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

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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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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].

Polymorphisms of genes involved in innate immunity are likely to influence the strength and nature of this defense system [24]. Moreover, IL28B polymorphisms were shown to be associated with lipid metabolism [25]. Thus, this genetic factor is thought to influence the natural course of HCV infection including liver fibrosis, inflammation activity, or steatosis. However, associations between IL28B polymorphisms and the state of background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC remain controversial. Single studies may have limited statistical power to detect the modest effects of IL28B polymorphisms on disease progression.

Thus, we conducted the present meta-analysis to integrate the results of eligible studies and provide statistically reliable evidence of the role of IL28B polymorphisms in patients with CHC.

Materials and Methods

2.1 Search strategy

An electronic search was conducted in MEDLINE, EMBASE, and the Cochrane Library for articles published prior to 30 April, 2012. Search terms included *IL28B*, *IL28*, *IL-28B*, *interleukin-28B*, *interleukin 28B*, *rs12979860*, and *rs8099917*. The search was limited to the English language.

2.2 Inclusion criteria

A study was included in the current analysis if it satisfied the following criteria: (1) It evaluated the associations between IL28B polymorphisms (rs12979860 or rs8099917) and liver fibrosis, inflammation activity, or steatosis. We also included studies that evaluated fibrosis or inflammation activity using the aminotransferase platelet ratio index or ALT. (2) It provided sufficient published data for estimating odds ratios (OR) with 95% confidence intervals (CIs). In case of multiple studies based on the same population, we selected the study with the largest number of participants. A study was excluded if (1) it dealt only with co-infection of HCV and human immunodeficiency virus, (2) it dealt only with patients with a specific condition such as a comorbid disease (e.g., thalassemia) or status after liver transplantation, or (3) it only used a recessive hereditary model (rs12979860 CC + CT vs. TT, or rs8099917 TT +TG vs. GG).

2.3 Data extraction

Two authors (M.S. and M.K.) independently screened titles and abstracts for potential eligibility and full texts for final eligibility. Disagreements were resolved by consultation with a third author (R.T.). The following information was extracted or calculated from each study: first author, year of publication, country of origin, ethnicity, sex, HCV genotype, and background liver information (fibrosis, inflammation activity, or steatosis) for each genotype. The analysis was based on the dominant model (CC vs. CT and TT in rs12979860; TT vs. TG and GG in rs8099917).

2.4 Definition

In some studies, mild or severe fibrosis or inflammation activity was not defined. To compare results among studies on these outcomes, we defined Ishak level F4 to F6; METAVIR, Ludwig Batts, and Inuyama level F3 to F4; and Knodell histology activity index as severe fibrosis. We also defined METAVIR A2 to A3 as severe inflammation activity.

2.5 Statistical analysis

The association of liver fibrosis, inflammation activity, or steatosis with the IL28B genotype in patients with CHC was assessed by summary ORs and corresponding 95% CIs. Heterogeneity among studies was examined with I^2 statistics interpreted as the proportion of total variation contributed by between-study variation [26]. If there was no or low statistical heterogeneity among studies ($I^2<50\%$ and $P>0.05$), the ORs and 95% CIs were calculated by the fixed-effects model. Otherwise, the random-effects model was adopted. When significant heterogeneity was observed, we performed a meta-regression analysis to investigate relationships between the effect of IL28B polymorphisms on liver fibrosis, inflammation activity, or steatosis; and continuous variables (proportion of patients with genotype 1 or 4 virus infection, proportion of males; and proportion of Caucasian, African-American, and Asian patients) to explore the possible reason for heterogeneity between studies [27,28]. To check for publication bias, we used the linear regression approach described by Egger et al. [29]. All calculations were performed using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ).

Results

3.1 Characteristics of articles

Figure 1 shows the literature search and study selection procedures. A total of 471 potentially relevant publications up to 30 April, 2012, were initially identified through MEDLINE, EMBASE, and the Cochrane Library, 443 of which were excluded because they did not meet our inclusion criteria. Therefore, 28 studies involving a total number of 10,024 patients were included in the meta-analysis. Study characteristics are shown in Table 1. There were 5616 males and 3974 females, and the sex was not reported in the remaining 434 patients (1 study). Nineteen studies (7542 patients) evaluated liver fibrosis according to rs12979860 polymorphism and 16 studies (5052 patients) according to rs8099917 polymorphism; four studies (2301 patients) evaluated inflammation activity according to rs12979860 polymorphism and eight studies (2904 patients) according to rs8099917 polymorphism; and four studies (962 patients) evaluated steatosis according to rs12979860 polymorphism and five studies (1308 patients) according to rs8099917 polymorphism.

3.2 Fibrosis

For rs12979860, the between-study heterogeneity was not significant ($I^2=25\%$, $P=0.147$); thus, the fixed-effects model was applied. The pooled results indicated that IL28B rs12979860 genotype CC was associated with an increased possibility of severe fibrosis (OR, 1.122; 95%CI, 1.003–1.254; $P=0.044$) (Fig. 2-a). For rs8099917, there was no or low heterogeneity ($I^2=31\%$, $P=0.111$), and IL28B rs8099917 genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference did not reach statistical significance (OR, 1.126; 95%CI, 0.988–1.284; $P=0.076$) (Fig. 2-b). Egger's test showed no evidence for publication biases for either rs12979860 ($P=0.839$) or rs8099917 ($P=0.342$). When restricted to studies in which only treatment-naïve patients were included, 12 studies (5865 patients) according to rs12979860 polymorphism and eight studies (3333 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2=0\%$, $P=0.615$) and rs8099917 ($I^2=16\%$, $P=0.304$). For rs12979860, fixed-effect model analyses showed a higher probability of severe fibrosis in genotype CC (OR, 1.184; 95%CI, 1.040–1.348; $P=0.010$) (Fig. 2-c), and for rs8099917, genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference was not statistically significant (OR, 1.154; 95%CI, 0.985–1.351; $P=0.076$) (Fig. 2-d). Egger's test showed no evidence of publication bias ($P=0.394$ for rs12979860 and $P=0.295$ for rs8099917).

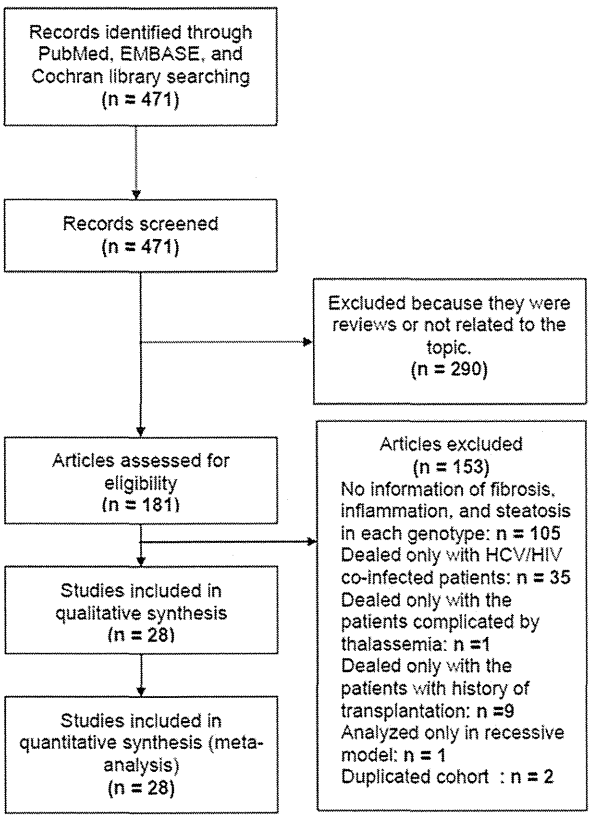

Figure 1. Literature search and study selection process. Twenty-eight individual studies that met all of the inclusion and exclusion criteria.
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3.3 Inflammation activity

The between-study heterogeneity was not significant ($I^2 = 35\%$, $P = 0.204$) for rs12979860. In the fixed-effects model, the pooled results indicated that IL28B rs12979860 genotype CC was associated with a higher possibility of severe inflammation activity (OR, 1.288; 95%CI, 1.050–1.581; $P = 0.015$) (Fig. 3-a). For rs8099917, there was no or low heterogeneity ($I^2 = 0\%$, $P = 0.598$), and IL28B rs8099917 genotype TT was also associated with a higher possibility of severe inflammation activity (OR, 1.324; 95%CI, 1.110–1.579; $P = 0.002$) (Fig. 3-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.448$) and rs8099917 ($P = 0.531$). When restricted to studies in which only treatment-naïve patients were included, three studies (2192 patients) according to rs12979860 polymorphism and two studies (1769 patients) according to rs8099917 polymorphism were extracted. Significant heterogeneities were found for rs12979860 ($I^2 = 53\%$, $P = 0.120$); thus, the random-effect model was applied. The pooled results indicated that IL28B rs12979860 genotype was not associated with inflammatory activity (OR, 1.340; 95%CI, 0.938–1.916; $P = 0.108$) (Fig. 3-c). For rs8099917, the between-study heterogeneity was not significant ($I^2 = 0\%$, $P = 0.585$). In the fixed-effects model, genotype TT tended to be associated with a higher possibility of severe inflammation activity (OR, 1.217; 95%CI, 0.978–1.515; $P = 0.079$) (Fig. 3-d). Egger's test showed no evidence of publication bias in rs12979860 ($P = 0.646$). For rs8099917, Egger's test was not applicable because only 2 studies were included. We also performed a meta-regression analysis for rs12979860 because significant heterogeneities were observed. Table 2 shows the results of these meta-regression analyses. Significant correlation was observed between rs12979860 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, 2.992 ± 1.497; $P = 0.046$).

3.4 Steatosis

Significant heterogeneities were found for rs12979860 ($I^2 = 86\%$, $P<0.001$) and rs8099917 ($I^2 = 52\%$, $P = 0.082$); thus, we applied the random-effects model for this outcome. The pooled results indicated that IL28B rs12979860 genotype CC was not associated with hepatic steatosis (OR, 1.062; 95%CI, 0.415–2.717, $P = 0.901$) (Fig. 4-a), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.580; 95%CI, 0.351–0.959; $P = 0.034$) (Fig. 4-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.238$) or rs8099917 ($P = 0.182$). We also performed a meta-regression analysis because significant heterogeneities were observed. Table 3 shows the results of these meta-regression analyses. In terms of the effect of rs12979860 on steatosis, significant correlations were observed between the proportion of patients with genotype 1 or 4 virus (slope, -4.947 ± 1.086; $P<0.001$), the proportion of Caucasian patients (slope, 7.361 ± 1.569; $P<0.001$), and the proportion of African-American patients (slope, -8.996 ± 1.918; $P<0.001$). We also observed a significant correlation between the effect of rs8099917 polymorphism on steatosis and the proportion of male patients (slope, 6.225 ± 2.530; $P = 0.014$) (Fig. 5). Finally, we observed significant correlations between rs8099917 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, -2.704 ± 1.277; $P = 0.034$), the proportion of Caucasian patients (slope, 1.168 ± 0.422; $P = 0.006$), and the proportion of Asian patients (slope, -1.049 ± 0.398; $P = 0.008$). When restricted to studies in which only treatment-naïve patients were included, two studies (495 patients) according to rs12979860 polymorphism and four studies (812 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.823$) and rs8099917 ($I^2 = 41\%$, $P = 0.166$). For rs12979860, fixed-effect model analyses showed that rs12979860 genotype CC was significantly associated with a higher possibility of severe steatosis (OR, 1.708; 95%CI, 1.047–2.787; $P = 0.032$) (Fig. 4-c), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.675; 95%CI, 0.474–0.960; $P = 0.026$) (Fig. 4-d). Egger's test showed no evidence of publication bias in rs8099917 ($P = 0.554$). For rs12979860, Egger's test was not applicable because only 2 studies were included.

Discussion

In the present study, we evaluated the association between IL28B polymorphisms and the background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC. The rs12979860 CC genotype was significantly associated with a higher probability of severe fibrosis (Fig. 2-c), and the rs8099917 TT genotype tended to be associated with a higher possibility of severe fibrosis (Fig. 2-d). The accumulation of liver inflammation promotes liver fibrosis, and these polymorphisms are associated with the effect of IFN-based treatment; therefore, past treatment might alter the results. Thus, we also analyzed studies involving only patients without a history of IFN-based treatment; however, the results were not changed.

The rs12979860 CC and rs8099917 TT genotypes were also associated with a higher possibility of severe inflammation activity. Genetic variations near the IL28B gene were originally reported as

Table 1. Main characteristics of all studies included in the meta-analysis.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Abe (2010)	[48]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	212	152	364	1/2			265	99
Honda (2010)	[49]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	58	33	91	1			60	31
Lotrich (2010)	[50]	Mixed (African-American/Caucasian), USA	rs12979860 C/T	F: Ishak	101	32	133	1/2	57	76		
Monte (2010)	[51]	Caucasian, Spain	rs12979860 C/T	F: Scheuer	166	117	283	1–4	129	154		
Thompson (2010)	[52]	Mixed (African-American/Caucasian/Asian/Hispanic), USA	rs12979860 C/T	F: METAVIR	986	642	1628	1	538	1090		
Bochud (2011)	[53]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F: Ishak, A: ALT S: Histological finding	163	79	242	1–3	90	150	150	92
Dill MT (2011)	[54]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	30	79	109	1–4	33	96	52	57
Fabris (2011)	[44]	Caucasian, Italy	rs12979860 C/T	F: Ishak	N.A	N.A	434	1–4	133	301		
Falleti (2011)	[55]	Caucasian, Italy	rs12979860 C/T	F: Ishak	357	272	629	1–4	205	424		
Kurosaki (2011)	[56]	Asian, Japan	rs8099917 T/G	F: METAVIR S: Histological finding	250	246	496	1			269	106
Lagging (2011)	[57]	Caucasian, Sweden	rs12979860 C/T rs8099917 T/G	F: Ishak S: Histological finding	169	83	252	1–4	93	159	153	99
Lin (2011)	[58]	Asian, Taiwan	rs12979860 C/T rs8099917 T/G	F: METAVIR	123	68	191	1	171	20	170	21
Lindh (2011)-1	[59]	Mixed (Caucasian/Asian), Sweden	rs12979860 C/T rs8099917 T/G	F: Batts Ludwig	67	43	110	1	38	72	66	44
Lindh (2011)-2	[60]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	204	137	341	2/3	150	191		
Marabita (2011)	[61]	Caucasian, Italy	rs12979860 C/T rs8099917 T/G	F: Ishak	129	118	247	1–4	88	159	131	116
Miyamura (2011)	[62]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	37	42	79	1			53	26
Moghaddam(2011)	[63]	Caucasian, Norway	rs12979860 C/T rs8099917 T/G	F: APRI score	166	115	281	3	129	152	201	80
Rueda (2011)	[64]	Caucasian, Spain	rs12979860 C/T	F, A: Scheuer	246	177	423	1–4	83	184		
Tillman (2011)	[35]	Mixed (African-American/Caucasian/Asian), USA	rs12979860 C/T rs8099917 T/G	S: Histological finding	215	110	325	1	88	237	97	67
Yu (2011)	[65]	Asian, Taiwan	rs8099917 T/G	F: Knodell and Scheuer	264	218	482	2			315	34
Asahina (2011)	[66]	Asian, Japan	rs12979860 C/T rs8099917 T/G	F: Inuyama	28	60	88	1	54	34	54	34

Table 1. Cont.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Bochud (2012)	[47]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	870	657	1527	1–4	534	993	855	672
Mach (2012)	[67]	Slav: Poland	rs12979860 C/T	F: Batts Ludwig	82	60	142	1	38	104		
Miyashita (2012)	[68]	Asian, Japan	rs8099917 T/G	F, A: Desmet	88	132	220	1/2			155	63
Ohnishi (2012)	[69]	Asian, Japan	rs8099917 T/G	S: Histological finding	83	70	153	1			116	37
Rembeck (2012)	[70]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	199	140	339	2/3	144	179		
Tolmane (2012)	[71]	Caucasian, Latvia	rs12979860 C/T	F: Knodell histology activity index S: Histological finding	84	58	142	1–3	41	80		
Toyoda (2012)	[72]	Asian, Japan	rs8099917 T/G	F, A: METAVIR	139	133	272	1			187	59

*Patients included in the original study.
Thus, patients without information regarding IL28B polymorphism were also included.
APRI, aminotransferase platelet ratio index.
doi:10.1371/journal.pone.0091822.t001

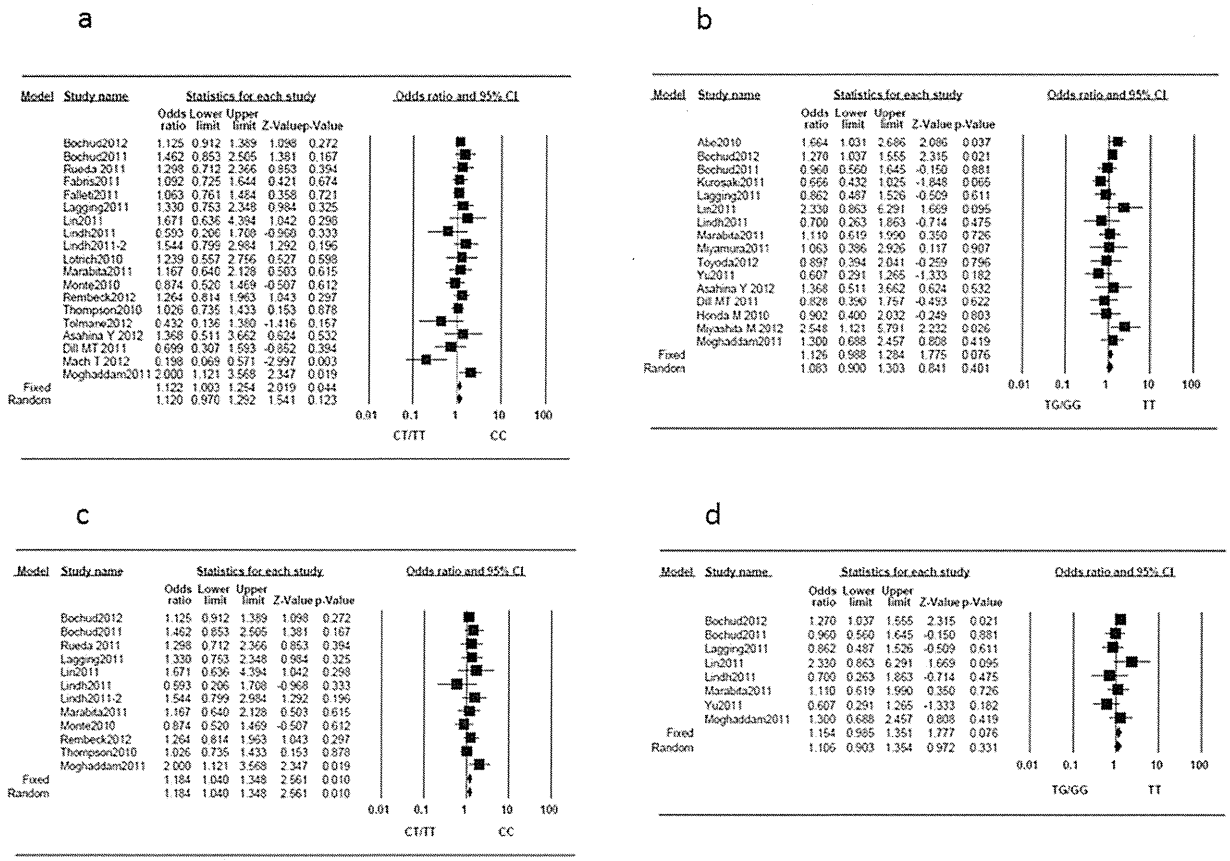

Figure 2. Forest plot of the IL28B genotypes and the risk of severe fibrosis. (a) rs12979860 in all patients, (b) rs8099917 in all patients, (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g002

strong predictors of a sustained viral response [17–20] or spontaneous clearance of HCV [21]. The level of IL28B gene transcripts is reportedly higher in patients homozygous for the IFN responsive allele [18,19]. Therefore, in patients with the rs12979860 CC and rs8099917 TT genotype, IL28B production, which induces expression of interferon-stimulated genes, including some inflammatory cytokines, was thought to be increased. This may be the underlying cause of the higher inflammation activity and progressed fibrosis in patients with the IFN responsive allele. In analysis with the studies involving only patients without a history of IFN-based treatment, rs12979860 CC and rs8099917 TT genotypes were associated with higher possibility of having severe inflammation activity; however, the differences did not reach to the significant level. Only three studies according to rs12979860 polymorphism and two studies according to rs8099917 polymorphism were included when restricted to studies with only treatment-naïve patients, and may be underpowered to detect the effects of IL28B polymorphisms on inflammation activity. The further analyses with larger sample are needed to confirm this association. Additionally, meta-regression analysis showed that the effect of the rs12979860 polymorphism was influenced by viral genotype distribution. This result may imply a different influence of rs12979860 polymorphism on immune response according to viral genotype in treatment-naïve patients.

IL28B polymorphisms were also shown to be associated with lipid metabolism [25]. In the present study, the rs8099917 TT genotype was significantly associated with a lower possibility of severe steatosis. This association still remained statistically significant after we restricted to studies in which only treatment-naïve patients were included. The lower hepatic steatosis in patients with the IFN responsive allele could be explained by a more efficient export of lipids from hepatocytes. Higher interferon expression was shown to lead to suppression of lipoprotein lipase, which would result in decreased conversion of VLDL to LDL and subsequent higher steatosis [30–33]. The difference in IL28B expression might cause an aberration of lipid metabolism in patients with CHC. We found no significant association of rs12979860 with steatosis. And when we restricted to treatment-naïve patients, rs12979860 CC genotype was significantly associated with a higher possibility of severe steatosis. Previous studies have shown that racial differences or viral genotypes make a difference in the effects of rs12979860 and rs8099917 polymorphisms [34,35]. This may explain the discrepancy between the effect of rs12979860 and rs8099917 on hepatic steatosis. However, only four studies (962 patients) were included in the analysis of rs12979860; or when it comes to the studies with only treatment-naïve patients, only two studies (495 patients) were extracted. Thus, we should not make any definite conclusion on this matter right now. Further studies with larger sample sizes are needed to identify their exact correlation.

According to the meta-regression analysis, the effect of rs8099917 polymorphisms on steatosis became smaller with the

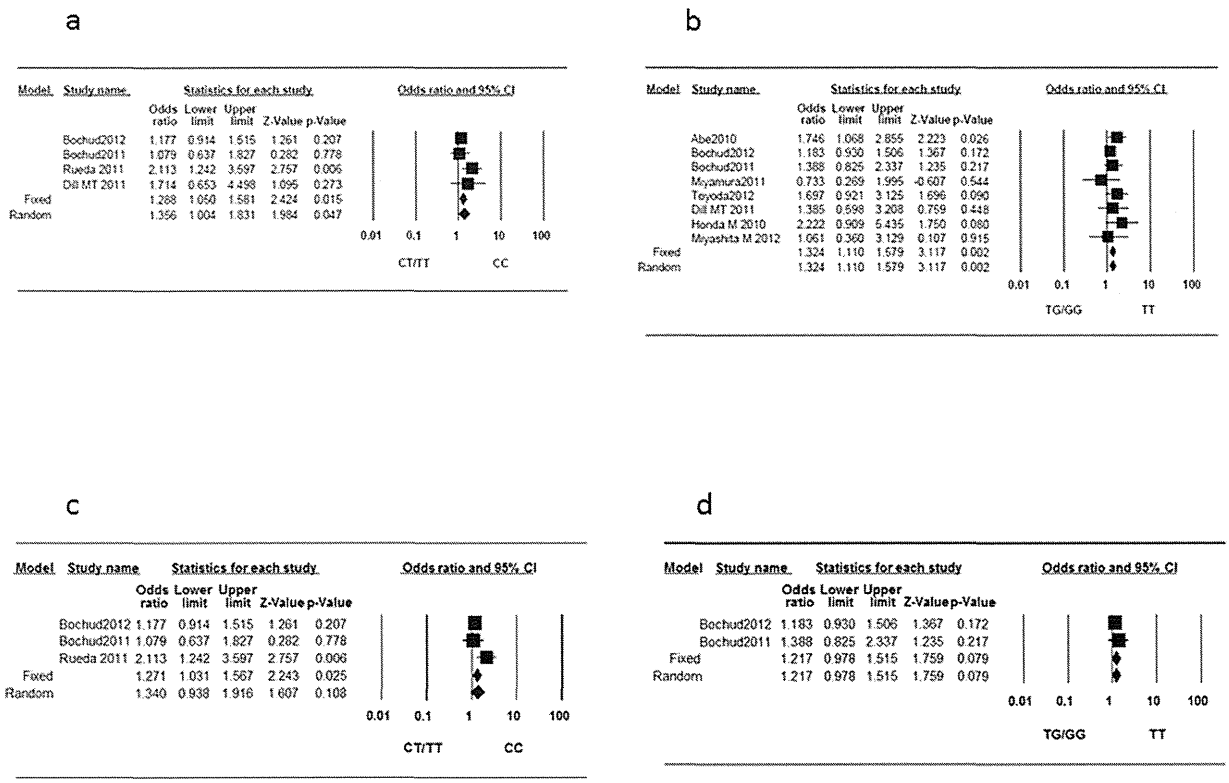

Figure 3. Forest plot of the IL28B genotypes and the risk of severe inflammation activity. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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increase in the male proportion (Fig. 5), suggesting that a sexual dimorphism might be involved in the effect of rs8099917 polymorphisms on the liver fat content. Although the present study cannot explain the interaction between the polymorphism and sex, immune systems responding to IFN are reportedly controlled by estrogenic sex hormones [36,37]. Differences in IL28B expression mediated by sex hormones could be a possible mechanism for the sexual dimorphism in the effect of rs8099917 polymorphisms on liver steatosis.

The rs738409 genotype within the patatin-like phospholipase domain containing 3 locus was also reported to be associated with hepatic steatosis in patients with CHC [38–40]. Notably, previous meta-analysis evaluating the effect of patatin-like phospholipase domain containing 3 polymorphisms on steatosis also reported a

Table 2. Meta-regression analysis between each continuous variable among the studies (only treatment- naïve patients were included) and the effect (log odds ratio) of IL28B polymorphisms on inflammation activity.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	**2.992**	**1.497**	**0.046**
Proportion of male patients, per 1% increase			
rs12979860	**−2.963**	**5.802**	**0.610**
Proportion of Caucasian patients, per 1% increase			
rs12979860†	–	–	–
Proportion of African-American patients, per 1% increase			
rs12979860†	–	–	–
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC genotype with severe inflammation activity are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform meta-regression analyses for these outcomes because only caucasian patients were included in all 3 studies included in this analysis.
doi:10.1371/journal.pone.0091822.t002

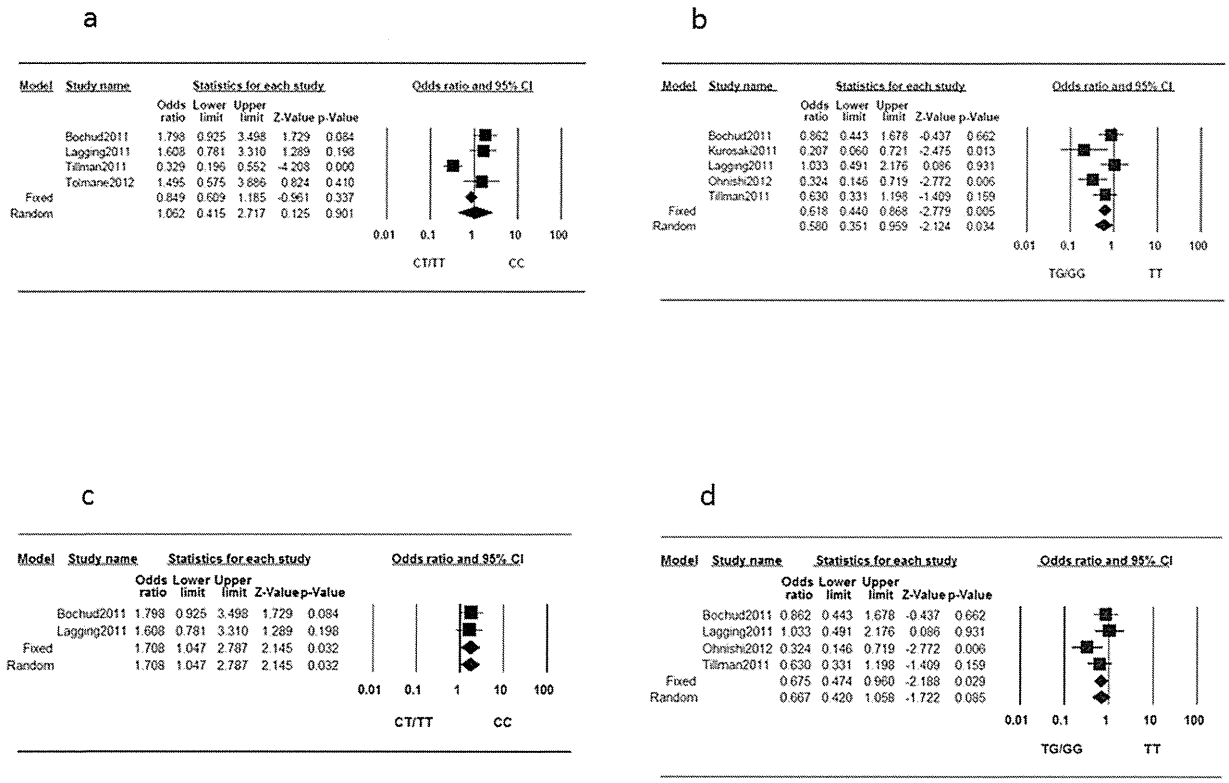

Figure 4. Forest plot of the IL28B genotypes and the risk of hepatic steatosis. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g004

Table 3. Meta-regression analysis between each continuous variable among the studies and the effect (log odds ratio) of IL28B polymorphisms on steatosis.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	−4.947	1.086	<0.001
rs8099917	−2.704	1.277	0.034
Proportion of male patients, per 1% increase			
rs12979860	−2.899	16.577	0.861
rs8099917	6.225	2.530	0.014
Proportion of Caucasian patients, per 1% increase			
rs12979860	7.361	1.569	<0.001
rs8099917	1.168	0.422	0.006
Proportion of African-American patients, per 1% increase			
rs12979860	−8.996	1.918	<0.001
rs8099917	0.142	2.147	0.947
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–
rs8099917	−1.049	0.398	0.008

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC or rs8099917 TT genotypes with severe steatosis are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform a meta-regression analysis for this outcome because only one patient was included in the corresponding studies.
doi:10.1371/journal.pone.0091822.t003

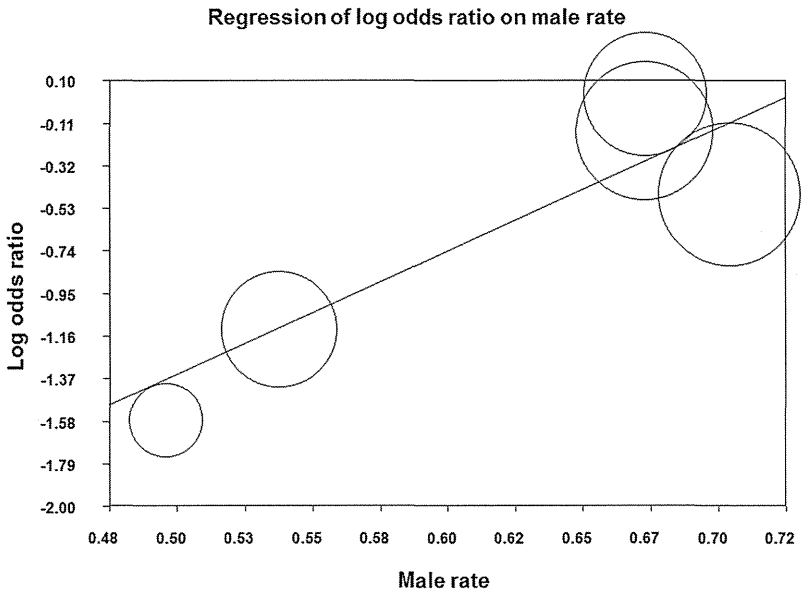

Figure 5. Meta-regression plot for log odds ratios in rates of patients with severe hepatic steatosis by proportion of males (%) in rs8099917.
doi:10.1371/journal.pone.0091822.g005

negative correlation between the male proportion and the effect of rs738409 on the liver fat content in nonalcoholic fatty liver disease [41]. Interestingly, the meta-regression analysis in the present study showed that the effect of the IL28B (rs12979860 and rs8099917) polymorphisms on steatosis was also influenced by racial and viral genotype distributions.

In the present study, we included studies that did not report the associations between IL28B genotypes and background liver diseases as study outcomes, but provided raw data that allowed us to calculate the OR for each outcome, which may have minimized potential publication bias. In fact, no publication bias was observed in the present study. The Human Genome Epidemiology Network highlighted the necessity of meta-analysis before evidence for a particular association can be regarded as strong [42]. The impact of IL28B genotypes on the disease progression found in the present meta-analysis may provide clinically important information in the follow-up of patients with CHC. The effect of IL28B polymorphisms on hepatocarcinogenesis, which is also crucial information in the HCC screening of patients with CHC, remains controversial [43–47]. Further analysis with larger sample sizes may be needed to elucidate the exact effect of IL28B polymorphisms on hepatocarcinogenesis.

A potential limitation of this study is inter-study variability in the outcome measure and the definition of "severe" among studies, where some discrepancies among studies exist. The studies without a pathological diagnosis, using laboratory data as surrogates, were also included. These studies may have diminished the accuracy of our research results concerning liver disease severity.

In conclusion, the present study highlighted the impact of IL28B polymorphisms on liver fibrosis, inflammation activity, and steatosis in patients with CHC. Disease progression appeared to be promoted in patients with rs12979860 CC or rs8099917 TT genotypes. The current findings may provide clinically important information in the follow-up of patients with CHC.

Supporting Information

Checklist S1 PRISMA 2009 Checklist.
(DOC)

Acknowledgments

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IWcYpT.

Author Contributions

Conceived and designed the experiments: MS RT NK. Performed the experiments: MS MK RT. Analyzed the data: MS RT. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS RT HY. Critical revision of manuscript: NF MT KK.

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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.

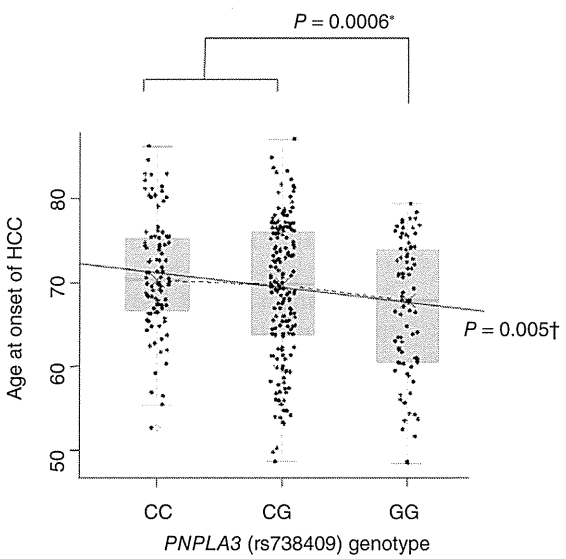

Figure 1 Box and whisker and dot plot: distributions of the age at onset of hepatocellular carcinoma (HCC) in each genotype. The dashed line connects the median value of each genotype, and the solid line shows the linear regression. The Jonckheere–Terpstra trend test showed a significant trend across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

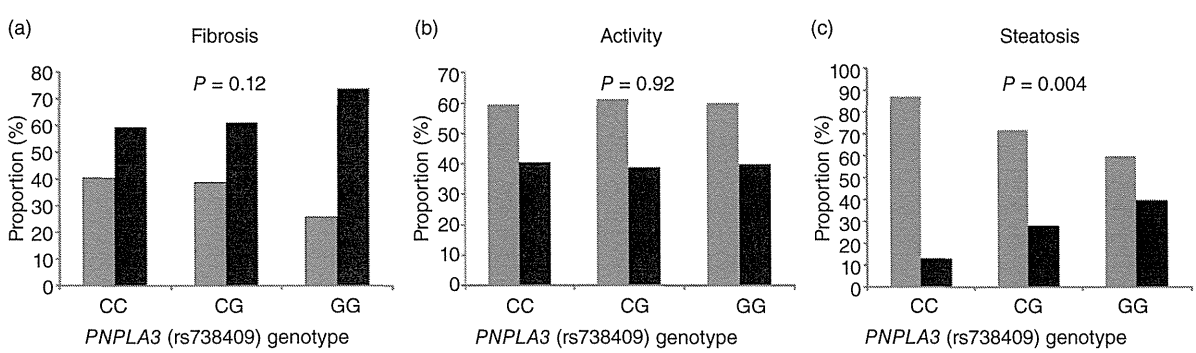

Figure 2 Bar plot: prevalence of fibrosis (F1–3 vs F4, a), necroinflammation (A1 vs A2–3, b) and steatosis (<5% vs ≥5%, c) in 235 patients with chronic hepatitis C. The proportions are shown on the Y axis. *P*-values of the frequency distributions are shown (Cochran–Armitage trend test). ■, F1–3; ■,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