J Gastroenterol (2015) 50:887–893
DOI 10.1007/s00535-014-1018-z

The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Effect of *PNPLA3* rs738409 variant (I148 M) on hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C

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Received: 27 August 2014 / Accepted: 9 November 2014 / Published online: 26 November 2014

Abstract
Background Host genetic factors have been suspected to influence histological liver damage in chronic liver disease. The nonsynonymous single-nucleotide polymorphism rs738409 C > G in the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*, also known as adiponutrin), encoding the I148 M protein variant, has been identified as a novel genetic marker for hepatic steatosis and fibrosis in nonalcoholic fatty liver disease and alcoholic liver disease. We aimed to determine whether the *PNPLA3* rs738409 variant was associated with hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C.
Methods In a cross-sectional study in Japan, we analyzed 276 patients with chronic hepatitis C who underwent liver biopsy. Genotyping for rs738409 was performed using the TaqMan genotyping assay.
Results The frequencies of the rs738409 CC, CG, and GG genotypes were 32.6, 46.4, and 21.0 %, respectively. Multivariate analysis revealed that the GG genotype was independently associated with the presence of steatosis [odds ratio (OR) 2.58, 95 % confidence interval (CI) 1.37–4.84, $p = 0.003$], severe necroinflammatory activity (OR 2.16, 95 % CI 1.12–4.16, $p = 0.02$), and advanced fibrosis (OR 2.10, 95 % CI 1.07–4.11, $p = 0.03$), after adjustment for age, sex, body mass index, and diabetes.
Conclusions The *PNPLA3* rs738409 variant influences histological liver damage in Japanese patients with chronic hepatitis C. The G allele homozygotes are at higher risk for hepatic steatosis, severe necroinflammation, and advanced fibrosis.

Keywords *PNPLA3* · Single-nucleotide polymorphism · Hepatitis C · Steatosis · Fibrosis

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Introduction

Chronic hepatitis C (CHC) is a leading cause of liver cirrhosis and hepatocellular carcinoma in many countries. Hepatic steatosis occurs in more than half of patients infected with hepatitis C virus (HCV) and appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [1–3]. Both viral and host factors, including HCV genotype 3, older age, higher body mass index (BMI), diabetes, and alcohol consumption, are thought to contribute to HCV-related steatosis, [2, 4, 5]. HCV genotype 3, which is directly responsible for steatosis, is far less frequent in Japan than in Europe or the United States.

The rate of progression of liver fibrosis varies among patients with CHC. Known risk factors for fibrosis progression include older age, male sex, higher BMI, steatosis, insulin resistance, alcohol consumption, and co-infection with human immunodeficiency virus. However, these factors remain poor predictors of fibrosis progression [6, 7].

Host genetic factors have been suspected to influence histological liver damage in chronic liver disease. The

nonsynonymous single-nucleotide polymorphism rs738409 C > G in the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*, also known as adiponutrin), encoding an isoleucine to methionine substitution at residue 148 (I148 M), was initially associated with hepatic fat content in the first genome-wide association study (GWAS) on nonalcoholic fatty liver disease (NAFLD) [8]. The GWAS showed that the G allele of rs738409 influenced hepatic fat content independently of BMI, insulin resistance, and dyslipidemia. There has been growing evidence that the rs738409 variant (I148 M) is associated with steatosis, steatohepatitis, fibrosis, and cirrhosis both in NAFLD [9–11] and alcoholic liver disease [12–14]. Our GWAS of Japanese patients with NAFLD revealed that the G allele of rs738409 is associated only with typical nonalcoholic steatohepatitis with fibrosis (i.e., type 4 in Matteoni's classification [15]—not with simple steatosis or with steatosis with lobular inflammation or ballooning degeneration (i.e., types 1–3) [16].

Recent studies have suggested that the rs738409 variant is associated with hepatic steatosis and fibrosis in European patients with CHC [17, 18]. The influence of the rs738409 variant may be different in different populations. For example, the effect of the rs738409 variant on hepatic fat content is more evident among Hispanic-Americans, in whom the G allele of rs738409 is more frequent, when compared with European-Americans and African-Americans [8]. In the present cross-sectional study, we aimed to examine whether the *PNPLA3* rs738409 C > G variant (I148 M) is associated with hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with CHC.

Methods

Patients

This study included a total of 276 Japanese patients with CHC who underwent liver biopsy between 2009 and 2012 at the Saiseikai Suita Hospital and the Hospital of Kyoto Prefectural University of Medicine. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV, and positive for serum HCV-RNA. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, the presence of other types of liver diseases (e.g., primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. None of the patients had received any antiviral therapy before the liver biopsy although many of the patients had received ursodeoxycholic acid and herbal medicines.

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

Laboratory tests

Clinical and laboratory data were collected at the time of liver biopsy. Body mass index (BMI) was calculated using the following formula: weight in kilograms/(height in meters)^2. Diabetes was defined as a fasting plasma glucose concentration of ≥126 mg/dl or a 2-h plasma glucose concentration of ≥200 mg/dl during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose [19].

Venous blood samples were taken in the morning of the day of liver biopsy after a 12-h overnight fast. The laboratory evaluation included a blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and albumin. These parameters were measured using standard clinical chemistry techniques. HCV genotype was determined according to the classification of Simmonds et al. [20], and the serum HCV-RNA level was quantified as described previously [5].

Histopathological examination

Histopathological examination of the liver was performed as described previously [5]. The degrees of inflammation and fibrosis were evaluated according to the METAVIR scoring system [21].

Genotyping

DNA was extracted from peripheral blood mononuclear cells using the Gentra Puregene kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. DNA concentration and purity were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Genotyping for rs738409 was performed using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 22 (IBM, Chicago, IL, USA) or R (http://www.r-project.org/). To evaluate the association between the rs738409 genotypes and clinical parameters, we used the Jonckheere–Terpstra trend test (for continuous variables) and the Cochran–Armitage trend test (for categorical variables). Logistic regression analysis was used for multivariate

Table 1 Patient characteristics

Characteristic	Total	*PNPLA3* rs738409			P^a
		CC	CG	GG	
n	276 (100)	90 (32.6)	128 (46.4)	58 (21.0)	
Age (years)	58.2 ± 13.0	59.5 ± 11.9	57.7 ± 13.3	57.1 ± 13.7	0.29
Male sex	112 (40.6)	36 (40.0)	50 (39.1)	26 (44.8)	0.61
BMI (kg/m^2)	23.0 ± 3.4	23.0 ± 3.7	22.9 ± 3.4	23.1 ± 2.8	0.49
Diabetes	13 (4.7)	4 (4.4)	6 (4.7)	3 (5.2)	0.84
Platelet count ($\times 10^4$/μl)	16.0 ± 5.8	15.5 ± 5.1	16.4 ± 6.3	16.0 ± 5.7	0.52
AST (IU/l)	58.7 ± 43.6	61.7 ± 53.7	58.2 ± 38.7	55.0 ± 36.4	0.95
ALT (IU/l)	67.4 ± 58.0	69.2 ± 61.0	68.4 ± 60.1	62.3 ± 48.4	0.92
γ-GTP (IU/l)	59.8 ± 79.4	62.8 ± 94.3	57.5 ± 69.9	60.3 ± 74.8	0.53
Albumin (g/dl)	4.0 ± 0.5	4.0 ± 0.5	4.0 ± 0.5	4.1 ± 0.4	0.27
HCV genotype					0.09
1	198 (71.7)	55 (61.1)	102 (79.7)	41 (70.7)	
2	76 (27.5)	33 (36.7)	26 (20.3)	17 (29.3)	
ND	2 (0.8)	2 (2.2)	0 (0)	0 (0)	
HCV RNA level (logIU/ml)	6.0 ± 1.1	6.0 ± 1.1	6.1 ± 1.0	6.1 ± 1.3	0.37
Liver histology					
Steatosis					
<1 %	139 (50.4)	67 (74.5)	53 (41.4)	19 (32.8)	
1–10 %	100 (36.2)	17 (18.9)	58 (45.3)	25 (43.1)	
11–33 %	26 (9.4)	3 (3.3)	15 (11.7)	8 (13.8)	
>33 %	11 (4.0)	3 (3.3)	2 (1.6)	6 (10.3)	
Activity grade[b]					
0	7 (2.5)	3 (3.3)	2 (1.5)	2 (3.5)	
1	120 (43.5)	42 (46.7)	60 (46.9)	18 (31.0)	
2	120 (43.5)	35 (38.9)	56 (43.8)	29 (50.0)	
3	29 (10.5)	10 (11.1)	10 (7.8)	9 (15.5)	
Fibrosis stage[b]					
0	10 (3.6)	3 (3.3)	4 (3.1)	3 (5.2)	
1	111 (40.2)	41 (45.6)	53 (41.4)	17 (29.3)	
2	83 (30.1)	26 (28.9)	40 (31.3)	17 (29.3)	
3	45 (16.3)	10 (11.1)	20 (15.6)	15 (25.9)	
4	27 (9.8)	10 (11.1)	11 (8.6)	6 (10.3)	

Values are mean ± standard deviation or numbers (%). Where no other unit is specified, values refer to numbers (%) of patients

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *ND* not determined, *γ-GTP* γ-glutamyl transpeptidase

[a] Jonckheere–Terpstra test or Cochran–Armitage trend test

[b] According to reference [21]

analysis. Values of $p < 0.05$ were considered significant. Post hoc power analysis was performed using nQuery Advisor (Statistical Solutions, Boston, MA, USA).

Results

The characteristics of the 276 study subjects with chronic hepatitis C and the frequency distribution of the *PNPLA3* rs738409 C > G polymorphism are summarized in Table 1. The frequencies of the rs738409 CC, CG, and GG genotypes were 32.6, 46.4, and 21.0 %, respectively, and were in Hardy–Weinberg equilibrium. The rs738409 genotype was not significantly associated with clinical or biochemical factors, including age, sex, BMI, diabetes, HCV genotype, platelet count, and levels of serum AST, ALT, γ-GTP, albumin, and HCV-RNA.

We assessed the impact of the rs738409 genotype on histological liver damage in a cross-sectional manner. The prevalence of steatosis (defined as ≥1 %), severe

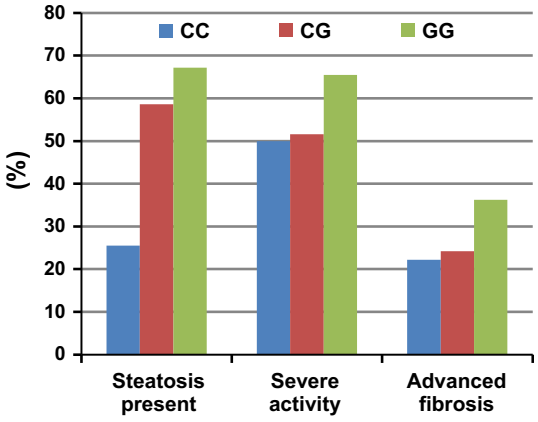

Fig. 1 The prevalence of steatosis (defined as ≥1 %), severe necroinflammatory activity (grade 2 or 3), and advanced fibrosis (stage 3 or 4) according to the *PNPLA3* rs738409 genotype

Table 2 Multivariable logistic regression analysis of the association of *PNPLA3* (rs738409 C>G), under a recessive inheritance model, with the presence of steatosis, severe necroinflammatory activity and advanced fibrosis

Variables	OR	95 % CI	*p*
Steatosis present (≥1 %)			
rs738409 (GG vs. CG + CC)	2.58	1.37–4.84	0.003
Age (years)	0.99	0.97–1.01	0.45
Sex (male vs. female)	1.19	0.70–2.02	0.51
BMI (kg/m^2)	1.19	1.10–1.30	<0.001
Diabetes (yes vs. no)	0.70	0.21–2.36	0.57
Severe necroinflammatory activity (grade 2 or 3)			
rs738409 (GG vs. CG + CC)	2.16	1.12–4.16	0.02
Age (years)	1.06	1.03–1.08	<0.001
Sex (male vs. female)	1.24	0.73–2.14	0.42
BMI (kg/m^2)	1.11	1.02–1.20	0.01
Diabetes (yes vs. no)	2.17	0.54–8.68	0.28
Advanced fibrosis (stage 3 or 4)			
rs738409 (GG vs. CG + CC)	2.10	1.07–4.11	0.03
Age (years)	1.07	1.04–1.10	<0.001
Sex (male vs. female)	1.31	0.72–2.40	0.38
BMI (kg/m^2)	1.14	1.04–1.24	0.006
Diabetes (yes vs. no)	1.77	0.52–6.05	0.37

BMI body mass index, *CI* confidence interval, *OR* odds ratio

necroinflammatory activity (grade 2 or 3), and advanced fibrosis (stage 3 or 4) according to the rs738409 genotype is shown in Fig. 1. Steatosis was present in 25.5, 58.6, and 67.2 %, severe necroinflammatory activity was found in 50.0, 51.6, and 65.5 %, and advanced fibrosis was found in 22.2, 24.2, and 36.2 % of patients with CC, CG, and GG genotypes, respectively.

To evaluate the effect of the rs738409 variant G allele, we used a recessive model of inheritance comparing G allele homozygotes (GG) with heterozygotes (CG) or C allele homozygotes (CC) (i.e., GG vs. CG + CC), according to previous reports [17, 18]. Multivariate analysis revealed that the GG genotype was independently associated with the presence of steatosis [odds ratio (OR) 2.58, 95 % confidence interval (CI) 1.37–4.84, $p = 0.003$], severe necroinflammatory activity (OR 2.16, 95 % CI 1.12–4.16, $p = 0.02$), and advanced fibrosis (OR 2.10, 95 % CI 1.07–4.11, $p = 0.03$), after adjustment for age, sex, BMI, and diabetes (Table 2). Besides the rs738409 GG genotype, a higher BMI was independently correlated with the presence of steatosis, severe necroinflammatory activity, and advanced fibrosis, and older age was independently associated with severe necroinflammatory activity and advanced fibrosis.

Discussion

In the present study, we showed that the *PNPLA3* rs738409 GG genotype was independently associated with the presence of steatosis, severe necroinflammatory activity, and advanced fibrosis in Japanese patients with CHC. Our results appear to be compatible with the following previous studies of European patients. The Swiss Hepatitis C Cohort Study [22] reported that the rs738409 G allele was associated with an increased risk of steatosis in Caucasian patients with HCV genotype non-3. The large Italian cross-sectional study [17] showed that the rs738409 GG genotype was associated with steatosis, fibrosis stage, cirrhosis, lower response to antiviral therapy, and hepatocellular carcinoma occurrence in CHC. In the cross-sectional and prospective study of Caucasian patients with CHC from Belgium, Germany, and France [18], the rs738409 GG genotype was associated with steatosis, fibrosis, and fibrosis progression.

However, to our knowledge, this is the first report to demonstrate the association of the *PNPLA3* rs738409 variant with advanced fibrosis in Japanese patients with CHC. Sato et al. [23] recently reported that the rs738409 GG genotype was associated with a higher prevalence of steatosis in Japanese patients with CHC, and Moritou et al. [24] showed that the rs738409 G allele tended to be associated with steatosis in such patients. In contrast, Nakamura et al. [25] reported that there was no correlation between the rs738409 genotype and steatosis and liver cirrhosis diagnosed by ultrasonography in Japanese patients with CHC. However, ultrasonography is a less accurate method for the diagnosis of steatosis and liver cirrhosis than the liver biopsy that was used in the present study.

Genotype frequency varies according to ethnicity. Importantly, our study, together with other studies of

Japanese patients with CHC [23–25], indicate that the frequency of the rs738409 GG genotype seems to be higher in Japanese patients (21.0–24.0 %) than in European patients (approximately 10 %) with CHC [17, 18]. If so, Japanese patients may be at higher risk for rapid progression of CHC than European patients.

Furthermore, several lines of evidence suggest the association of the rs738409 GG genotype with an increased risk of hepatocellular carcinoma in patients with CHC [17, 23, 26–28], although the association seems to be less pronounced in CHC than in alcoholic liver disease [26, 28].

Alcohol consumption is known to promote the development of steatosis and the progression of fibrosis in CHC. Müller et al. [29] found a distinct effect of the rs738409 genotype on steatosis and fibrosis in German patients with CHC according to the amount of alcohol intake; that is, while the rs738409 GG genotype was associated with steatosis only in abstainers (<30 g alcohol/day), it was associated with liver cirrhosis only in at-risk drinkers (>30 g alcohol/day). Valenti et al. [30] confirmed that the rs738409 GG genotype was associated with steatosis only in abstainers; however, they found that it was associated with cirrhosis in both abstainers and at-risk drinkers. Further studies are needed to examine the interaction between a moderate amount of alcohol intake and the rs738409 variant in CHC. In the present study, we excluded patients who consumed more than 20 g of alcohol per day to eliminate the confounding effect of alcohol on steatosis and fibrosis.

Although the rs738409 GG genotype was associated with severe necroinflammatory activity and advanced fibrosis, it was not related with a higher ALT level or a lower platelet count. The reason for this discrepancy is unknown. However, studies have shown that necroinflammatory activity grade is not well correlated with ALT levels in CHC [31, 32]. We may have failed to show the association of the GG genotype with a lower platelet count in part because the proportion of patients with advanced fibrosis (stage 3 or 4) was relatively low (26 %) and therefore the mean platelet count was not very low ($16.0 \times 10^4/\mu l$) in our patients.

Liver transplantation provides a unique opportunity to assess whether the effect of the rs738409 variant is localized in the liver or in other tissues, because transplantation creates a chimeric individual. A recent study of patients who underwent liver transplantation for hepatitis C in the United States showed that donor, but not recipient, rs738409 GG or CG genotype was associated with increased risk of fibrosis progression, retransplantation, or death after liver transplantation [33]. This finding indicated that the liver is indeed the site where the effect of the variant occurs. However, neither donor nor recipient rs738409 genotype was associated with hepatic steatosis during follow-up biopsies. These observations suggested that the rs738409 variant in the liver is responsible for fibrosis progression but not for steatosis. The rs738409 variant may influence the development of fibrosis and steatosis through different pathways.

PNPLA3 encodes a 481-amino acid protein that contains a highly conserved patatin-like domain at the N-terminal. PNPLA3 is a membrane-bound protein and is most highly expressed in the liver, followed by the skin and adipose tissue in humans [34, 35]. PNPLA3 expression is highly regulated by nutritional stimuli at both the transcriptional and posttranslational levels through the transcription factors SREBP-1c and liver X receptor [35–37]. Studies have found that levels of PNPLA3 were very low in the liver during fasting and were increased with carbohydrate feeding [35, 36].

Despite the strong clinical association of the *PNPLA3* rs738409 variant (I148 M) with liver diseases, the biochemical function of *PNPLA3* and the underlying mechanism by which the I148 M variant affects liver injury remain controversial. Some investigators have proposed that *PNPLA3* shows lipase activity and that the I148 M variant results in a loss of function [34, 38–40], while other authors have suggested that *PNPLA3* plays a role in lipid synthesis and that the I148 M variant exerts a gain-of-function effect [37, 41, 42]. Interestingly, Pirazzi et al. [40] reported that the wild-type *PNPLA3* has retinyl-palmitate lipase activity in human hepatic stellate cells, and that the lipase activity is markedly reduced in the I148 M variant. Because hepatic stellate cells are key players in fibrogenesis in chronic liver disease, *PNPLA3* may possibly be involved in the activation and transformation of hepatic stellate cells in response to hepatic injury and the development of liver fibrosis. Contrary to expectations, *PNPLA3*-deficient mice have not shown any obvious phenotype [43, 44]. However, it must be noted that there are differences in tissue-specific expression of *PNPLA3* between humans and mice [35].

Certain limitations should be considered in the interpretation of our findings. The cross-sectional study design hinders the ability to draw inferences regarding the causality of the rs738409 variant in histological liver damage. Although none of our patients had received any antiviral therapy before the liver biopsy, many of these patients had received ursodeoxycholic acid and herbal medicines. A past history of these treatments for CHC might have slightly influenced our results. A post hoc power analysis was performed using the actual sample size, based on the Chi-square test. Our study had sufficient power (more than 80 %) to detect a clinically meaningful effect size (odds ratio $\geq$2.4).

The *PNPLA3* rs738409 variant (I148 M) has now been associated with the progression of chronic liver diseases

with different etiologies, including NAFLD, alcoholic liver disease, and CHC. Moreover, the association appears to be common in different populations. Elucidation of the physiological functions of *PNPLA3* and the pathological effects of the I148 M variant will reveal the common underlying mechanisms involved in chronic liver diseases and may hopefully lead to identification of therapeutic targets for these diseases.

Acknowledgments This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan (H20-hepatitis-008 to Takeshi Okanoue, H24-hepatitis-general-006 to Takeshi Okanoue).

Conflict of interest The authors declare that they have no conflict of interest.

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