Bertolini L, et al. Increased risk of CKD among type 2 diabetics with nonalcoholic fatty liver disease. J Am Soc Nephrol 2008;19:1564-70.
[29] Chang Y, Ryu S, Sung E, et al. Nonalcoholic fatty liver disease predicts chronic kidney disease in nonhypertensive and nondiabetic Korean men. Metabolism 2008;57:569-76.
[30] Yilmaz Y, Alahdab YO, Yonal O, et al. Microalbuminuria in nondiabetic patients with nonalcoholic fatty liver disease: association with liver fibrosis. Metabolism 2010 [Epub ahead of print].
[31] Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. Atherosclerosis 2007;191:235-40.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease

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Abstract

Background Hyperlipidemia, insulin resistance, and oxidative stress can heavily contribute to the initiation and progression of nonalcoholic fatty liver disease (NAFLD). Currently, there is no established treatment for this disease. Recently, several studies have shown that ezetimibe (EZ), a lipid-lowering drug, attenuates liver steatosis in an experimental NAFLD model. This study was designed to assess the efficacy of long-term EZ monotherapy in patients with NAFLD.

Methods A total of 45 patients with newly diagnosed liver biopsy-proven NAFLD were treated with EZ (10 mg/day) for 24 months. NAFLD-related biochemical parameters, imaging by computerized tomography, and liver biopsy were studied before and after treatment.

Results Ezetimibe therapy significantly improved NAFLD-related metabolic parameters including visceral fat area, fasting insulin, homeostasis model assessment of insulin resistance (HOMA-R), triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-Ch), oxidative-LDL, the net electronegative charge modified-LDL, profiles of lipoprotein particle size and fatty acids component, and estimated desaturase activity. EZ therapy also significantly lowered serum alanine aminotransferase and high-sensitivity C-reactive protein levels, whereas no significant changes were found in serum type IV collagen 7S, adiponectin, leptin, and resistin levels. Histological features of steatosis grade ($P = 0.0003$), necroinflammatory grade ($P = 0.0456$), ballooning score ($P = 0.0253$), and NAFLD activity score (NAS) ($P = 0.0007$) were significantly improved from baseline. However, the fibrosis stage was not significantly ($P = 0.6547$) changed.

Conclusion The results in this study suggest that the long-term EZ therapy can lead to improvement in metabolic, biochemical, and histological abnormalities of NAFLD. Therefore, EZ may be a promising agent for treatment of NAFLD.

Keywords Ezetimibe · NAFLD · Insulin resistance · Lipid metabolism · Fatty acid metabolism

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury in the world [1–3]. NAFLD is a metabolic condition which encompasses a wide spectrum of liver disease ranging from

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simple steatosis to nonalcoholic steatohepatitis (NASH). Although the exact intricacies of the molecular and cellular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated, hyperlipidemia, insulin resistance, and oxidative stress are major contributors to the initiation and progression of NAFLD [4–6]. A two-hit hypothesis has been proposed, whereby steatosis (first hit) sensitizes the liver to a variety of metabolic injuries (second hit) that lead to necrosis, inflammation, and fibrosis [6]. Several investigators have suggested that NASH is the hepatic manifestation of the metabolic syndrome [2–7]. While there are few proven beneficial therapies for NASH, its association with insulin resistance has provided the rationale for evaluation of medical therapies that increase insulin sensitivity. Indeed, several pilot studies have shown that treatment with the biguanides and the thiazolidinediones, two classes of insulin-sensitizing drugs, can lead to improvements in biochemical and histological features of NASH [8–16].

Ezetimibe (EZ) is a useful lipid-lowering agent that inhibits the absorption of dietary and biliary cholesterol by selectively binding to the intestinal cholesterol transporter Niemann–Pick C1-like 1 [17, 18]. Several recent studies in an experimental NAFLD model have shown that EZ monotherapy not only protects against diet-induced hyperlipidemia, but also attenuates liver steatosis in an experimental NAFLD model [19–21].

In the present study, we investigated the efficacy of long-term EZ monotherapy in patients with NAFLD.

Patients and methods

Patients

The study protocol was approved by the ethical committee of Saiseikai Suita Hospital and the Kyoto Prefectural University of Medicine, and informed consent was obtained from all subjects prior to enrollment in the study. A total of 45 patients who had been newly diagnosed histologically as having NAFLD at Saiseikai Suita Hospital and Kyoto Prefectural University Hospital between 2007 and 2009 were evaluated in this study.

All liver biopsy specimens were examined by two experienced pathologists blinded to the patients' clinical or laboratory data or liver biopsy sequence. Histological features of samples were interpreted as outlined by Brunt et al. [22]. The stage of fibrosis was classified as follows: stage 0 = no fibrosis, stage 1 = zone 3 predominant pericellular fibrosis, stage 2 = zone 3 fibrosis plus periportal fibrosis, stage 3 = bridging fibrosis, stage 4 = cirrhosis. Necroinflammation was graded 0 (absent) to 3 (1, occasional ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). The grade of steatosis was defined as mild (≤33%), moderate (34–65%), and advanced (≥66%). The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2) as reported by Kleiner et al. [23], and as shown in Table 1 was used to classify NAFLD into "not NASH" (NAS ≤2), "borderline NASH" (NAS = 3–4), and "definite NASH" (NAS ≥5).

Prior to evaluation of liver histology we excluded patients with an alcohol intake exceeding 20 g/day and those who reported any sign, symptom, and/or history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, previous use of anti-diabetic medication including insulin-sensitizing agents such as metformin and pioglitazone. All patients received EZ (10 mg/day) for 24 months.

Estimation of energy and nutrient intake

All patients were asked to adhere to a dietary plan tailored to their energy requirements and metabolic control by a registered dietitian and/or physician, using the current Japan Diabetes Society recommendations. The patients

Table 1 Baseline anthropometrics and demographics

Male/female	24/21
Age (years)	50.2 ± 9.4
Body mass index (kg/m^2)	26.9 ± 3.3
Waist circumference (cm)	92.3 ± 5.7
Visceral fat area (cm^2)	155.9 ± 38.9
Subcutaneous fat area (cm^2)	170.9 ± 51.3
Obesity[a] (%)	41 (91.1)
Hyperlipidemia[b] (%)	45 (100)
Hypertension[c] (%)	23 (48.9)
75 g oral glucose tolerance test	
NGT (normal glucose tolerance; %)	7 (15.6)
IGT (impaired glucose tolerance; %)	28 (62.2)
Diabetes (%)	10 (22.2)
NAFLD activity score (NAS)	
NAS ≤2	4 (11.1)
NAS 3–4	3 (8.9)
NAS ≥5	38 (80.0)

NAFLD nonalcoholic fatty liver disease

[a] Obesity was defined as a body mass index of ≥25.1

[b] Hyperlipidemia diagnosed if serum total cholesterol level was ≥220 mg/dl and/or serum triglyceride level was ≥160 mg/dl on at least two occasions

[c] Hypertension was diagnosed if the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions

recorded their daily dietary intake in a diary by using the calorie and lipid list in the Japan Diabetes Society recommendations guidebook. The dietary diary was collected every month, and the results were reported back to the subjects the following month. In addition, daily activity and physical condition were recorded every month using a checklist; and depending on the report, the physician checked the patient's condition and provided appropriate advice.

Clinical and laboratory investigations

The intra-abdominal visceral (VSA) and subcutaneous fat areas (SFA) were determined at the umbilical level by a computed tomography (CT) scanning technique (TSX-012A, X-Vigor, Toshiba Co. Ltd, Tokyo, Japan) using a method described previously [24].

Blood samples were obtained in the morning after an overnight fast. Plasma glucose (PG) was measured by the glucose oxidase method and HbA1c was determined by high-performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan). Plasma insulin immunoreactive insulin (IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and plasma glucose levels by the following equation: HOMA-R = fasting IRI (μU/ml) × fasting PG (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch), and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Serum type IV collagen 7S was measured by a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by nephelometry using a latex particle-enhanced immunoassay (Dade Behering, Tokyo, Japan). Serum oxidized LDL (oxLDL) was measured by an enzyme-linked immunoassay (ELISA) kit (Kyowa Medex Co., Ltd., Tokyo, Japan). The net electronegative charge modified-LDL (emLDL) was analyzed by using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System™; Helena Labs, Saitama, Japan). The percentage frequency of emLDL was calculated on a computer from the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera according to the following formula: emLDL density = $[b - a/a] \times 100\%$.

Serum lipoproteins were also analyzed by an HPLC system according to the procedure described by Okazaki et al. [25], while lipoprotein particle size was determined based on individual elution times that corresponded to peaks on the chromatographic pattern of cholesterol fractions. In this study, we defined 3 VLDL, 4 LDL, and 5 HDL subclasses according to lipoprotein particle size, expressed as diameter.

Analysis of fatty acid composition in plasma cholesterol esters (CEs) was as follows: total lipid was extracted from plasma by using the method of Bligh and Dyer [26], followed by separation of the CEs by thin-layer chromatography using silica gel plates (Silica Gel 60, Merck, Darmstadt, Germany) and a solvent system of petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). The spot corresponding to CEs was scraped from the plate and transmethylated with 2 ml of acetyl chloride/methanol (5:50, v/v) at 90°C for 2 h. Heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were quantified by using a model GC14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 25-m × 0.5-mm capillary column (HR-SS-10, Shinwa Chemical Industries, Ltd., Kyoto, Japan). Desaturase and elongase activities were estimated as the ratio product to the precursor of individual fatty acids in plasma CEs according to the following: D9-16D = 16:1n-7/16:1, D9-18D = 18:1n-9/18:1, D6D = 18:3n-6/18:2n-6, and D5D = 20:4n-6/20:3n-6.

Statistical analysis

All statistical analyses were performed using Statview version 5.0 (Abacus Concepts, Berkeley, CA, USA). Data were summarized by frequencies and percentages for categorical variables, and means ± SD for continuous variables. Comparison of pre- and posttreatment of EZ data was carried out using nonparametric Wilcoxon signed rank test. A P value less than 0.05 was considered statistically significant.

Results

Effect of ezetimibe on clinical and laboratory parameters

Compared to baseline, VFA level reduced significantly from 155.9 ± 38.9 to 146.5 ± 34.8 ($P < 0.05$) at the end of the study (Table 2). There were no significant changes in body mass index (BMI), waist circumference, and SFA at the end of the study.

Mean ALT level decreased significantly by the end of the study from 62 ± 25 to 49 ± 23 ($P < 0.01$), whereas the AST level did not. The mean level of fasting insulin level and HOMA-R decreased significantly (both $P < 0.05$), although mean HbA1c and fasting glucose levels remained unchanged at the end of the study. Regarding lipid metabolism, the mean levels of TG, T-Ch, LDL-Ch, oxLDL, and