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Original Article

Impact of *PNPLA3* polymorphisms on the development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Aim: The *PNPLA3* rs738409 C>G polymorphism (encoding for I148M) has recently been identified as a susceptibility factor for steatosis-mediated liver damage. We evaluated the influence of this polymorphism on hepatocarcinogenesis in patients with chronic hepatitis C (CHC) virus infection.

Methods: We genotyped the rs738409 single nucleotide polymorphism in 358 hepatitis C-associated hepatocellular carcinoma (HCC) patients and correlated the age at onset of HCC and the interval between hepatitis C virus (HCV) infection and the development of HCC in patients with each genotype.

Results: The frequencies of CC, CG and GG genotypes were 27.9% (100/358), 49.2% (176/358) and 22.9% (82/358), respectively, and were in Hardy–Weinberg equilibrium. The median age at onset of HCC for the GG genotype was significantly younger compared to for non-GG genotypes (67.81 vs 69.87 years, $P < 0.001$), and the median interval between HCV infection and the development of HCC was significantly shorter in patients with the GG genotype (39.96 vs 40.85 years, $P = 0.008$). *PNPLA3* GG genotype was also associated with a higher aspartate aminotransferase level (69.5 vs 59.0 IU/L, $P = 0.02$), lower prothrombin time (73.0% vs 78.0%, $P = 0.008$) and a higher prevalence of histological steatosis (40.0% *vs.* 22.2%, $P = 0.01$) at the time of HCC onset.

Conclusion: The *PNPLA3* genotype GG may be associated with accelerated hepatocarcinogenesis in CHC patients through increased steatosis in the liver.

Key words: fibrosis, hepatocarcinogenesis, risk allele, rs738409, steatosis

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major health burden, with 130–170 million people infected, representing nearly 3% of the world's population.[1] HCV infection is one of the major causes of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC).[2]

In epidemiological studies of chronic HCV infection (CHC), age, duration of infection, alcohol consumption, co-infection with HIV, low CD4 count, male sex and HCV genotype 3 have been shown to be associated with histological activity.[3–8] We also reported higher body mass index (BMI) as an independent risk factor for HCC development in CHC patients.[9] Although these factors explain part of the extreme variability seen in fibrosis progression among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in CHC.[10–12] Recently, two genome-wide association studies (GWAS) carried out in Japan reported genetic factors, MICA locus (rs2596542) and DEPDC5 locus (rs1012068), associated with HCV-related HCC.[13,14]

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Because of the global epidemic of obesity, non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the most common liver disorder worldwide.[15–18] Liver steatosis also has gained increasing attention as a modifier of CHC progression. In fact, hepatic steatosis is a common histological feature of CHC, seen in more than half of patients, and has been associated with fibrosis progression and increased risk of HCC via overproduction of reactive oxygen species.[19–21]

Adiponutrin encoded by *PNPLA3* has been reported to have both lipolytic and lipogenic properties.[22] Recently, independent GWAS identified a single nucleotide polymorphism (SNP; rs738409 C>G) in the *PNPLA3* gene on chromosome 22, encoding an isoleucine to methionine substitution (p.I148M) of patatin-like phospholipase A3 as a genetic determinant of liver fat content or disease severity.[23,24] A recent meta-analysis showed that this polymorphism has been related, in NAFLD, to inflammatory activity and progression of fibrosis.[25] The previous basic research showed that the *PNPLA3* I148M impairs hydrolytic activity against triacylglycerol *in vitro* and is thought to lead to accumulation of triacylglycerol.[26] Other studies using mice showed that the inactivation of *PNPLA3* has no effect on hepatic fat accumulation,[27] but the overexpression of *PNPLA3* I148M causes an increase in hepatic triacylglycerol content.[28] The rs738409 polymorphism was also found to be associated not only with elevated liver enzymes or prevalence of fatty liver histology in healthy subjects,[29,30] but also with disease severity and fibrosis in NAFLD,[25,31,32] alcoholic liver disease[33,34] and CHC.[35,36] However, the influence of *PNPLA3* (rs738409 C>G) polymorphism on HCV-related HCC still remains controversial.[34,36,37] In the present study, we focused on the association between the rs738409 SNP and the age at onset of HCC and the interval between HCV infection and the development of HCC to evaluate the influence of the *PNPLA3* polymorphism on hepatocarcinogenesis in CHC patients.

METHODS

Patients

THIS RESEARCH PROJECT was approved by the ethics committees of the University of Tokyo (no. 400). The patients analyzed in the present study were derived from a HCV study cohort of the University of Tokyo Hospital. All patients visited the liver clinic at our institution between August 1997 and August 2009 and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. We enrolled patients who had developed HCC and received initial therapy for HCC at our institution by 31 January 2010, and with samples available for genotyping. Exclusion criteria were positivity for hepatitis B surface antigen and presence of biliary disease. We also excluded patients without information on BMI, daily alcohol intake, HCV genotype and HCV viral load. Finally, 358 patients were enrolled, and all subjects were Japanese. We analyzed the association of rs738409 C>G polymorphism with the age at onset of HCC and the interval between HCV infection and the development of HCC. Because we lacked knowledge of the exact date of hepatitis C seroconversion, the duration of HCV infection was estimated indirectly, based on the year of the first transfusion.

Diagnosis of HCC

Hepatocellular carcinoma was diagnosed by dynamic computed tomography, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC. When the diagnosis of HCC was ambiguous, an ultrasound-guided tumor biopsy was performed, and a pathological diagnosis was made based on the Edmondson and Steiner criteria.[38]

Genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the *PNPLA3* rs738409 C/G polymorphism was performed by polymerase chain reaction (PCR) using the TaqMan predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA), as recommended by the manufacturer. Allele-specific primers were labeled with fluorescent dye (6-carboxyfluorescein or hexachloro-6-carboxyfluorescein) and used in the PCR reaction. Aliquots of the PCR products were genotyped using an allele-specific probe of the SNP on a real-time PCR thermocycler (MX3000P; Stratagene, La Jolla, CA, USA). Samples were subjected to 45 cycles of denaturation for 15 s at 95 °C, annealing of primers for 30 s at 60 °C and elongation for 30 s at 60 °C.

Study end-point

We analyzed the relationship between host factors, including *PNPLA3* (rs738409 C>G) polymorphisms, sex, BMI, alcohol consumption and HCV genotype, and the age at onset of HCC or the interval between HCV infection and the development of HCC (the primary end-points of this study). We also examined the relationship between rs738409 polymorphisms and clinical

findings at the onset of HCC (the secondary end-point), such as biochemical markers and histological findings. The histological grade of disease activity and the histological stage of fibrosis were assessed using the reproducible METAVIR scoring system as follows: grades A1 to A3 for the degree of necroinflammatory activity (A1 = mild to A3 = marked), and stages F0 to F4 for the degree of fibrosis (F0 = no fibrosis to F4 = cirrhosis).[39,40] The presence of steatosis was studied as a qualitative (<5% vs ≥5%) variable.

Statistical analysis

Continuous variables are presented as medians with 1st and 3rd quartiles, whereas categorical variables are expressed as frequencies (%). Categorical data were analyzed using the χ^2-test, and stepwise logistic regression analyses were used to adjust the influence of the *PNPLA3* genotype by other covariates such as sex, BMI (<25 or not) and alcohol consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using Student's *t*-test or the non-parametric Wilcoxon rank sum test as appropriate. Because the age at onset of HCC and the length of time between HCV infection and the development of HCC (the primary end-points of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis for multivariate analyses. We evaluated the association between the rs738409 mutant G allele and each outcome using a recessive model of inheritance, comparing G allele homozygotes (GG genotype) with patients carrying one copy or no copies of the G allele (CG or CC genotypes) because this was suggested to be the most appropriate one by studies of the impact of rs738409 on CHC liver damage.[36,41] The Jonckheere–Terpstra trend test for continuous variables and the Cochran–Armitage trend test for categorical variables were used to evaluate the increasing or decreasing tendency of the findings across rs738409 CC, CG and GG genotypes. All statistical analyses were two-sided, and the threshold of the reported *P*-values for significance was less than 0.05. All statistical analyses were performed using the R version 2.13.1 software (http://www.r-project.org).

RESULTS

Patient characteristics

PATIENT CHARACTERISTICS ARE shown in Table 1. Frequencies of the rs738409 CC, CG and GG genotypes were 27.9% (100/358), 49.2% (176/358)

Table 1 Clinical characteristics and genotype distributions of the subjects ($n = 358$)

Parameter	Values
Median age at onset of HCC, years	69.76 (63.88–75.35)
Male sex	200 (55.9%)
BMI >25	67 (18.7%)
Alcohol consumption (>50 g/day)	75 (20.9%)
PNPLA3 genotype	
CC	100 (27.9%)
CG	176 (49.2%)
GG	82 (22.9%)
G allele frequency	0.47
HCV genotype	
Genotype 1	271 (75.7%)
Genotype 2	87 (24.3%)

Continuous variables are presented as medians with 1st and 3rd quartiles, and categorical variables as numbers and frequency (%).
BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

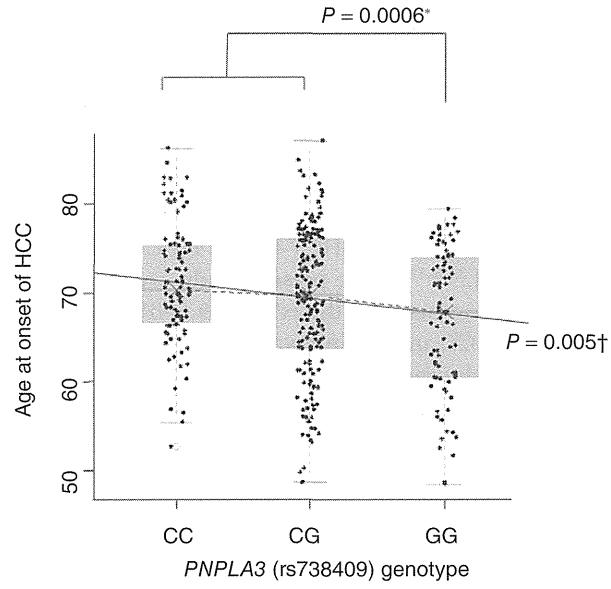

Figure 1 Box and whisker and dot plot: distributions of the age at onset of hepatocellular carcinoma (HCC) in each genotype. The dashed line connects the median value of each genotype, and the solid line shows the linear regression. The Jonckheere–Terpstra trend test showed a significant trend across the CC, CG and GG alleles ($P = 0.005$). **P*-values after adjustment for sex, body mass index and alcohol consumption. †*P*-value by the Jonckheere–Terpstra trend test.

and 22.9% (82/358), respectively. The SNP genotype distribution was in Hardy–Weinberg equilibrium (*P*-value was non-significant). The median age at onset of the HCC patients was 69.76 years, and approximately 55% were male.

Primary end-point

Table 2 shows the age at onset of patients with HCC and the associations among rs738409 genotypes, sex, BMI, alcohol consumption, HCV genotype and HCV viral load. The median ages (1st–3rd quartile) at onset in patients with HCC for the rs738409 GG and non-GG (CC/CG) genotypes were 67.8 years (range, 60.6–74.0) and 69.9 years (range, 65.2–75.6), respectively. The median age was significantly younger in patients with the rs738409 GG genotype than in those with non-GG genotype ($P = 0.004$). In multivariate analysis, early age at onset of HCC was independently associated with rs738409 GG genotype ($P < 0.001$), male sex ($P = 0.004$) and higher BMI ($P = 0.03$). The median ages at onset of patients with HCC for the CC and CG genotypes were 70.3 and 69.7 years, respectively. The Jonckheere–Terpstra trend test showed a significant trend across the GG, CG and CC alleles ($P = 0.005$; Fig. 1). One hundred and sixty-six patients had histories of blood transfusion. The median (1st–3rd quartile) intervals between blood transfusion and the onset of HCC in patients with rs738409 GG and non-GG (CC/CG) genotypes were 39.96 (range, 33.43–45.84) and 40.85 years (range, 33.52–46.76), respectively. In multivariate analysis, the median interval between blood transfusion and the onset of HCC was significantly shorter in patients with rs738409 GG genotype ($P = 0.008$) and male sex ($P < 0.001$) (Table 3).

Secondary end-point

Table 4 shows the clinical findings and associations between the rs738409 genotypes at the time of HCC onset. The rs738409 GG genotype was significantly associated with a higher aspartate aminotransferase (AST) level (69.5 vs 59.0 IU/L, $P = 0.02$), a lower prothrombin time (72.95% vs 78.00%, $P = 0.008$) and a higher prevalence of histological steatosis (40.00% *vs.* 22.16%, $P = 0.01$) compared to the non-GG genotype after adjustment for sex, BMI and alcohol consumption. There were no significant associations between rs738409 genotype and histological stage of fibrosis or histological grade of disease activity. Figure 2 shows the

Table 2 Factors associated with the age at onset of HCC ($n = 358$)

Variable	Median	1st–3rd quartile	*P*-value	
			Univariate	Multivariate†
PNPLA3 genotype			0.004	<0.001
GG	67.81	60.58–73.97		
CC/CG	69.87	65.20–75.62		
Sex			<0.001	0.004
Male	68.59	62.09–74.20		
Female	71.81	65.98–76.26		
BMI			0.07	0.03
>25	68.95	63.05–73.50		
≤25	70.49	64.32–75.57		
Alcohol consumption			0.02	0.11
>50 g/day	68.25	59.75–73.35		
≤50 g/day	70.12	64.80–75.47		
HCV genotype			0.2	
Genotype 1	69.87	64.35–75.53		
Genotype 2	68.65	63.50–74.17		
Viral load			0.09	0.06
High‡	70.57	65.08–75.82		
Low§	68.89	63.75–74.59		

†Stepwise regression analysis for the age at onset of hepatocellular carcinoma (HCC; the dependent variable) using *PNPLA3* genotype, sex, body mass index (BMI), alcohol consumption, hepatitis C virus (HCV) genotype and HCV viral load as independent variables.
‡At or above the median value.
§Below the median value.

Table 3 Factors associated with the time between HCV infection and the development of HCC ($n = 166$)

Variable	Median	1st–3rd Quartile	*P*-value	
			Univariate	Multivariate†
PNPLA3 genotype			0.47	0.008
GG ($n = 40$)	39.96	33.43–45.84		
CC/CG ($n = 126$)	40.85	33.52–46.76		
Sex			0.04	<0.001
Male	38.54	31.95–44.93		
Female	42.45	35.67–47.25		
BMI			0.75	–
>25 kg/m^2	37.94	32.91–45.60		
≤25 kg/m^2	40.85	33.70–46.87		
Alcohol consumption			0.26	–
>50 g/day	40.13	28.55–45.33		
≤50 g/day	40.87	33.79–46.76		
HCV genotype			0.09	–
Genotype 1	41.46	34.20–46.92		
Genotype 2	37.80	28.70–45.44		
Viral load			0.008	0.11
High‡	41.81	35.18–48.28		
Low§	38.53	30.79–45.12		

†Stepwise regression analysis of age at onset of hepatocellular carcinoma (HCC; the dependent variable) using *PNPLA3* genotype, sex, body mass index (BMI), alcohol consumption, hepatitis C virus (HCV) genotype, HCV viral load and the age at blood transfusion as independent variables.
‡At or above the median value.
§Below the median value.

histological findings for CC, CG and GG genotypes. The increment in the G allele was significantly associated with a higher prevalence of steatosis, as demonstrated by the Cochran–Armitage trend test (CC 13.11% vs CG 28.45% vs GG 40.00%, respectively; $P = 0.004$).

DISCUSSION

IN THIS STUDY, we found that the risk allele of *PNPLA3*, which was strongly correlated with significant liver steatosis, also may be a risk factor for hepatocarcinogenesis in CHC patients. Median age at onset of HCC was significantly younger ($P < 0.001$), and the median interval between blood transfusion and the onset of HCC was significantly shorter ($P = 0.008$) in patients with the rs738409 GG genotype than in those with non-GG genotypes after adjustment for sex, BMI, alcohol consumption, HCV genotype and HCV viral load.

Earlier age at HCC onset or shorter time between HCV infection and the development of HCC in the GG genotype was thought to be caused by the acceleration of liver fibrosis. The patients with the rs738409 GG genotype may reach the stage of advanced cirrhosis and develop HCC in their early age or shorter time after HCV infection. Previous studies reported hepatic steatosis as a risk factor for progressed fibrosis and HCC in CHC patients.[4,42] The *PNPLA3* polymorphism was originally reported as a determinant of liver fat content,[23] and a significant association between rs738409 SNP and histological evidence of steatosis (≥5%) was identified in the present study. The *PNPLA3* polymorphism was thought to affect the susceptibility to HCC in CHC patients via alteration of lipid accumulation in the liver.

Although this was not confirmed histologically, the *PNPLA3* GG genotype was also significantly associated with higher AST level and tended to be associated with a higher prevalence of progressed histological fibrosis compared to the non-GG genotypes (74.0% vs 60.5%, $P = 0.11$) at the time of HCC onset. Moreover, the GG genotype was associated with a lower prothrombin time, which suggests depressed liver function. Increased lipid accumulation in the *PNPLA3* GG genotype may enhance the risks of hepatic inflammation, fibrosis and impairment of liver function in CHC patients.

Table 4 Associations between *PNPLA3* genotype and clinical findings at the time of HCC onset ($n = 358$)

Variable	Median/number (1st–3rd quartile)		*P*-values	
	GG	Non-GG	*P*-value	Adjusted *P*-value†
Platelet count ($\times 10^4/\mu L$)	10.05 (7.73–12.78)	10.30 (7.68–13.35)	0.53	–
AST (IU/L)	69.5 (49.0–88.5)	59.0 (43.0–83.5)	0.048	0.02§
ALT (IU/L)	59.0 (42.0–93.3)	55.0 (37.0–86.3)	0.29	–
TB (mg/dL)	0.8 (0.6–1.1)	0.8 (0.6–1.1)	0.85	–
Albumin (g/dL)	3.7 (3.3–3.9)	3.7 (3.4–3.9)	0.41	–
PT (%)	73.0 (67.3–79.0)	78.0 (69.0–90.0)	0.004	0.008§
Viral load (log IU/mL)	4.73 (4.51–4.94)	4.75 (4.35–5.20)	0.90	–
LDL cholesterol (mg/dL)	77.2 (63.1–90.3)	74.7 (57.6–93.6)	0.77	–
Triglyceride (mg/dL)	82.0 (59.0–108.0)	87.0 (66.0–114.0)	0.32	–
Fasting plasma glucose (mg/dL)	100.0 (88.5–116.0)	103.0 (91.3–121.8)	0.20	–
Plasma insulin (μg/mL)	12.0 (8.0–18.0)	12.0 (9.0–19.0)	0.67	–
Histological findings ($n = 235$)				
Fibrosis				
F0–3	13	73	0.11	–
F4	37	112		
Activity				
A0–1	30	112	0.93	–
A2–3	20	73		
Steatosis‡				
<5%	30	144	0.02	0.01¶
≥5%	20	41		

†Adjusted for sex, BMI and alcohol consumption (independent variables). The dependent variables of each *P*-value are the items in the leftmost fields of the corresponding row (e.g. platelet count, AST, ALT).
‡Odds ratio (95% CI) for the GG allele was 2.43 (1.24–4.77), and the 95% CI of each proportion is shown in parentheses for this outcome.
§*P*-value by stepwise regression analysis.
¶*P*-value by stepwise logistic regression analysis.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; HCC, hepatocellular carcinoma; LDL, low-density lipoprotein; PT, prothrombin time; TB, total bilirubin.

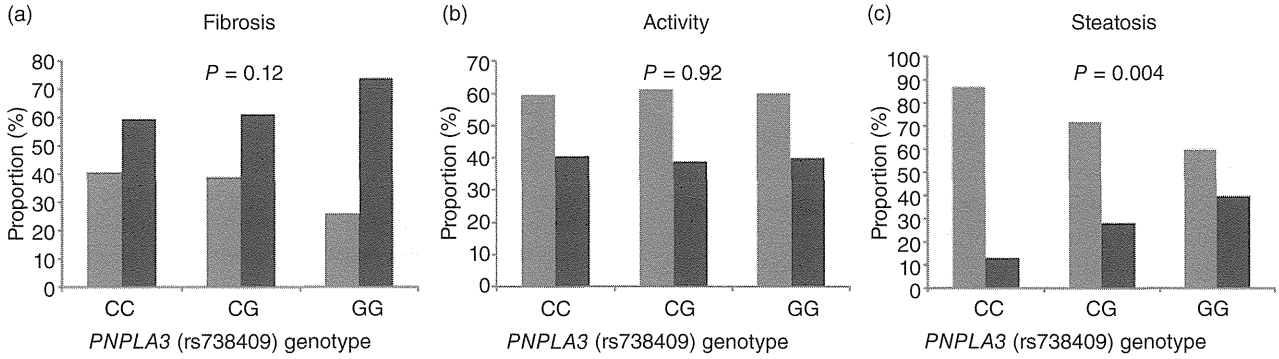

Figure 2 Bar plot: prevalence of fibrosis (F1–3 vs F4, a), necroinflammation (A1 vs A2–3, b) and steatosis (<5% vs ≥5%, c) in 235 patients with chronic hepatitis C. The proportions are shown on the Y axis. *P*-values of the frequency distributions are shown (Cochran–Armitage trend test). ■, F1–3; ■, F4; ■, A1; ■, A2–3; ■, <5%; ■, ≥5%.

One study investigated the impact of the *PNPLA3* polymorphism on liver steatosis and fibrosis in CHC patients.[36] In this study, the cumulative incidence of HCC during the follow-up period was significantly higher in patients with the GG genotype.[36] The *PNPLA3* polymorphism is also associated with susceptibility to HCC in patients with other causes of hepatitis.[34,43] Our data suggest that the *PNPLA3* rs738409 polymorphism may provide important information that will assist identification of patients at particular risk for HCC.

In the present study, early age at onset of HCC was also independently associated with male sex and higher BMI, and the median interval between blood transfusion and the onset of HCC was significantly associated with male sex. These results are consistent with previous reports of male sex and higher BMI as independent risk factors for HCC development in CHC patients.[9,44,45]

A limitation of the present study is its retrospective design. The histology samples at the time of initial treatment were obtained via ultrasound-guided aspiration at the time of percutaneous tumor ablation or surgical resection. To minimize the risk of bleeding, ultrasound-guided aspiration was not performed for patients with a platelet count of less than 6 ($\times 10^4/\mu L$). Therefore, the histological samples were collected from a biased group of patients. Another limitation is the cross-sectional study design and the lack of controls without HCC. We are unable to confirm whether the age at onset of HCC (primary outcome of the present study) is an adequate indicator of susceptibility to HCC from the current study alone. Further prospective study is needed to validate the current results.

In conclusion, the *PNPLA3* rs738409 C>G polymorphism may play a significant role in hepatocarcinogenesis in CHC patients. Thus, this genetic factor should be taken into consideration when determining a treatment strategy intended to prevent the future development of HCC in CHC patients.

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