an important element.[11,12] And, the localization of OCT is restricted to mitochondria.[13] Taken together, in this study we measured the serum OCT level in NASH patients and evaluated its clinical significance in NASH.

PATIENTS AND METHODS

ONE HUNDRED AND twenty Japanese patients histologically diagnosed as having NASH at Tokyo Women's Medical University between 1995 and 2008 were evaluated along with 80 healthy subjects serving as controls. All liver biopsy specimens were examined using hematoxylin-eosin, Mallory, and silver reticulin as stains. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 3 and 2, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis.[2,14,15] The diagnosis of NASH was established based on the following criteria: (i) histologically macrovesicular steatosis affecting at least 10% of hepatocytes and steatohepatitis including ballooning cells or perisinusoidal/pericellular fibrosis in zone 3;[2,14,15] (ii) intake of less than 20 g of ethanol per day, as confirmed by physicians and family members of the patients; and (iii) 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, and metabolic liver diseases. NASH patients were divided into three groups: (i) NASH patients without liver cirrhoisis (LC) and hepatocellular carcinoma (HCC), (ii) cirrhotic NASH (NASH-LC) without HCC, and (iii) NASH with HCC (NASH-HCC). Eighteen NASH-LC patients were proven as LC by liver biopsy. Nine NASH-HCC patients were diagnosed histologically or by detection of consistent findings on at least two radiologic modalities.

All patients underwent liver tests for measurement of the following laboratory parameters: AST, ALT, platelet count, hepatitis B serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen), hepatitis C virus (HCV) serology (antibody to HCV and HCV-RNA polymerase chain reaction), and autoantibodies (antinuclear antibody (ANA), anti-smooth muscle antibody, and anti-mitochondrial antibody). The upper normal limit of the ALT level was set at 30 U/L in our hospital. In all NASH patients, diet and exercise were directed.

All control subjects were Japanese and were confirmed to have normal liver function and no viral hepatitis infection by blood test. In addition, fatty liver was ruled out by ultrasound. This group was formed by enrolling volunteers. Informed consent was obtained from all patients and healthy controls before their entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's research committee.

Serum OCT levels were measured by ELISA as reported previously.[8,9] Briefly, 50 μL of the HRP-conjugated F(ab') fraction of anti-OCT monoclonal IgG (secondary antibody, Mo5B11), and 50 μL of standard solution or sample diluted 10-fold with assay buffer (250 mmol/L glycine-buffer pH 9.4, containing 0.1% bovine serum albumin, 50 mmol/L NaCl and 0.1% ProClin950) were added to an antibody-coated dish (first antibody, Mo3B11). After mixing, the dish was incubated for 2 h and then washed with washing solution (10 mmol/L phosphate-buffer pH 7.4, containing 0.1% BSA, 150 mmol/L NaCl and 0.1% ProClin950). Then, a substrate solution (200 μg/mL 3, 3', 5, 5'-teramethylbenzidine containing 0.001% H_2O_2) was added. After the coloring reaction (20 min) was terminated by adding a stop solution (0.5 mol/L H_2SO_4), absorbance at 450 nm was measured with a microplate reader.

Statistic analysis

Data were expressed as mean ± standard deviation (SD). Statistical comparison among the four groups (control, NASH, NASH-LC, NASH-HCC) was conducted using Dunn's test, with $P < 0.05$ considered statistically significant. The comparison between F0-2 patients and F3-4 patients was performed by Mann–Whitney *U*-test. The correlations between serum OCT levels and serum ALT levels or serum AST levels or platelet count were confirmed by Spearman's correlation test.

RESULTS

DETAILS CONCERNING THE patients and control subjects are shown in Table 1. Serum levels of OCT, AST and ALT were measured. Table 2 shows the mean serum AST, ALT, OCT levels, platelte counts, and the ratios of AST : ALT, OCT : AST and OCT : ALT in 93 NASH patients without HCC and LC, 18 NASH-LC patients and 9 NASH-HCC patients. There were significant associations between serum OCT levels and serum AST levels or ALT levels in all patients and controls (ALT, $r = 0.774$, $P < 0.01$; AST, $r = 0.843$, $P < 0.01$). The AST and ALT levels of NASH-HCC were slightly increased compared to those of NASH-LC and NASH. The ALT level of NASH-LC was slightly decreased compared to those of

Table 1 Patient profiles

	N	Age (years)	Sex (M : F)	BMI (kg/m^2)
Control	80	21–59	43:37	ND
NASH	93	18–81	46:47	27.8 ± 5.3
NASH-LC	18	43–87	6:12	27.4 ± 5.7
NASH-HCC	9	61–77	7:2	27.5 ± 3.0

BMI, body mass index (mean ± SD); ND, not done; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NASH-HCC, non-alcoholic steatohepatitis with hepatocellular carcinoma; NASH-LC, liver cirrhosis induced by NASH.

NASH and NASH-HCC. However, regarding AST and ALT, the differences among the three groups (NASH, NASH-LC, NASH-HCC) were not significant. In contrast, the serum OCT levels in NASH were higher than those of controls, and gradually increased with development of liver disease from NASH to LC and HCC. In addition, the ratios of OCT : ALT and OCT : AST were significantly increased in parallel with the progression of NASH, LC and HCC. Especially, serum OCT levels and the ratios of OCT : ALT and OCT : AST were markedly increased in HCC. Regarding the comparison between NASH-LC and NASH-HCC, the serum OCT level and the ratio of OCT : AST of NASH-HCC were significantly higher than those of NASH-LC (both, $P < 0.05$). The ratio of OCT : ALT of NASH-HCC tended to be higher than that of NASH-LC ($P < 0.1$), but the ratios of AST/ALT in NASH-LC and NASH-HCC were almost equal. Regarding the relationships between OCT or ratios and platelet counts, there was a significant association between the ratio of OCT : ALT and platelet count ($r = -0.285$, $P < 0.01$). However, the relationships between platelet counts and OCT/AST ratio or OCT were not significant. Further, there were no significant relationships between body mass index and both ratios or OCT. Among all NASH patients, 42 patients had a normal range of ALT, and among these 42 patients, 13 (31%) had OCT over 43 ng/mL (mean ± 1.96 SD in control = 43.2 ng/mL). Of these 13 patients, eight had F3 or F4 fibrosis.

Figure 1 shows the association between liver fibrosis and serum OCT levels, the ratios of OCT : AST and OCT : ALT. The serum OCT levels and the OCT : ALT ratios were increased in parallel with liver fibrosis. In the comparison between F0-2 patients and F3-4 patients, OCT and both ratios in F3-4 patients were significantly higher than those of F0-2 patients (F0-2 patients (mean) OCT, 56.1; OCT : ALT, 1.20; OCT : AST, 1.69; F3-4 patients OCT, 144.8; OCT : ALT, 2.08; OCT : AST, 2.16).

Table 2 Serum levels of liver-specific markers and their ratios in NASH

Disease	OCT (ng/mL)	AST (U/L)	ALT (U/L)	AST : ALT	OCT : AST	OCT : ALT	Plt (×10^4/μL)
Control ($n = 80$)	20.6 ± 12.6	18.4 ± 4.3	16.3 ± 7.0	1.22 ± 0.33	1.09 ± 0.62	1.30 ± 0.87	N.D.
NASH ($n = 93$)	73.1 ± 68.8[a]	41.7 ± 39.6[a]	68.4 ± 99.4[a]	0.77 ± 0.30[a]	1.75 ± 0.86[a]	1.37 ± 1.00	22.4 ± 6.8
NASH-LC ($n = 18$)	82.8 ± 74.3[a]	42.9 ± 20.9[a]	41.2 ± 21.3[a,b]	1.13 ± 0.41[b]	1.84 ± 0.93[a]	2.02 ± 1.15[b]	13.5 ± 6.4[b]
NASH-HCC ($n = 9$)	398.3 ± 583.8[a,b,c]	93.3 ± 90.7[a]	105.8 ± 114.8[a]	1.05 ± 0.38[b]	3.41 ± 1.41[a,b,c]	3.44 ± 1.62[a,b]	12.8 ± 4.3[b]

Data are expressed as mean ± standard deviation (SD). [a]$P < 0.05$ versus control. [b]$P < 0.05$ versus NASH. [c]$P < 0.05$ versus NASH-LC.
ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; NASH, non-alcoholic steatohepatitis; NASH-HCC, non-alcoholic steatohepatitis with hepatocellular carcinoma; NASH-LC, liver cirrhosis induced by NASH; ND, not done; OCT, ornithine carbamoyltransferase; Plt, platelate.

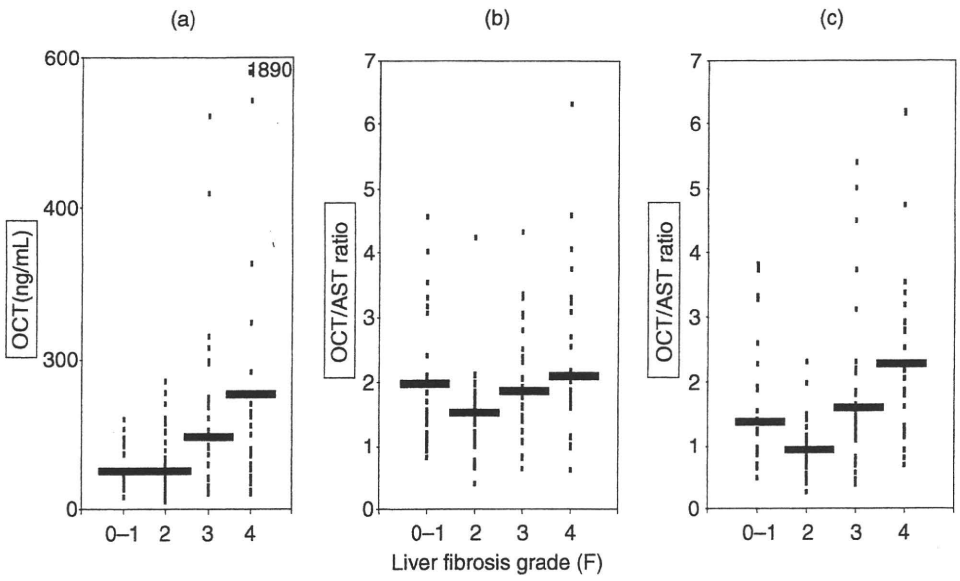

Figure 1 The relationship between liver fibrosis grade (F) and serum ornithine carbamoyltransferase (OCT) levels (a), OCT : alanine aminotransferase (ALT) ratio (b), OCT : aspartate aminotransferase ratio (c). The serum OCT levels and the ratio of OCT : ALT levels were increased in parallel with liver fibrosis. Bars express mean.

DISCUSSION

THIS IS THE first report of the measurement of OCT in NASH patients. An ideal biomarker should be simple, accurate, specific, inexpensive and readily available. OCT is highly liver-specific and a relatively abundant protein. Murayama and Watanabe[8,9] reported that OCT levels tended to be higher in HCC than in LC or CH patients induced by other etiologies, mainly viral hepatitis. It was confirmed that in NASH patients, OCT and the ratios of OCT : ALT and OCT : AST were increased in parallel with disease progression. Without liver biopsy, we can only speculate about the activity and progression of NASH by routine laboratory examinations. As the next step, we need to perform longitudinal analyses of serum OCT levels in NASH. Regarding HCC, the ratios of OCT : ALT and OCT : AST were markedly increased in NASH-HCC, suggesting that the increase of these ratios in HCC was a common phenomenon in various liver diseases. The reason why both ratios were increased in HCC is still unclear. One possibility is that cancer cells, expressing Fas-Ligand, might have induced apoptosis of hepatocytes.[16] Then, in apoptotic cell death, mitochondoria-related proteins were released. Another possibility is that the expression of enzymes might change in HCC. In any event, the ratios of OCT : ALT and OCT : AST were markedly increased in NASH-HCC, and there was significant association between fibrosis grade and OCT : ALT. Therefore, we believe that OCT and the ratios of OCT : ALT and OCT : AST are useful for monitoring the progression of NASH. We need to compare the sensitivity and specificity between these ratios and other tumor markers such as AFP or PIVKA-II.

Aminotransferase levels do not necessarily reflect the activity and progression of NASH. Fracanzani *et al.*[4] reported that more than half of NAFLD patients with persistently normal ALT have a potentially progressive liver disease. In our study, about 30% of NASH patients with a normal range of ALT show the elevation of OCT. In addition, the majority of these patients had severe fibrosis. Hashimoto *et al.*[17] reported that serum ALT levels in severe fibrosis of NASH were decreased. Taken together, in NASH patients with a normal rage of ALT and severe fibrosis, OCT might be a useful marker. It is unclear why OCT is frequently elevated in NASH patients with a normal rage of ALT and severe fibrosis. One possibility is that even if necrosis is minimum in NASH patients with severe fibrosis, mitochondria damage might continue.

The localization of OCT is restricted to mitochondria, which are mainly expressed in cytosol.[13] Some studies have reported that in alcoholic liver disease and NASH, oxidative stress induced endoplasmic reticulum stress and mitochondrial damage.[10,11,18,19] In

our preliminary data, the OCT : AST ratio of NASH was higher than that of viral hepatitis, suggesting that OCT might be a more useful marker in NASH, compared to in viral hepatitis.

As for the relationship between OCT and fibrosis grade, OCT levels and OCT : ALT ratios were increased in parallel with liver fibrosis. The relationship between the ratio of OCT : ALT and liver fibrosis was confirmed by that between the ratio of OCT : ALT and platelet count, which is associated with liver fibrosis grade. Murayama *et al.* reported in a chronic liver damage model that OCT was elevated compared to AST and ALT. The mechanism for this is still unknown. However, the expression of enzymes of parenchymal cells might change during the course of disease progression, as Murayama *et al.* reported that serum OCT levels were influenced by the state of Kupffer cell activation.[20]

AST and ALT do not necessarily reflect the activity and progression of NASH. In contrast, OCT testing is simple, inexpensive, and highly liver-specific, and serum OCT levels and the ratios of OCT : ALT and OCT : AST were elevated in concert with the progression of NASH. Thus, it was concluded that OCT might be a useful marker for revealing the progression of NASH.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Influence of adiponectin gene polymorphisms in Japanese patients with non-alcoholic fatty liver disease

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Abstract
Purpose Single nucleotide polymorphisms (SNPs) of the adiponectin gene have been reported to be associated with insulin resistance and the prevalence of type-2 diabetes. We investigated the SNPs of adiponectin in non-alcoholic fatty liver disease (NAFLD) patients.
Methods One hundred nineteen patients histologically diagnosed as having NAFLD and 115 control subjects were examined. Adiponectin SNP sites were investigated at +45 of exon 2 and at +276 of intron 2; these sites have been thought to be associated with diabetes or insulin resistance.
Results Regarding the +276 SNP, the frequency of G/G tended to be higher in NAFLD patients than in controls, but not significantly. Among females only, however, the G/G frequency was significantly higher in NAFLD patients. As for the +45 SNP, in the severe fibrosis group, the frequency of G/G homozygotes was significantly higher than that in the mild fibrosis group, and G/G homozygotes of the +45 SNP proved by multivariate analysis to be an independent factor in severe fibrosis. In NAFLD patients with adiponectin +45 G/G, homeostasis model assessment-insulin resistance was significantly higher than in NAFLD patients without adiponectin +45 G/G.
Conclusion Adiponectin SNPs were found to be associated with the progression of liver fibrosis and insulin resistance, suggesting that adiponectin SNPs might play roles in the occurrence and progression of NAFLD.

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Keywords NAFLD · Adiponectin · SNP · Insulin resistance

Introduction

Non-alcoholic fatty liver disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function tests. Its spectrum ranges from simple steatosis, which is usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis [1, 2]. Patients with NAFLD usually have insulin resistance syndrome, but Stefan et al. reported that hyperinsulinemia alone is not a major driving force for fatty liver [3, 4]. NASH's etiology remains unclear. Most investigators agree that its development requires underlying steatosis followed by a "second hit" that induces inflammation, fibrosis, or necrosis [2]. The interaction of cytokines with oxidative stress and lipid peroxidation has been postulated to play a key role in NASH [2, 5, 6].

Adiponectin is one of the adipo-cytokines associated with insulin resistance and type-2 diabetes [7, 8]. Several papers have reported a significant decrease in the serum levels of adiponectin in NASH patients [9, 10]. And the expression of adiponectin was lower in NASH liver than in those with simple steatosis [11]. Adiponectin is associated with liver fibrosis and inflammation [12, 13], suggesting that it might be associated with the pathogenesis of NASH.

Cytokine production rates vary among individuals [14]. Some of these differences may be related to polymorphisms in the cytokine genes themselves, or to polymorphisms in genes that regulate cytokine gene transcription. We have recently reported the relationships between tumor necrosis factor (TNF) gene polymorphisms and the progression to NASH [15]. Single nucleotide polymorphisms

(SNPs) of the adiponectin gene at +45 of exon 2 and +276 of intron 2 have been reported to be associated with insulin resistance and the prevalence of type-2 diabetes [16]. Other SNP sites of the adiponectin gene were reported [17, 18]. But many papers reported that adiponectin SNPs at +45 of exon 2 and at +276 of intron 2 were associated with diabetes, insulin resistance, and atherosclerosis, especially in East Asia [16, 19–21]. We therefore investigated these SNPs of the adiponectin gene, comparing them with the respective clinical and pathological findings.

Patients and methods

We evaluated 119 Japanese patients who were histologically diagnosed as having NAFLD at Tokyo Women's Medical University between 1995 and 2006, along with 115 healthy subjects who served as controls. The diagnosis of NAFLD was established based on the following criteria: (1) Histologically macrovesicular steatosis affecting at least 10% of hepatocytes. NAFLD activity score (NAS) was used to classify NAFLD into simple steatosis (NAS 0–2) or borderline (NAS 3–4) and steatohepatitis (NASH) (NAS $\geq$ 5) [22]. (2) Intake of less than 20 g of ethanol per day, as confirmed by physicians and family members with the patients. (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, and metabolic liver diseases.

Body mass index (BMI) was calculated by the standard formula: weight(kg)/[height(m)^2]. The diagnosis of type II diabetes mellitus (DM) was based on WHO criteria. Homeostasis model assessment-insulin resistance (HOMA-IR; fasting serum insulin μU/ml × fasting glucose mg/dl/405) was measured in NAFLD patients without DM [23]. None of the patients received drug treatment for NAFLD before the liver biopsy. A complete history was obtained, and physical examinations were performed in all patients. All patients underwent liver tests for measurement of the following laboratory parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, hepatitis B serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen), hepatitis C serology (antibody to hepatitis C virus and hepatitis C RNA polymerase chain reaction), and autoantibodies [antinuclear antibody (ANA), antismooth muscle antibody, and antimitochondrial antibody].

All liver biopsy specimens were examined using hematoxylin–eosin, Mallory, and silver reticulin staining. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis with focal or extensive portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis [2, 24, 25]. Fibrosis was divided into mild (F0–2) and severe (F3–4) groups.

All control subjects were Japanese and matched for age and gender with NAFLD patients. Basically, control subjects consisted of hospital staff and medical students. To match age and gender to NAFLD patients, we recruited older volunteers from acquired relatives of hospital staff and patients. To exclude the possibility of latent fatty liver, control subjects with alcoholism or obesity (BMI < 25) were excluded. All control subjects were confirmed by a blood test to have normal liver function and no viral hepatitis infection. Informed consent was obtained from all patients and healthy controls before their entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's research committee.

Genomic DNAs were obtained from peripheral blood leukocytes by standard phenol–chloroform extraction and amplified by the PCR method. Screening for adiponectin polymorphisms was determined with a fluorescent allele-specific DNA primer assay system, as described previously [26]. Adiponectin SNP sites were investigated at +45 of exon 2 and +276 of intron 2; both sites have been associated with diabetes or metabolic diseases [16].

Serum adiponectin levels were measured using enzyme-linked immunosorbent assays (human adiponectin ELISA kit, Otuka, Tokyo, Japan).

Statistical analysis

Analyses were performed with Dr SPSS (SPSS Institute, Tokyo, Japan). Data are presented as mean ± standard deviation (SD) or frequencies. Student's *t* test was used to compare the two groups with respect to normally distributed continuous variables, and the Mann–Whitney *U* test was used for skewed continuous variables. Normality was evaluated by the Shapiro–Wilks test. To evaluate the genotype between mild fibrosis (F0–2) and severe fibrosis (F3–4), univariable and multivariable logistic regression models were used. In subgroup analysis, the heterogeneity of the genotype prevalence was also evaluated in the model. Influences of profile, linearity, interaction, and collinearity in multivariate generalized linear models were examined using regression diagnostic analysis. Two-tailed *p* values less than 0.05 were considered to indicate a statistically significant difference.

Results

Table 1 shows the clinical data for NAFLD patients. There is no difference in age or gender between NAFLD patients and control subjects.

Table 2A shows data on the +276 SNP. The frequency of G/G tended to be higher in NAFLD patients (56.8%) than in the controls (51.3%), but the difference was not significant. In NASH patients (NAS ≥ 5) only, the G/G frequency was 56.8%, and there was no difference between NASH and other types of NAFLD (simple steatosis and borderline). Regarding the +45 SNP, the frequency of T/T tended to be lower (45.4%) in NAFLD patients than in the controls (51.3%), but the difference was not significant (Table 2B). In NASH patients only, the frequency of T/T homozygotes (47.7%) was not significantly different than in other types of NAFLD. BMI differs between NAFLD patients and control subjects. To exclude the influence of BMI, we compared non-obese control subjects and non-obese NAFLD patients (BMI < 25). In the 31 NAFLD patients without obesity, the frequency of G/G at +276 was 61.3% and the frequency of T/T at +45 was 48%. The tendency is almost the same, suggesting that BMI did not influence genotype data.

Table 1 Patient profiles

	NAFLD	Control
No.	119	115
Age (mean ± SD)	50.3 ± 17.8	47.5 ± 18.7
Gender (M/F)	65/54	58/57
Obesity (BMI > 25)	88 (73.9%)	0 (0%)
DM	53 (44.5%)	2 (1.7%)
HL	75 (63.0%)	12 (10.4%)
HT	29 (24.4%)	10 (8.7%)
BMI (kg/m^2)	28.3 ± 5.2	21.9 ± 2.0
AST	55.1 ± 31.7	18.5 ± 4.2
ALT	79.9 ± 58.9	16.2 ± 7.0
Plt	23.9 ± 28.8	ND
NASH (NAS ≥ 5)	88 (73.4%)	
F0–2	72 (60.5%)	
F3–4	47 (39.4%)	

ND not done

We compared the frequency of SNPs between females and males (Table 3A, B). Regarding the +276 SNP, in females only the frequency of the G allele (77.8%) was significantly higher than in female controls (64.9%). In contrast, in males only the frequency of the G allele did not differ. Regarding the +45 SNP, in females the frequency of the G allele (34.3%) tended to be higher than that of female controls (24.6%). In contrast, in males only, the frequency of the G allele was almost the same.

Tables 4A and B show the association between liver fibrosis and adiponectin SNPs. Regarding the +276 SNP, the G/G frequency of the severe-fibrosis group was 66.0%, indicating a higher frequency with fibrosis severity. Regarding the +45 SNP, the frequency of the G allele was significantly higher in the severe fibrosis group than in the mild fibrosis group (41.5 vs 27.1%, respectively; $p = 0.02$). Also, in the severe fibrosis group the frequency of G/G homozygotes (23.4%) was significantly higher than in

Table 2 Results of adiponectin gene SNPs

	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
Control (n = 115)	59	47	9	165/230	1		
	51.3%	40.9%	7.8%	71.7%			
NAFLD (n = 118)	67	47	4	181/236	1.30	0.85–1.97	NS
	56.8%	39.6%	3.4%	76.7%			
NASH (n = 88)	50	35	3	135/176	1.30	0.83–2.04	NS
	56.8%	39.8%	3.4%	76.7%			
	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	T/G	G/G				
(B) +45 T/G SNP of exon 2							
Control (n = 115)	59	44	12	66/230	1		
	51.3%	38.2%	10.4%	28.7%			
NAFLD (n = 119)	54	52	13	78/238	1.21	0.82–1.80	NS
	45.4%	43.7%	10.9%	32.8%			
NASH (n = 88)	42	36	10	56/176	1.16	0.76–1.78	NS
	47.7%	40.9%	11.4%	31.8%			

OR odds ratio, *CI* confidence interval, *NS* not significant

Table 3 Results of adiponectin gene SNPs in female and male patients

	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
Male control ($n = 58$)	36	19	3	91/116	1		
	62.0%	32.8%	5%	78.4%			
Male NAFLD ($n = 64$)	34	29	1	97/114	0.86	0.47–1.57	NS
	53.1%	45.3%	1.6%	75.8%			
Female control ($n = 57$)	23	28	6	74/114	1		
	40.4%	49.1%	10.5%	64.9%			
Female NAFLD ($n = 54$)	33[a]	18	3	84/108	1.89	1.04–3.43	0.03
	61.1%	33.3%	5.5%	77.8%			
	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	G/T	G/G				
(B) +45 T/G SNP of exon 2							
Male control ($n = 58$)	27	22	9	40/116			
	46.6%	37.9%	15.5%	34.5%	1		
Male NAFLD ($n = 65$)	31	27	7	41/130			
	47.7%	41.5%	10.8%	31.5%	0.88	0.51–1.49	NS
Female control ($n = 57$)	32	22	3	28/114	1		
	56.1%	38.6%	5.3%	24.6%			
Female NAFLD ($n = 54$)	23	25	6	37/108	1.60	0.89–2.87	NS
	42.6%	46.3%	11.1%	34.3%			

OR odds ratio, *CI* confidence interval, *NS* not significant

[a] The frequency of G/G homozygotes; female NAFLD vs female control, $p = 0.029$ by χ^2 test

the mild fibrosis group (2.8%) ($p < 0.01$). In addition, we compared the mild and severe fibrosis groups by multivariate analysis (Table 5). Gender, age, BMI, HOMA-IR, serum adiponectin level, +45 SNP, +276 SNP, DM, AST, ALT, hypertension, platelet, triglyceride, and total cholesterol were analyzed. In this multivariate analysis, G/G homozygotes of the +45 SNP proved to be an independent factor of severe fibrosis.

We next investigated the influence of SNPs on insulin resistance, as shown in Tables 6A and B. We measured HOMA-IR as an indicator of insulin resistance in non-diabetes patients, because in diabetes patients HOMA-IR does not represent correct insulin resistance. HOMA-IR tended to be higher in NAFLD patients with adiponectin +276 G/G than in those without it, but the difference was not significant (Table 6A). The frequency of HOMA-IR was significantly higher in NAFLD patients with adiponectin +45 G/G than in those without it (Table 6B; G/G, 5.67 ± 2.73; G/T or T/T, 3.40 ± 2.26, $p = 0.02$). Also, in patients with adiponectin +45 G/G, the frequency of HOMA > 4 was significantly increased by χ^2 test (frequency of HOMA > 4: 75% in G/G, 26% in G/T or T/T; $p < 0.01$).

We measured adiponectin levels of peripheral blood in 83 NAFLD patients. Tables 7A and B show the association between adiponectin SNPs and serum adiponectin levels. The level in NAFLD patients with both +45 G/G and BMI ≥ 25 tended to be lower than in patients with other genotypes, but we did not detect a significant difference between genotypes and serum adiponectin levels.

Among NAFLD patients, there was no significant association between genotypes and DM or obesity.

Regarding haplotypes, the frequencies of +45 G/G and +276 G/G were investigated. The frequencies of +45 G/G and +276 G/G (11.0%) were not particularly different from those of control subjects (10.4%). The frequencies of +45 G/G and +276 G/G in severe fibrosis (23.4%) are significantly higher than those in patients with mild fibrosis (2.8%).

Discussion

The present results suggested that adiponectin SNP is associated with the progression of liver fibrosis and insulin resistance.

Table 4 The association between liver fibrosis and adiponectin SNPs

Fibrosis grade	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
F0–2 ($n = 71$)	36	32	3	104/142	1		
	50.7%	45.1%	4.2%	73.2%			
F3–4 ($n = 47$)	31	15	1	77/94	1.65	0.87–3.15	NS
	66.0%	31.9%	2.1%	81.9%			
Fibrosis grade	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	T/G	G/G				
(B) +45 T/G SNP of exon 2							
F0–2 ($n = 72$)	35	35	2	39/144	1		
	48.6%	48.6%	2.8%	27.1%			
F3–4 ($n = 47$)	19	17	11[a]	39/94	1.91	1.10–3.31	0.02
	40.4%	36.2%	23.4%	41.5%			

OR odds ratio, *CI* confidence interval, *NS* not significant

[a] The frequency of G/G homozygotes; F3–4 vs F0–2, $p < 0.01$ by χ^2 test

Table 5 Multivariate analysis of clinical data and two adiponectin gene polymorphisms between mild fibrosis group and severe fibrosis group

	OR	95% CI	*p* value
Adiponectin +45 SNP (G/G)	71.7	3.67–1399	0.005
Age	1.16	1.07–1.26	0.001
Gender (male 0, female 1)	0.027	0.003–0.26	0.002
DM	8.12	1.49–44.2	0.015
BMI	1.28	1.06–1.55	0.011

Factors: age, gender, BMI, DM, hypertension, HOMA-IR, AST, ALT, platelets, triglyceride, total cholesterol, adiponectin +45 SNP(G/G), adiponectin +276 SNP(G/G), serum adiponectin level

Table 6 Association between HOMA-IR and adiponectin gene SNPs in patients without DM

Genotype	No.	HOMA-IR (mean ± SD)	*p* value
(A) +276 T/G SNP of intron 2			
G/G	28	4.06 ± 2.45	
G/T or T/T	20	3.25 ± 2.38	NS
(B) +45 T/G SNP of exon 2			
G/G	8	5.67 ± 2.73	
G/T or T/T	48	3.40 ± 2.26	0.02

NS not significant by Mann–Whitney test

Adiponectin is associated with insulin resistance and is an important factor in the pathogenesis of DM [7, 8]. In addition, adiponectin levels predict the severity of liver disease in NAFLD, even in the absence of diabetes and obesity [27, 28]. Hara et al. [16] have reported that in

Table 7 The association between serum adiponectin levels and adiponectin SNPs

Genotype	BMI > 25			BMI < 25		
	No.	Adiponectin	*p* value	No.	Adiponectin	*p* value
(A) +276 T/G SNP of intron 2						
G/G	34	6.89 ± 3.49		13	6.54 ± 4.17	
G/T	22	6.80 ± 3.10	NS	10	8.84 ± 5.12	NS
T/T	2	9.15 ± 1.48	NS	2	7.15 ± 2.76	NS
(B) +45 T/G SNP of exon 2						
G/G	7	5.70 ± 2.41		0		
G/T	25	6.72 ± 3.17	NS	11	6.64 ± 3.7	NS
T/T	27	7.53 ± 3.53	NS	13	8.15 ± 4.98	NS

Adiponectin was expressed as mean ± standard deviation (SD)

NS not significant

diabetes patients, the frequency of G/G at the +276 SNP is significantly higher (DM 58.3%, control 49.2%), and the frequency of T/T at the +45 SNP is significantly lower than in controls (DM 42.7%, control 52.3%). Almost the same percentages were observed in all NAFLD patients, but we did not observe a significant difference. To determine whether or not the difference between NAFLD and controls was significant, many more patients would be needed. In addition, this study did have a limitation with regard to the control subjects. Although none of the control subjects had abnormal liver function and none were obese, and although abdominal ultrasound examinations were performed in approximately 70% of the controls and no clear fatty liver disease was apparent, we cannot

completely rule out the possibility that the control group included those with mild steatosis, because we did not perform liver biopsies on the control subjects.

Sample size is a limitation of this study. However, we performed a liver biopsy in all NAFLD patients. Romeo et al. [29] performed an SNP study of many NAFLD patients but did not perform liver biopsies. On this point, our study provides important information.

We have already reported that significantly more male NAFLD patients overate and got less exercise than female patients, based on a questionnaire survey [30]. The frequencies of the adiponectin SNP sites differed significantly between NAFLD patients and controls only among females. Considering the survey data, it was speculated that genomic background might have more influence in female NAFLD and that lifestyle might have more influence in male NAFLD. Other papers have reported associations between adiponectin SNPs and insulin resistance, especially in women [31, 32]. Although only females showed significant differences, the numbers of female NAFLD and control subjects were small. In other reports, G/G frequencies at +276 among healthy women were 37.1–48.8% [31, 33], demonstrating that our female control data are reliable. When we compare the G/G frequency at +276 among female NAFLD patients to data on female control subjects in other papers, we find p values of 0.02–0.06. Therefore, these data suggest that our hypothesis is reasonable.

Recently, Musso et al. [34] reported that adiponectin SNPs at +45 and +276 modulate the acute adiponectin response to dietary fat and are associated with the presence of NAFLD in an Italian population. In addition, +45 TT and +276 GT/TT carriers had significantly increased prevalence and severity in NAFLD than in the other genotypes. Their results are not consistent with our own. But in Japan and other countries, many papers have reported that +276 GG and +45 GG are associated with low adiponectin, diabetes, insulin resistance, metabolic syndrome, and coronary artery diseases [19–21, 31, 33, 35]. This difference might be explained by ethnic differences. Even in NAFLD, the ethnic differences are well documented [36]. Differences similar to those found in the SNP study have frequently been observed. Regarding the TNF promoter SNPs in NASH, different results between Japanese and Italian populations have been reported [15, 37]. Adiponectin has been reported to be associated with insulin resistance and liver fibrosis [7, 12, 13]. In the present study, significant associations were found between adiponectin +45 SNP G/G and insulin resistance or liver fibrosis. Some papers have reported that insulin resistance is important for the pathogenesis of NASH [2, 3], but the relationship between insulin resistance and NAFLD has been controversial [4]. In our patients, about 16% did not have insulin resistance (HOMA < 2), suggesting that the etiologies of NAFLD were formed from several factors. Therefore, the difference in adiponectin SNPs between NAFLD and controls might not be so large. Anyway, our data indicate that adiponectin +45 SNP might play an important role in the pathogenesis and progression of NAFLD. Shimada et al. [38] reported that age and DM were proven to be risk factors for severe fibrosis. In the present study, it was important that adiponectin +45 SNP was added as an independent factor of severe fibrosis in NAFLD.

The serum adiponectin levels in NAFLD patients with both +45 G/G and BMI ≥25 tended to be lower than in those with other genotypes, but we did not detect a significant difference. Basically, adiponectin production was influenced by the volume of visceral fat. To clarify this association, we must measure serum adiponectin levels in patients who have almost equal BMI. However, it is difficult to compare subjects who have the same BMI. Another possibility is that adiponectin gene SNPs might link with the expression of other genes except adiponectin genes.

Recently, it was reported that adiponectin receptor SNPs might affect insulin sensitivity and liver fat [39]. The SNPs of both adiponectin and adiponectin receptor might play important roles. Finally, we hope that genomic analysis, including that of adiponectin genes, as well as lifestyle surveys will clarify the pathogenesis and progression of NAFLD, leading to therapy.

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Original Article

Treatment of nonalcoholic steatohepatitis with colestimide

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Aim: To clarify the usefulness of colestimide in patients with nonalcoholic steatohepatitis (NASH) with hyperlipidemia.

Methods: In an open-label randomized controlled trial, 17 NASH patients with hyperlipidemia received colestimide (3 g/day) for 24 weeks. There were 21 control patients. All patients received lifestyle modification therapy. Efficacy was assessed based on metabolic profile, insulin resistance, transaminases, serum hepatic fibrosis markers, adipokine levels, visceral fat on computed tomography (CT), and the fatty liver grade on CT. NASH patients with moderate to severe steatosis by histology were also evaluated separately.

Results: Baseline clinical characteristics of the two groups were similar. Both groups experienced a significant decrease of BMI with no difference between them. However, visceral fat decreased significantly more in the colestimide group ($P = 0.046$). Aspartate aminotransferase (AST) showed a significantly greater decrease in the colestimide group compared with the control group ($P = 0.042$). In patients with moderate to severe histological steatosis, there were significant differences between the two groups regard to HbA_{1c}, transaminases, and hyaluronic acid ($P = 0.018$ for HbA_{1c}, $P = 0.003$ for AST, $P = 0.042$ for alanine aminotransferase, and $P = 0.042$ for hyaluronic acid). Steatosis significantly improved in patients in the colestimide group who had fatty liver on CT ($P = 0.049$). In the colestimide group, abdominal distension and/or constipation were seen, but mostly tolerable, no other clinical or laboratory adverse events associated with the use of this medicine were not observed.

Conclusions: Colestimide seems to increase the efficacy of lifestyle modification in NASH patients with hyperlipidemia. Its beneficial effects were more prominent in NASH patients with moderate to severe histological steatosis.

Key words: hyperlipidemia, lipid-lowering agents, nonalcoholic steatohepatitis

INTRODUCTION

RECENTLY, OBESITY AND lifestyle-related diseases such as type 2 diabetes mellitus (DM), hypertension, and dyslipidemia have become a leading public health problem because of their dramatic increase.[1,2] It is now established that nonalcoholic fatty liver diseases (NAFLD) are the hepatic manifestation of the metabolic syndrome. In Japan, 10–30% of adults are currently diagnosed as having NAFLD at annual health checks,[3,4] and nonalcoholic steatohepatitis (NASH) is estimated to affect 1–3% of Japanese adults. Accordingly, NASH will eventually become the most important cause of end-stage liver disease.

The pathogenesis of NASH is not clearly understood. However, development of NASH has been suggested to occur by the "two-hit" mechanism, with fatty liver being the first hit and subsequent hepatocellular injury being the second hit.[5] Insulin resistance may be the most important factor in the development of fatty liver (first hit). Insulin resistance, excess intracellular fatty acids, imbalance of cytokine production, oxidative stress, lipid peroxidization, iron overload, depletion of adenosine triphosphate, and mitochondrial dysfunction may then induce hepatocellular injury (second hit) in persons with fatty liver. Accordingly, the pathogenesis of NASH is multifactorial. Treatment has focused on the modification of risk factors such as weight reduction and on pathogenesis-oriented pharmacotherapy, including insulin sensitizers, lipid-lowering agents, and antioxidants, and hepatoprotectants. The significant association of NASH with insulin resistance is the rationale for treating these patients with insulin sensitizers and promising results have been obtained, but insulin sensitizers cannot be used in patients without type 2 DM in Japan.[6–8]

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In this study, we focused on NASH patients with hyperlipidemia. Several small-scale trials have already explored the potential value of statins, fibrates, and probucol for NASH.[9–13] These trials demonstrated the safety of the drugs and some efficacy, but the hepatotoxic potential of these agents remains controversial.[14] Accordingly, we investigated the use of colestimide for NASH patients with hyperlipidemia.

Colestimide is an anion-exchange resin with a unique mode of action.[15–17] Chloride ions in the resin are displaced by negatively charged bile acids within the lumen of the intestinal tract. As a result, resorption of bile acids decreases and fecal excretion increases, retarding enterohepatic circulation and causing a decrease of bile acids. In response to the low bile acid level, the liver begins to convert cholesterol to bile acids, thereby reducing the cholesterol level. The decrease of cholesterol in turn causes an increase in hepatic expression of low-density lipoprotein cholesterol (LDL) receptors, which leads to increased uptake of LDL from the blood. The final result is a decrease of the blood LDL cholesterol level. Thus, colestimide lowers cholesterol without being absorbed in the body, which is the most important benefit of this drug. Colestimide has been available in Japan since 1999 as a lipid-lowering drug. The most frequent side effects of colestimide are gastrointestinal symptoms, such as abdominal distention due to increase of gas and constipation, but no serious side effects have been reported, because the resin is not absorbed.[15–17] For the same reason, the occurrence of liver dysfunction is very uncommon compared with statins, fibrates, and probucol.[18] We performed an open-label randomized controlled trial that compared colestimide plus lifestyle modification with lifestyle modification alone.

According to our data, 66% of the NASH patients were obese, 46% had type 2 DM, 57% had hyperlipidemia and 34% had hypertension.[19] Interestingly, the prevalence of type 2 DM showed a positive correlation with the stage of fibrosis. In contrast, the prevalence of hyperlipidemia showed a positive correlation with the grade of steatosis. Thus, we not only investigated NASH patients, in general, but also focused on NASH patients with moderate to severe histological steatosis.

METHODS

THIS WAS AN open-label randomized controlled trial that compared the efficacy and safety of colestimide plus lifestyle modification versus lifestyle modification alone in NASH patients with hyperlipidemia. Between June 2004 and January 2006, a total of 40 patients with liver biopsy-proven NASH and hyperlipidemia were entered into this randomized controlled trial. The patients were recruited from among those referred to our hospital. This study was conducted in compliance with the Declaration of Helsinki and was approved by the ethics committee of our hospital. All patients gave written informed consent to participation.

Subjects

The inclusion criteria were as follows:

1. Over 18 years of age.
2. NASH proven by liver biopsy within 6 months before this trial. A diagnosis of NASH was based on the following criteria: (i) detection of steatohepatitis on liver biopsy, (ii) intake of less than 100 g of ethanol per week (as confirmed by the attending physician and family members in close contact with the patient), and (iii) 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 such as Wilson's disease and hemochromatosis).[20–22] All patients were negative for hepatitis B surface antigen and for antibody to hepatitis C virus and/or hepatitis C RNA by the polymerase chain reaction. The liver biopsy specimens of the patients were examined to determine the stage of fibrosis and the severity of steatosis. The NAFLD activity score (NAS) was calculated and patients with a NAS ≥5 were diagnosed as having NASH.[23]
3. Hyperlipidemia, which was diagnosed from elevation of total cholesterol (>220 mg/dL) and/or LDL (>140 mg/dL) and/or triglycerides (>150 mg/dL) on at least 3 occasions.

Exclusion criteria consisted of:

1. Treatment with lipid-lowering medications during the 6 months prior to the study.
2. Liver failure; hepatic coma, diuretic-resistant ascites, or bacterial peritonitis.
3. Hepatocellular carcinoma.
4. Any life-threatening disease.
5. Pregnancy or breast-feeding.
6. Treatment with any drugs that have been associated with steatohepatitis (e.g. corticosteroids, high-dose estrogens, methotrexate, or amiodarone).
7. Hormone replacement therapy for menopause.
8. Treatment with other drugs for NASH (including vitamin E, pioglitazone, and ursodeoxycholic acid) within 6 months from the entry of this study.

Study design

All patients of our hospital were randomly assigned patient identification (ID) numbers by persons unrelated to this study. Then according to this patient ID, the patients with even numbers received treatment with colestimide plus lifestyle modification (colestimide group) and patients with odd numbers received lifestyle modification alone (control group). Colestimide was administered at a dose of 3 g/day.

All patients received lifestyle modification therapy, with advice about diet and exercise from their doctors. Each patient also saw a dietitian before the study and every 2 months during the study to monitor their cholesterol intake. Overweight or obese patients were instructed to reduce their calorie intake to the theoretical ideal, which was calculated as ideal body mass index (BMI) × 25–30 kcal per day, in order to attain a healthy weight. Obesity was defined as a BMI of more than 25 according to the Japanese Obesity Association criteria. Diagnosis of type 2 DM was based on the following Japanese criteria: random blood glucose >200 mg/dL or fasting glucose >126 mg/dL or hemoglobin (Hb) A1c >6.5% on 2 occasions, or current treatment for DM. Hypertension was diagnosed if the patient was receiving antihypertensive therapy or had a blood pressure of more than 140/90 mmHg on at least 3 occasions.

Patients were evaluated at least every 8 weeks during the trial.

A complete history was obtained, physical examination was performed (including measurement of body weight), and the following laboratory paremeters were determined: lipid profile (triglycerides, high-density lipoprotein cholesterol [HDL], LDL), fasting plasma glucose, immunoreactive insulin, homeostasis model of insulin resistance (HOMA-IR), HbA1c, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (gGTP), hyaluronic acid, and platelet count. High sensitive-C reactive protein (hs-CRP) and iron parameters were assessed at the start and end of the study. Serum adipokine levels (tumor necrosis factor-α [TNF-α], transforming growth factor-β [TGF-β], interleukin-6 [IL-6], resistin, leptin, adiponectin, and high-molecular-weight adiponectin [HMW-adiponectin]) were retrospectively measured in 15 patients from the colestimide group and 11 patients from the control group.

Ultrasonography and computed tomography (CT) were performed within 6 months before this trial and at the end of the trial. CT was done with a multi-detector row helical scanner. On non-enhanced scans, visceral fat and the liver/spleen attenuation ratio were measured. The presence of steatosis was indicated by a ratio of less than 0.9.[24]

Patients recorded their intake of colestimide for assessment of compliance. Patients were also asked to report any new drugs they were prescribed by other doctors.

A second liver biopsy was performed at the end of the study in patients who agreed to this procedure.

Efficacy was assessed by determining the change from baseline to 24 weeks of the metabolic profile, insulin resistance, transaminases, serum hepatic fibrosis makers, adipokine levels, and visceral fat and the fatty liver grade on CT.

We also evaluated the subgroup of NASH patients with histological evidence of moderate to severe steatosis after patients were stratified for the extent of steatosis on liver biopsy.

Statistical analysis

Analysis was performed with the SPSS statistical package (SPSS, Chicago, IL, USA). The χ^2-test was used for comparison of prevalences. Changes within each group during the study were evaluated by the paired *t*-test (for BMI, visceral fat, the fatty liver grade on CT, triglycerides, LDL, HDL, HOMA-IR, HbA_{1c}, AST, ALT, gGTP, and hyarulonic acid). Between-group comparison of the changes of all parameters from baseline to 24 weeks was done with the Mann–Whitney U-test. Pearson's correlation coefficients were calculated to assess the strength of associations.

RESULTS

NINETEEN PATIENTS WERE randomized to the colestimide group and 21 patients to the control group. However, two patients dropped out of the colestimide group because they stopped treatment due to side effects (abdominal distension associated with increased intestinal gas and constipation) after 4 and 6 weeks, respectively, and stopped attending our hospital at 6 and 10 weeks after starting the study. These 2 patients were included in the evaluation of safety, but not in the evaluation of efficacy. During this trial, no patient developed cancer or cardiovascular events. There were 17 patients in the colestimide group with a median age of 46 years (11 men and 6 women) and 21 patients in the control group with a median age of 46 years (13 men and 8 women). The two groups were similar with respect to their baseline clinical and biochemical char-

acteristics (Table 1). Moderate to severe steatosis was detected in 16 patients from the colestimide group and 18 patients from the control group. Table 1 also shows a comparison of the two subgroups of NASH patients with moderate to severe steatosis. Both subgroups were similar with respect to their baseline clinical and biochemical characteristics.

Metabolic response

During the study period, BMI decreased significantly in both groups ($P = 0.006$ in the colestimide group and $P = 0.005$ in the control group). No difference of BMI was observed between the two groups (Table 2). These changes were also seen in the subgroups of NASH patients with moderate to severe steatosis (Table 3).

Visceral fat also showed a statistically significant decrease in both groups at the end of the study ($P = 0.001$ in the colestimide group and 0.003 in the control group). Comparing the two groups, the colestimide group showed a significantly greater decrease of visceral fat ($P = 0.046$, Table 2).

The colestimide group also showed significant decrease of LDL compared with the control group ($P = 0.004$, Table 2).

In both groups in all NASH patients, fasting plasma glucose, immunoreactive insulin, and HOMA-IR were unchanged. However, the colestimide group showed a significant decrease of HbA1c at the end of the study ($P = 0.048$). The difference of HbA1c between the colestimide and control groups was not significant (Table 2). Among patients with moderate to severe steatosis, however, the difference of HbA_{1c} between the colestimide and control groups was statistically significant ($P = 0.018$, Table 3).

Transaminases, gGTP and hyaluronic acid

Colestimide treatment for 24 weeks was associated with a gradual decrease of serum transaminase levels (Fig. 1). AST levels decreased significantly in both groups in all NASH patients ($P = 0.004$ in the colestimide group and $P = 0.035$ in the control group), while ALT and gGTP level only decreased in the colestimide group ($P = 0.002$ for ALT and $P = 0.041$ for gGTP). Only the decrease of AST showed a significant difference between the colestimide and control groups ($P = 0.042$, Table 2). Linear regression analysis showed a significant association between the decreases of BMI and transaminases in the control group ($r = 0.488$; $P = 0.025$ for AST; $r = 0.785$; $P < 0.001$ for ALT), but these associations were not found in the colestimide group.

The hyaluronic acid level was only decreased significantly in the colestimide group ($P = 0.028$), however there was no significant differences between the colestimde and control groups in all NASH patients (Table 2). Among the patients with moderate to severe steatosis, there were statistically significant differences of AST, ALT, and hyaluronic acid between the colestimide and control groups (Table 3; $P = 0.003$ for AST; $P = 0.042$ for ALT; $P = 0.042$ for hyaluronic acid).

Other biochemical markers

At the end of the study, hs-CRP, iron parameters, and serum adipokine levels showed no significant differences between the groups.

Hepatic steatosis measured by CT

Concerning hepatic steatosis assessed from the liver/spleen attenuation ratio on CT scans, 10 patients from the colestimide group and 17 patients from the control group were diagnosed as fatty liver. Among them, only patients in the colestimide group showed a significant improvement of the liver/spleen attenuation ratio (from 0.79 ± 0.06 to 0.87 ± 0.1, $P = 0.049$). However, the difference between the colestimide and control groups was not significant.

Liver histology

A second liver biopsy was only performed in two patients (one from the colestimide group and the other from the control group). In these two patients, there was a similar histological severity of fibrosis compared with that at the start of the study, but steatosis was improved in the patient from the colestimide group. The NAS decreased from 7 to 4 in the colestimide group and from 7 to 6 in the control group.

Compliance and adverse effects

The compliance rate with the study medication was 89% (17/19). Two patients who were randomized to the colestimide group withdrew from the study due to mild abdominal distension and a mild increase of intestinal gas. These symptoms resolved within a few days after they stopped taking colestimide, but they stopped attending our hospital, subsequently. In the colestimide group, 29% of the patients noted abdominal distension and/or constipation, but these side effects were mostly tolerable. Other clinical or laboratory adverse events associated with the use of colestimide were not observed.

Table 1 Baseline clinical characteristics of the study population

Variable	All NASH patients $n = 38$			NASH patients with moderate to severe steatosis $n = 34$		
	Colestimide $n = 17$	Control $n = 21$	‡	Colestimide $n = 16$	Control $n = 18$	‡
Male†	65%	62%	NS	69%	61%	NS
Age (years)	46 ± 22.45	47 ± 26.89	NS	47 ± 21.24	46 ± 25.75	NS
BMI (kg/m^2)	25.90 ± 3.97	26.68 ± 4.28	NS	26.04 ± 4.06	27.00 ± 4.42	NS
Hypertension†	24%	10%	NS	25%	11%	NS
Type 2 Diabetes mellitus†	12%	33%	NS	13%	33%	NS
Visceral fat area (cm^2)	161.65 ± 42.83	187.90 ± 60.70	NS	162.06 ± 44.20	196.17 ± 60.77	NS
Fatty liver on CT†	59%	81%	NS	63%	83%	NS
Histological moderate to severe steatosis†	94%	86%	NS	100%	100%	NS
Histological moderate to severe fibrosis†	6%	9%	NS	6%	6%	NS
Triglycerides (mg/dL)	263.88 ± 345.75	210.90 ± 117.58	NS	262.13 ± 357.01	209.72 ± 112.64	NS
LDL (mg/dL)	161.04 ± 34.32	148.63 ± 23.11	NS	163.20 ± 34.38	151.08 ± 23.33	NS
HDL (mg/dL)	50.59 ± 19.32	49.48 ± 14.14	NS	49.69 ± 19.58	46.56 ± 10.57	NS
HOMA-IR	3.13 ± 1.86	4.48 ± 6.03	NS	3.23 ± 1.88	4.78 ± 6.43	NS
HbA1c (%)	5.59 ± 1.02	5.69 ± 0.58	NS	5.63 ± 1.04	5.73 ± 0.61	NS
AST (IU/37°C)	54.35 ± 29.41	43.90 ± 19.26	NS	56.00 ± 29.55	41.72 ± 19.00	NS
ALT (IU/37°C)	94.88 ± 61.75	73.62 ± 48.47	NS	98.69 ± 61.68	75.83 ± 52.03	NS
gGTP (IU/L)	112.40 ± 116.18	110.67 ± 117.88	NS	116.38 ± 118.80	121.94 ± 123.76	NS
Hyaluronic acid (ng/mL)	46.18 ± 51.29	45.75 ± 52.07	NS	46.38 ± 52.96	42.60 ± 52.29	NS
Platelet (/10 000/μL)	19.95 ± 4.48	20.21 ± 3.99	NS	20.58 ± 4.57	22.99 ± 5.85	NS
Serum iron (μg/dL)	20.28 ± 4.59	22.90 ± 6.46	NS	105.06 ± 34.41	91.53 ± 17.59	NS
Ferritin (ng/mL)	92.32 ± 15.30	90.55 ± 10.30	NS	224.00 ± 180.15	218.64 ± 84.06	NS
TNF-α (pg/mL)	1.20 ± 0.36§	1.79 ± 0.91¶	NS	1.20 ± 0.37§	1.57 ± 0.57¶	NS
TGF-β1 (ng/mL)	36.95 ± 7.88§	33.84 ± 11.22¶	NS	36.91 ± 8.18§	36.13 ± 8.70¶	NS
IL-6 (pg/mL)	1.89 ± 1.33§	4.12 ± 5.15¶	NS	1.96 ± 1.35§	2.64 ± 1.67¶	NS
Resistin (ng/mL)	3.95 ± 2.21§	5.24 ± 2.53¶	NS	4.01 ± 2.28§	5.49 ± 2.51¶	NS
Leptin (ng/mL)	8.30 ± 4.57§	10.41 ± 9.93¶	NS	8.50 ± 4.67§	9.20 ± 9.58¶	NS
Adiponectin (μg/mL)	8.79 ± 6.17§	6.46 ± 2.37¶	NS	9.11 ± 6.27§	6.45 ± 2.50¶	NS
HMW-adiponectin (μg/mL)	4.23 ± 3.92§	4.34 ± 5.10¶	NS	4.39 ± 4.02§	2.93 ± 2.19¶	NS

†Prevalences are expressed in percetage. ‡Comparison between groups. §$n = 15$. ¶$n = 11$.
Data except prevalences are expressed as mean ± standard deviation. NS, not significant.

Table 2 Effect of 24 weeks of treatment on clinical and biochemical parameters in all study population

	Colestimide $n = 17$ (Baseline)-(After 24 weeks)	Control $n = 21$ (Baseline)-(After 24 weeks)	Comparison between Groups
BMI (kg/m^2)	0.96 ± 0.97	0.98 ± 1.08	NS
Visceral fat (cm^2)	23.59 ± 23.73	12.90 ± 15.93	$P = 0.046$
Triglycerides (mg/dL)	44.59 ± 67.09	35.45 ± 44.68	NS
LDL (mg/dL)	46.79 ± 28.24	19.68 ± 18.79	$P = 0.004$
HDL (mg/dL)	−3.50 ± 8.42	−3.21 ± 8.70	NS
HOMA-IR	0.26 ± 0.87	0.19 ± 0.81	NS
HbA1c (%)	0.21 ± 0.22	0.09 ± 0.13	NS
AST (IU/37°C)	18.71 ± 23.26	6.2 ± 13.87	$P = 0.042$
ALT (IU/37°C)	26.09 ± 28.23	10.55 ± 27.31	NS
gGTP (IU)	11.49 ± 30.04	10.21 ± 34.08	NS
Hyaluronic acid (ng/mL)	8.76 ± 30.74	7.21 ± 27.21	NS

Data are expressed as mean ± standard deviation. NS, not significant.

DISCUSSION

IN THE PRESENT study, NASH patients with hyperlipidemia received colestimide for 24 weeks and showed a significant decrease of BMI, visceral fat, HbA1c, transaminases, gGTP, hyaluronic acid in addition to improvement of the lipid profile. These improvements, except for the changes of BMI, visceral fat and gGTP, were more prominent among the patients with moderate to severe steatosis and the differences were statistically significant on comparison with the control group.

In NASH patients who were diagnosed fatty liver by CT measurement of the liver/spleen attenuation ratio, only the colestimide group showed a decrease of the hepatic fat content.

The reduction of transaminases levels with colestimide treatment is consistent with the results of previous studies on lipid-lowering therapy for NASH patients.[9–13] Lipid overload may play a central role in NASH with respect to the development of steatosis, necroinflammatory changes, and fibrogenesis. In this study, most of the patients showed improvement of liver function without improvement of insulin resistance. Moreover, when we focused on patients with hyperlipidemia (eliminating those with type 2 DM), the improvement of transaminases in the colestimide group was more marked (Fig. 2). Colestimide was initially thought to only have an influence on bile acids and cholesterol. However, animal studies have shown that the drug not only improves serum lipids, but also improves obesity,

Table 3 Effect of 24 weeks of treatment on clinical and biochemical parameters in NASH with moderate to severe steatosis

	Colestimide $n = 16$ (Baseline)-(After 24 weeks)	Control $n = 18$ (Baseline)-(After 24 weeks)	Comparison between Groups
BMI (kg/m^2)	0.96 ± 0.97	0.94 ± 1.10	NS
Visceral fat (cm^2)	22.31 ± 23.81	11.40 ± 16.82	NS
Triglycerides (mg/dL)	41.75 ± 64.68	34.22 ± 45.08	NS
LDL (mg/dL)	46.41 ± 29.63	21.33 ± 18.62	$P = 0.017$
HDL (mg/dL)	−3.75 ± 10.21	−4.25 ± 8.13	NS
HOMA-IR	0.28 ± 0.91	0.14 ± 1.25	NS
HbA1c (%)	0.23 ± 0.21	0.03 ± 0.13	$P = 0.018$
AST (IU/37°C)	19.81 ± 23.49	2.94 ± 10.14	$P = 0.003$
ALT (IU/37°C)	27.81 ± 30.04	9.44 ± 29.05	$P = 0.042$
gGTP (IU)	11.40 ± 29.51	10.12 ± 39.50	NS
Hyaluronic acid (ng/mL)	9.63 ± 31.54	6.69 ± 27.74	$P = 0.042$

Data are expressed as mean ± standard deviation. NS, not significant.

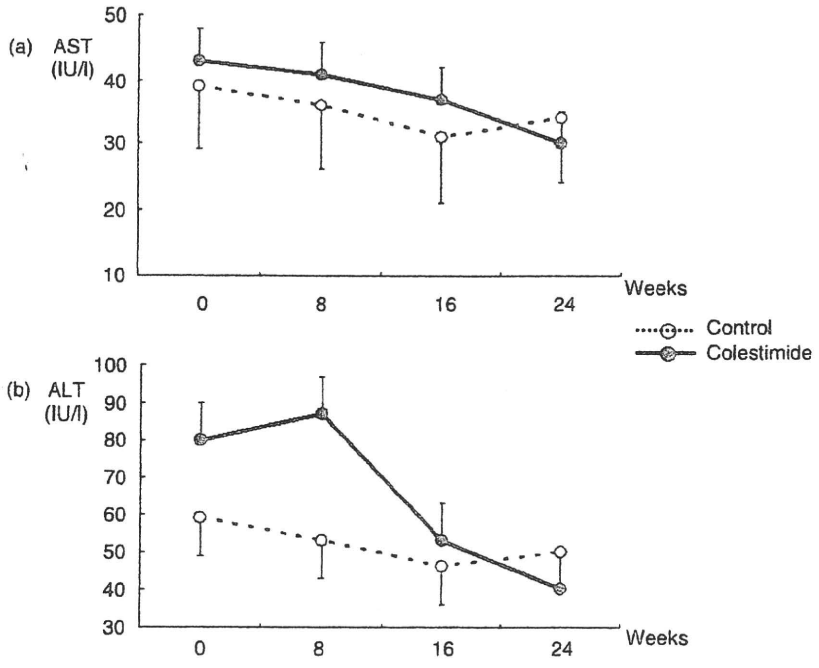

Figure 1 Changes in the level of transaminases during therapy. Each circle and bar express the mean serum level and SD at each time point in the colestimide group (black circles) and the control group (white circles). aspartate aminotransferase (AST) levels (a) and alanine aminotransferase (ALT) levels (b) gradually decreased in the colestimide group.

insulin resistance, type 2 DM, and fatty liver.[15,16] In addition, it has recently been reported that colestimide lowers cholesterol and improves with glycemic control in patients who have hypercholesterolemia combined with Type 2 DM.[17] In our study, the colestimide group showed significant decreases of visceral fat in all patients and HbA1c in patients with moderate to severe steatosis in comparison with the control group, which might represent additional effects of this drug. Decreasing visceral fat is the mainstay of treatment for NASH, so further studies are needed to confirm this effect of colestimide.[25]

In the present study, serum adipokine levels showed no significant changes in either group. Among subjects with overweight or obesity, however, the levels of adiponectin and HMW-adiponectin increased significantly in the colestimide group ($P = 0.036$ and $P = 0.032$, respectively). These changes might have been due to the decrease of visceral fat.

Concerning the histological effect of colestimide therapy for NASH, a second biopsy was only performed in two patients in this study. Currently, histologic examination is the most reliable means of assessing changes in NASH. However, liver biopsy is painful and has some risks. Moreover, NASH usually progresses very slowly, so the second biopsy at the end of this study was optional for ethical reasons. As a result, we did not obtain enough biopsy samples to properly assess the histological effects of colestimide therapy. Changes of transaminases are not sensitive or specific enough for assessing the effects of treatment for NASH.[26,27] Progression of fibrosis may occur despite normal transaminase levels.[27] However, our patients who received colestimide

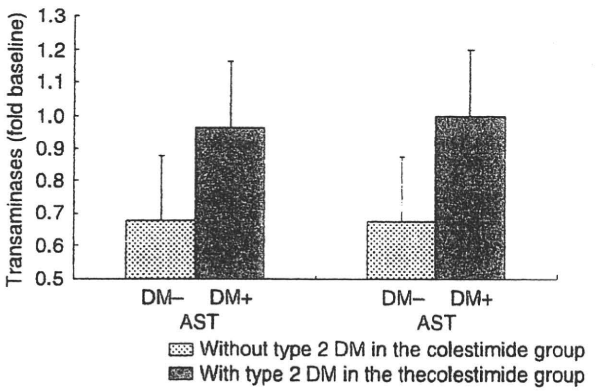

Figure 2 Changes of transaminases in the colestimide group in relation to the presence/absence of type 2 diabetes mellitus (DM). Each column and bar express the mean and SD of baseline fold after 24 weeks of treatment. The decreases of transaminases were more marked in patients without type 2 DM.

also showed a decrease of hyaluronic acid, which is an excellent indicator of fibrosis in NASH.[28,29] Accordingly, the decrease of transaminases might have resulted from improvement of NASH.

The limitations of this study include its short duration and small number of subjects, as well as the lack of histological assessment after treatment. However, our results may serve as "proof of concept" that colestimide can have the additional benefit to lifestyle modification for NASH patients with hyperlipidemia.

In summary, colestimide shows potential for the treatment of NASH, but an adequately powered, randomized, controlled trial with histological endpoints and long-term follow-up is needed to confirm our findings.

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