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Ⅳ．研究成果の刊行物・別刷

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Original Article

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

Masaya Sato,[1] Naoya Kato,[2] Ryosuke Tateishi,[1] Ryosuke Muroyama,[2] Norie Kowatari,[2] Wenwen Li,[2] Kaku Goto,[2] Motoyuki Otsuka,[1] Shuichiro Shiina,[1] Haruhiko Yoshida,[1] Masao Omata[3] and Kazuhiko Koike[1]

[1]*Department of Gastroenterology, Graduate School of Medicine,* [2]*Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, and* [3]*Yamanashi Prefectural Hospital Organization, Kofu, Japan*

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]

Correspondence: Dr Naoya Kato, Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Email: kato-2im@ims.u-tokyo.ac.jp

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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.

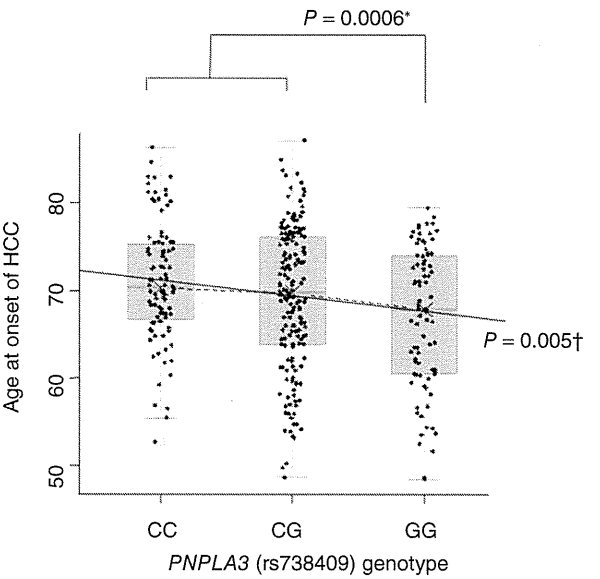

Figure 1 Box and whisker and dot plot: distributions of the age at onset of hepatocellular carcinoma (HCC) in each genotype. The dashed line connects the median value of each genotype, and the solid line shows the linear regression. The Jonckheere–Terpstra trend test showed a significant trend across 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.

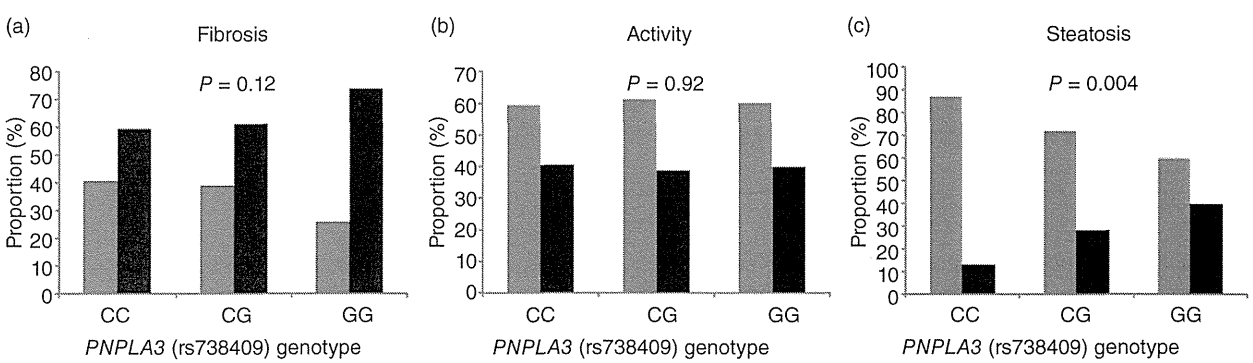

Figure 2 Bar plot: prevalence of fibrosis (F1–3 vs F4, a), necroinflammation (A1 vs A2–3, b) and steatosis (<5% vs ≥5%, c) in 235 patients with chronic hepatitis C. The proportions are shown on the Y axis. *P*-values of the frequency distributions are shown (Cochran–Armitage trend test). ■, F1–3; ■,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

IL28B minor allele is associated with a younger age of onset of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

Masaya Sato · Naoya Kato · Ryosuke Tateishi · Ryosuke Muroyama · Norie Kowatari · Wenwen Li · Kaku Goto · Motoyuki Otsuka · Shuichiro Shiina · Haruhiko Yoshida · Masao Omata · Kazuhiko Koike

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Abstract
Background IL28B polymorphisms were shown to be associated with a response to peg-interferon-based treatment in chronic hepatitis C (CHC) and spontaneous clearance. However, little is known about how this polymorphism affects the course of CHC, including the development of hepatocellular carcinoma (HCC). We evaluated the influence of IL28B polymorphisms on hepatocarcinogenesis in CHC patients.
Methods We genotyped the rs8099917 single-nucleotide polymorphism in 351 hepatitis C-associated HCC patients without history of IFN-based treatment, and correlated the age at onset of HCC in patients with each genotype.
Results Frequencies of TT, TG, and GG genotypes were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The mean ages at onset of HCC for TT, TG, and GG genotypes were 69.9, 67.5 and 66.8, respectively. In multivariate analysis, IL28B minor allele (TG and GG genotypes) was an independent risk factor for younger age at onset of HCC ($P = 0.02$) in males ($P < 0.001$) with higher body mass index (BMI; $P = 0.009$). The IL28B minor allele was also associated with a lower probability of having aspartate aminotransferase-to-platelet ratio index (APRI) >1.5 (minor vs. major, 46.7 vs. 58.6 %; $P = 0.01$), lower AST (69.1 vs. 77.7 IU/L, $P = 0.02$), lower ALT (67.8 vs. 80.9 IU/L, $P = 0.002$), higher platelet count (12.8 vs. 11.2 $\times$ 10^4/μL, $P = 0.002$), and higher prothrombin time (79.3 vs. 75.4 %, $P = 0.002$).
Conclusions The IL28B minor allele was associated with lower inflammatory activity and less progressed fibrosis of the liver; however, it constituted a risk factor for younger-age onset of HCC in CHC patients.

Keywords rs8099917 · Hepatocarcinogenesis · Interferon-λ · Risk allele · Fibrosis

Abbreviations

AFP	α-Fetoprotein
APRI	Aminotransferase platelet ratio index
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IL28B	Interleukin 28B
PCR	Polymerase chain reaction
peg-IFN	peg-Interferon
RIG- I	Retinoic acid-inducible gene-I
SNP	Single-nucleotide polymorphism
SVR	Sustained viral response
TLR3	Toll-like receptor 3

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Currently, patients with chronic

M. Sato · R. Tateishi · M. Otsuka · S. Shiina · H. Yoshida · K. Koike
Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

N. Kato (✉) · R. Muroyama · N. Kowatari · W. Li · K. Goto
Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
e-mail: kato-2im@ims.u-tokyo.ac.jp

M. Omata
Yamanashi Prefectural Hospital Organization, Kofu, Japan

hepatitis C (CHC) are treated with a combination of peg-interferon (peg-IFN) and ribavirin [2, 3]. Recently, HCV nonstructural 3/4A serine protease inhibitors combined with PEG-IFN and RBV were reported to achieve higher sustained viral response (SVR) rates in genotype 1 patients compared to conventional PEG-IFN/RBV. These triple therapies are considered to be the next standard of care for patients with CHC virus infection [4, 5].

Genetic variations near the interleukin 28B (IL28B) gene, encoding the type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [6–8] and also spontaneous clearance of HCV [9]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via the retinoic acid-inducible gene-I (RIG-I) and toll like receptor 3 (TLR3) and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [10, 11].

Genetic polymorphisms of genes involved in innate immunities are likely to influence the strength and nature of this defense system [12]. Besides its antiviral properties, IFN-λ exhibits antitumor activity; in fact, several experimental studies in cell lines and in animal models demonstrated that the activation of type III IFN induces apoptosis [13] and antitumor activities [14–16]. Thus, this genetic factor is thought to influence the natural course of HCV infection, including the development of HCC. However, little is known about the influence of IL28B polymorphisms on hepatocarcinogenesis in patients with CHC.

In the present study, we examined the association between the rs8099917 single-nucleotide polymorphism (SNP) at the IL28B locus with the age at onset of HCC and other clinical findings in patients with CHC who had no history of receiving IFN-based treatment.

Materials and methods

Patients

The patients analyzed in the present study were derived from an HCV study cohort of the University of Tokyo Hospital. In this cohort, we enrolled the patients who visited the liver clinic at our institute between August 1997 and April 2009, and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. All patients underwent laboratory blood tests at the time of enrollment in our cohort. The result of the blood tests were recorded with the information on alcohol consumption and BMI of each patient. The patients who were positive for hepatitis B surface antigen and had a history of biliary disease were excluded. All subjects in our cohort were Japanese, and this research project was approved by the ethics committees of the University of Tokyo (No. 400).

From this cohort, we examined the patients who had developed new-onset HCC and received initial therapy in our institute by January 31, 2010, and with available sample for genotyping. We excluded the patients with a history of receiving IFN-based treatment. Finally, 351 patients were enrolled for this study, and the association between the age at onset of HCC and the IL28B genotype was analyzed. Patient follow-up and Diagnosis of HCC was performed as previously described [17, 18].

IL28B genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the IL28B rs8099917 T/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 (FAM or HEX) 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). Samples were subjected to 50 cycles of denaturation for 15 s at 92 °C, annealing of primers for 30 s at 60 °C, and elongation for 30 s at 60 °C.

Study endpoint

We analyzed the relationship between the age at onset of HCC (the primary endpoint of this study) and host factors, including the IL28B genotypes, sex, BMI, alcoholic consumption, and HCV genotype. We also examined the relationship between IL28B genotypes and the clinical findings at the time of enrollment in our cohort (the secondary endpoint), such as the biochemical markers and presence of liver fibrosis. Liver biopsies were only available in a small number of patients (48); liver fibrosis was assessed using the aspartate aminotransferase platelet ratio index (APRI), and an APRI of >1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4) [19].

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD) while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the Chi square test, and stepwise logistic regression analyses were used to adjust the influence of IL28B genotype by other covariates such as sex, BMI (<25

or not), and alcoholic consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using the Student's t test or nonparametric Wilcoxon rank-sum test as appropriate. Since the age at onset of HCC (the primary endpoint of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis to adjust the influence of IL28B genotype by sex, BMI (<25 or not), and alcoholic consumption (<50 g/day or not). All statistical analyses were two-sided, and the threshold of the reported P values for significance was accepted as <0.05. All statistical analyses were performed using R 2.13.1 software (http://www.r-project.org).

Results

Patient characteristics

Patient characteristics are shown in Table 1. Frequencies of the rs8099917 TT, TG, and GG genotype were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The SNP genotype distribution was in Hardy–Weinberg equilibrium (P value was not significant). We defined the IL28 major genotype as homozygous for the major sequence (TT) and the IL28B minor genotype as homozygous (GG) or heterozygous (TG) for the minor sequence. The mean age at onset of the HCC patients was 69.3 years, and approximately 60 % were male. The mean age at the time of enrollment was 67.2 years and the follow-up period was 27.9 months in average.

Table 1 Clinical characteristics and genotype distributions in the study cohort ($n = 351$)

Parameter	Values
Mean age at onset of HCC, in years	69.26 ± 8.07
Mean age at the time of enrollment, in years	67.16 ± 8.32
Male sex	200 (57.0 %)
BMI >25	70 (20.0 %)
Alcohol consumption (>50 g/day)	75 (21.4 %)
IL28B genotype	
TT	261 (74.3 %)
TG	87 (24.8 %)
GG	3 (0.9 %)
T allele frequency	0.87
HCV genotype	
Genotype 1	240 (68.4 %)
Genotype 2	91 (25.9 %)
Not tested	20 (5.7 %)

Continuous variables were represented as the mean ± standard deviation (SD) and categorical variables were as number and frequencies (%)

Primary endpoint

Table 2 shows the age at onset of patients with HCC and the associations among IL28B genotypes, sex, BMI, alcohol consumption, and HCV genotype. The mean age at onset in patients with HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). In multivariate analysis, the age at onset of HCC was significantly younger in patients with the IL28B minor genotype ($P = 0.02$, Fig. 1), independently of male sex ($P < 0.001$) and higher BMI ($P = 0.009$). The characters of HCC, such as sizes (2.56 vs. 2.40 cm, $P = 0.41$) or the numbers (1.94 vs. 2.23, $P = 0.54$) at diagnosis were not significantly different between IL28B major and minor genotypes. We also analyzed the interval between blood transfusion and the onset of HCC in 161 patients who have histories of blood transfusion which had been the major cause of HCV infection in Japan [20]. The mean interval between blood transfusion and the onset of HCC for the IL28B major and minor genotypes were 39.09 ± 9.99 and 38.86 ± 9.27 years, respectively ($P = 0.9$; data not shown).

Secondary endpoint

Table 3 shows the clinical findings and associations between the IL28B genotypes at the time of enrollment in our cohort. The IL28B major genotype was significantly associated with a higher probability of having an APRI >1.5 (58.62 vs. 46.67 %, $P = 0.01$; Fig. 2), a lower platelet count (11.15 vs. 12.80 × 10^4/μL, $P = 0.002$), a higher AST level (77.69 vs. 69.12 IU/L, $P = 0.02$), a higher ALT level (80.92 vs. 67.79 IU/L, $P = 0.002$), and a lower prothrombin time (75.40 vs. 79.27 %, $P = 0.002$) compared to the IL28B minor genotype after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment of our cohort. A lower γ-GTP level was significantly associated with the IL28B major genotype in univariate analysis, and alcoholic consumption, sex, and age were stronger factors associated with the γ-GTP level. Thus, after adjustment for these factors, the IL28B genotype was not extracted as a significant factor associated with the γ-GTP level. Histological assessments of liver fibrosis were performed in 248 patients at the time of initial therapy. The prevalence of histologically proved liver cirrhosis (F4) was 65.6 % (118/180) in patients with major genotype and 51.5 % (35/68) in those with minor genotype. The prevalence of liver cirrhosis was significantly higher in patients with major genotype after adjustment for sex, BMI, alcoholic consumption, and the age at the time of initial therapy for HCC ($P = 0.045$, data not shown).

Table 2 Factors associated with the age at onset of HCC

Variable	Mean	Standard deviation (SD)	*P* value	
			Univariate	Multivariate[a]
IL28B genotype			0.02	0.02
Major (TT)	69.88	7.97		
Minor (TG/GG)	67.48	8.17		
Sex			<0.001	<0.001
Male	67.94	8.48		
Female	71.02	7.16		
BMI			0.01	0.009
>25	66.87	9.11		
≤25	69.86	7.70		
Alcohol consumption			0.11	–
>50 (g/day)	67.78	9.37		
≤50 (g/day)	69.67	7.65		
HCV genotype			0.29	–
Genotype 1	69.65	7.59		
Genotype 2	68.22	8.79		

[a] Stepwise regression analysis for the age at onset of HCC (the dependent variable) using IL28B genotype, sex, BMI, alcohol consumption, and HCV genotype as independent variables

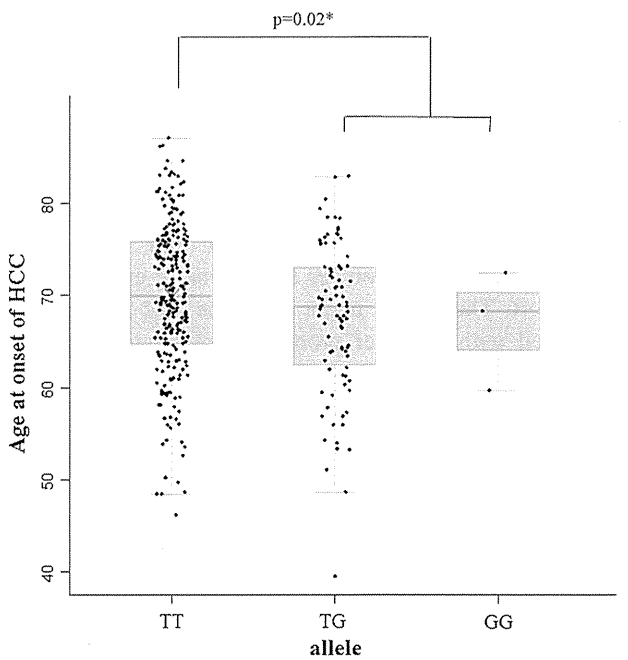

Fig. 1 *Box and whisker and dot plot* distributions of the age at onset of HCC in each genotype. The mean age at onset of HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and was significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). *P values after adjustment for sex, BMI, and alcoholic consumption

Discussion

In the present study, we evaluated the association between the IL28B polymorphism and the age at onset of HCC in patients with CHC. The IL28B minor genotype was significantly associated with younger age at onset of HCC with well known risk factors for the development of HCC such as male gender and higher BMI [21] without prior IFN-based treatment. Our previous study analyzing a susceptibility locus for HCV-induced HCC using a genome-wide association study (GWAS) could not detect the significant association between IL28B genotypes and the development of HCC in a cross-sectional distribution analysis between patients with and without HCC in more than 3,000 samples [22]. Also, IL28B alleles were not identified as a susceptibility locus for HCV-induced HCC in another GWAS study [23]. The cross-sectional distribution analyses may have underestimated the susceptibility to HCC because it could not take into consideration the future development of HCC and the duration after the past onset of HCC. Moreover, although GWAS would provide an effective and unbiased approach for revealing risk alleles for genetically complex non-Mendelian disorders, the risk of multiple comparisons made in a GWAS have resulted in reports of false positive results (Type 1 errors), and if the correction is overly conservative or the power is inadequate, false negative results (Type 2 errors) [24–26]. The relation between IL28B polymorphism and the susceptibility to HCC is still controversial. A previous study from Japan reported that the rs8099917 TT genotype was associated with a lower incidence of HCC even in non-responders to IFN based treatment [27] that was in agreement with the present study. Another study from Italy evaluating the association between genome frequency and the presence of cirrhosis due to hepatitis C, hepatitis B, alcohol use, and other factors also showed a higher prevalence of the IL28B minor allele in patients with HCC

Table 3 Associations between the IL28B genotype and clinical findings at the time of enrollment in our cohort

Variable	Mean/proportion (standard deviation; SD)		*P* values	
	Major (TT)	Minor (TG/GG)	*P* value	Adjusted *P* value[¶]
APRI >1.5[a]	58.62 % (52.38–64.66)	46.67 % (36.07–57.69)	0.07	0.01[‖]
Platelet count ($\times 10^4/\mu L$)	11.15 (5.00)	12.80 (5.43)	0.01	0.002**
AST (IU/L)	77.69 (45.14)	69.12 (38.16)	0.12	0.02**
ALT (IU/L)	80.92 (60.45)	67.79 (41.78)	0.17	0.002**
T.B (mg/dL)	0.90 (0.40)	0.83 (0.39)	0.02	–
Alb (g/dL)	3.69 (0.46)	3.71 (0.46)	0.9	–
ALP (IU/L)[b]	236.4 (81.75)	216.4 (58.96)	0.08	0.11**
γGTP (IU/L)[c]	76.83 (65.34)	87.23 (42.92)	0.005	–
PT (%)[d]	75.40 (13.36)	79.27 (13.13)	0.02	0.002**

[¶] Adjusted for sex, BMI, alcoholic consumption, and the age at enrollment (independent variables). The dependent variables of each *P* values are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, platelet count, AST, ALT and so on)

[‖] *P* value by stepwise logistic regression analysis

** *P* value by stepwise regression analysis

[a] Odds ratio (95 % CI) for major allele was 1.88 (1.13–3.11), and 95 % confidence interval (CI) of each proportion is parenthesized for this outcome

[b] Missing in 115 patients

[c] Missing in 112 patients

[d] Missing in 4 patients

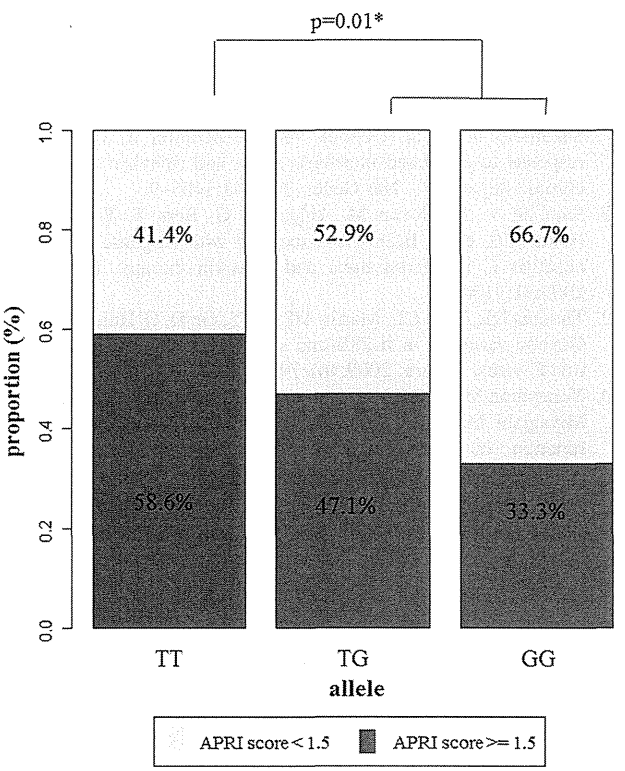

Fig. 2 *Bar plot* the proportion of having an AST-to-platelet ratio (APRI) score >1.5 in each allele. **P* values after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment

compared to those without HCC [28]. However, other studies showed no relation between IL28B polymorphism and the susceptibility to HCC [29–32]. Some studies have reported the HCV genotype 1 as a risk factor associated with HCC in patients who had CHC [33–35]; however, we could not find a significant association between the HCV genotype and hepatocarcinogenesis in the present study. Our data showed no relationship between the duration of HCV infection in the patients with a history of blood transfusion. The mean age of blood transfusion was not significantly different between patients with major and minor genotypes (28.99 in major genotype vs. 27.60 in minor genotype, $P = 0.18$). Moreover, older age at HCV infection was reported to be associated with more rapid disease progression [36]. Thus, the difference in the duration of HCV infection may have little effect on the result of the present study. The IL28B genotype may have a critical role in the onset of HCC. Moreover, only about 45 % of all patients in the present study have the history of blood transfusion; hence, further analysis with larger samples may be indicated.

Previous studies evaluating patients with chronic HCV infection showed severer histological inflammatory activity and fibrosis, as well as higher ALT levels and APRI scores in patients homozygous for the IL28B major alleles [29, 32, 37, 38]. Similarly, in the present study, the IL28B

major genotype was significantly associated with a higher probability of having an APRI >1.5 and a higher ALT level; and the prevalence of histologically proved liver cirrhosis (F4) was significantly higher in patients with major genotype at the age at the time of initial therapy for HCC. Given the association between the IL28B major allele and the severe inflammatory activity or progressed fibrosis, the IL28B allele is thought to be associated with the susceptibility to HCC via a mechanism that is independent of controlling an activity of HCV infection.

Recent experimental studies have suggested that IFN-λ has an antitumor activity. In esophageal cancer cell lines expressing IFN-λ receptor complexes, IFN-λ1 suppressed growth via the induction of the G1 phase arrest or apoptosis [39]. An antitumor activity of IFN-λ was also shown in the B16 melanoma, BNL hepatoma, Colon 26, and neuroendocrine BON1 tumor cells [40–43]. One probable explanation for the paradoxical result of the present study is that the more aggressive inflammatory activity of patients with IL28B major genotype may reflect a stronger immune response to the virus, which may also have anti-tumor effects. However, the innate immune responses and antitumor activity via IFN-λ, as well as the mechanism underlying the association of the IL28B genotype, have not been elucidated. Further studies are needed to determine the functional role of the IL28B gene in relation to the course of chronic HCV infection, including hepatocarcinogenesis.

Because of the retrospective design, this study is limited by the absence of some important clinical details such as information about the histological findings of fibrosis and inflammation. Although the APRI is a useful index for the prediction of fibrosis, the limitation of this score has been reported in previous studies [44, 45]. Prospectively designed studies are needed to confirm our findings. However, observing chronic HCV-infected patients without antiviral treatment would be nearly impossible in the future. In this regard, the present study may have important implications.

In conclusion, the IL28B minor genotype was associated with a younger age of onset of HCC in patients with CHC, and this association was completely independent of the response to IFN-based treatment. Hepatocarcinogenesis appeared to be suppressed in patients who had CHC with the IL28B major genotype, despite higher inflammatory activity and progressed fibrosis of liver. The current findings may provide a clinically important information in the follow-up or HCC screening of cirrhotic patients.

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Conflict of interest None of the authors have any conflicts of interest.

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Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis

Masaya Sato[1], Mayuko Kondo[1], Ryosuke Tateishi[1]*, Naoto Fujiwara[1], Naoya Kato[2], Haruhiko Yoshida[1], Masataka Taguri[3], Kazuhiko Koike[1]

1 Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, **2** Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan, **3** Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Kanagawa, Japan

Abstract

Background & Aims: IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression.

Methods: A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses.

Results: We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003–1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988–1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050–1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110–1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351–0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415–2.717; P = 0.901).

Conclusions: IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs8099917 TT genotypes.

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* E-mail: tateishi-tky@umin.ac.jp

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, coinfection with human immune deficiency virus, low CD4 count, male gender, and HCV genotype 3 have been shown to be associated with histological activity [2–7]. Although these factors explain part of the extreme variability seen in the progression of fibrosis among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in chronic hepatitis C (CHC) [8–10]. Two genome-wide association studies recently reported the susceptible loci for the progression of liver cirrhosis [11,12].

Currently, patients with CHC are treated with a combination of peg-interferon (peg-IFN) and ribavirin [13,14]. Telaprevir and boceprevir, two protease inhibitors, were recently approved for patients with genotype 1 in combination with peg-IFN and ribavirin. This combination has been shown to lead to substantial improvement in the sustained virologic response rate [15,16]. Genetic variations near the interleukin 28B (IL28B) gene, encoding type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [17–20] and with spontaneous clearance of HCV [21]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via retinoic acid-inducible gene-I and toll-like receptor 3 and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [22,23].