Research Article

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Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients

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Background & Aims: A common genetic variation at the IL28 locus has been found to affect the response of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. An allele associated with a favorable response (rs8099917 T), which is the major allele in the majority of Asian, American, and European populations, has also been found to be associated with spontaneous eradication of the virus.
Methods: As no studies have yet analyzed the effect of the polymorphism on biochemical and inflammatory changes in chronic infection, we analyzed a cohort of patients with chronic hepatitis C ($n = 364$) for the effect of the IL28 polymorphism on viral, biochemical, and histological findings.
Results: We found that the proportion of HCV wild type core amino acids 70 and 91 was significantly greater ($p = 1.21 \times 10^{-4}$ and 0.034) and levels of gamma-GTP significantly lower ($p = 0.001$) in patients homozygous for the IL28 major allele. We also found that inflammation activity and fibrosis of the liver were significantly more severe in patients homozygous for the IL28 major allele ($p = 0.025$ and 0.036, respectively). Although the higher gamma-GTP levels were also associated with higher inflammatory activity and fibrosis, multivariate analysis showed that only the IL28 allele polymorphism, sex, alcohol consumption, and liver fibrosis were independently associated with gamma-GTP levels ($p = 0.001$, 0.0003, 0.0013, and 0.0348, respectively).
Conclusions: These results suggest that different cytokine profiles induced by the IL28 polymorphism resulted in different biochemical and inflammatory conditions during chronic HCV infection and contribute to the progression of liver diseases.
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Introduction

Hepatitis C virus infection is one of the major causative agents of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. The best current therapeutic regimen is pegylated interferon and ribavirin combination therapy [2,3]. Although the eradication rate of the virus has been improved by extending the treatment period from the standard 48 to 72 weeks for genotype 1b infected patients, active viral replication still remains in nearly half of these patients [4].

Recent studies have identified both host and viral factors predictive of interferon therapy. Among the viral factors, a forty amino acid stretch in the NS5 region has been found to be predictive of response to interferon monotherapy [5,6]. More recently, Akuta et al. identified amino acid substitutions in the core region (core aa70 and 91) that are predictive for the effect of interferon and ribavirin combination therapy [7,8].

Among the host factors, many common polymorphisms in the human genome, including single nucleotide polymorphisms (SNP), have been identified [9–13]. We recently reported that a SNP in the MAPKAPK3 gene is associated with response to interferon therapy [14]. More recently, three groups of researchers found that several SNPs in the IL28 locus are related to the effectiveness of combination therapy [15–17]. We also performed a genome wide association study and confirmed that variation at the IL28 locus is related to the effectiveness of combination therapy (Chayama K, personal communication).

Keywords: IL28; SNP; Histological activity; Inflammation; gamma-GTP.
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Abbreviations: HCV, hepatitis C virus; SNP, single nucleotide polymorphism; ISDR, interferon sensitivity determining region; BMI, body mass index.

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These viral and host factors must influence the natural course of viral infection. Host immune cells produce interferon and other cytokines in response to viral infection. For RNA viruses such as HCV, cellular sensors such as RIG-I detect the double stranded RNA and activate a pathway to produce cytokines, including alpha and beta interferons that trigger an antiviral response to eradicate the virus [18]. Genetic polymorphism of genes involved in innate immunity is likely to influence the strength and nature of this defense. In fact, a polymorphism in the IL28 locus has been reported to correlate with spontaneous eradication of HCV [19]. However, little is known about how these factors affect the course of chronic infection of the virus. In this study, we focused on histological findings in the liver. We also analyzed viral and biochemical factors in patients chronically infected with HCV. We found that histological aspects of the liver (fibrosis and activity), HCV core amino acid substitutions, and gamma-GTP are associated with the polymorphism.

Materials and methods

Study subjects

We analyzed a cohort of 364 consecutive adult patients with chronic hepatitis C virus infection who visited Hiroshima University hospital and received liver biopsies between December 2002 and November 2008 and who agreed to provide blood samples for the human genome study. All patients included in the study had positive HCV viremia in serum for more than six months, assessed using a commercial quantitative polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test, v2.0; Roche Diagnostics, Branchburg, NJ). Patients with decompensated liver disease were excluded, as were patients co-infected with hepatitis B virus, or human immunodeficiency virus and patients with apparent auto-immune hepatitis and alcoholic liver disease. All patients provided written informed consent for the genomic analysis. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committees of Hiroshima University and Riken. The patient profiles are listed in Table 1. Using criteria reported by Desmet et al. [20], liver biopsy samples were evaluated by two pathologists. To verify consistency and accuracy, one of the pathologists independently re-evaluated samples analyzed by the other, and both pathologists were blind with respect to the IL28 polymorphism. We excluded insufficient or inconclusive biopsy samples, including those that were less than 10 mm^2 in size and containing less than 10 portal tracts. The amount of alcohol consumed was calculated according to the frequency of consumption and the alcohol concentration of beverages consumed. We estimated alcohol concentrations as follows: 5% for beer, 17% for sake, 25% for Japanese vodka, and 43% for whiskey; 1 ml of alcohol was considered equivalent to 0.886 g. The amount of alcohol consumed was divided into three categories: none, light (0–20 g/day), moderate (21–50 g/day). Heavy drinkers (more than 50 g/day) were excluded from the study.

Table 1. Characteristics of patients.

Characteristics of patients	
Age [median (range)]	59 (20–82)
Sex (male/female)	212/152
BMI [median (range)]	23 (16–39)
Alcohol consumption (Unavailable/none/0–20 g/day/21–50 g/day)	64/110/65/125
Hb [median (range)] mg/dl	14 (8–18)
Platelet [median(range)] $\times 10^4/mm^3$	14 (4–41)
ALT [median (range)]IU/L	62 (2–611)[c]
gamma-GTP [median (range)] IU/L	50 (7–680)
Genotype (1b/2a or 2b/1b + 2b/undertermined)	260/84/1/19
Fibrosis (F0/F1/F2/F3/F4)	4/116/141/66/37
Activity (A0/A1/A2/A3)	1/102/206/51
Virus titer [median (range)]kIU/l	1400 (<0.5–26,000)
Core 70[a] (wild/mutant/undertermined)	120/77/167
Core 91[a] (wild/mutant/undertermined)	107/88/169
ISDR[b] mutation (0/1/>2/undertermined)	58/70/48/188

[a] Hepatitis C virus core amino acid 70R and 91L are presented as wild type. Substituted amino acids are considered mutants.
[b] Interferon sensitivity determining region. Number of amino acids substituted from the prototype genotype 1b sequence were calculated.
[c] ALT levels of two patients remained around 2 IU/L even though AST and gamma-GTP levels were comparable to other chronic hepatitis C patients (peaking above 100 IU/L and returning to normal following SVR), probably due to deficiency of the ALT enzyme. These values were omitted from analysis of ALT.

Genotyping

Genotyping of some of the samples was performed as part of a genome wide association study using the Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, Inc., CA) at Riken Yokohama Institute. Genotyping of the remaining samples was performed using TaqMan assay or Invader assay as described previously [21,22].

Analysis of amino acid sequences in the core and ISDR region

HCV RNA was extracted from 100 µl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 µl of H_2O. The RNA was then reverse transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 µl of reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer and 1.25 U of LA Taq (Takara Bio Inc.) with a buffer supplied by the manufacturer. One microliter of 10×-diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession Number D90208) [23]. Amino acids at positions 70 and 91 of the core region that were identical to the prototype (arginine and leucine, respectively) were considered wild type.

Statistical analysis

χ^2 and Mann–Whitney U-tests were applied to detect significant associations. Simple and multiple regression analyses were used to examine the association between serum gamma-GTP levels and the values of other markers. When the data were not normally distributed, Box-Cox power transformation was performed to remove skewness, followed by linear regression analyses. All of the statistical analyses were two sided, and $p < 0.05$ was considered significant. All statistical analysis was performed using the PASW Statistics 18 program (SPSS Inc., IL).

Results

IL28 locus genotypes and viral and biochemical markers

We compared viral and biochemical markers with IL28 genotypes. First we analyzed the relationship between IL28 genotypes

Table 2. Primers used in this study.

Core region	
Outer forward	5′-GCC ATA GTG GTC TGC GGA AC-3′
Outer reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
Inner forward	5′-GCT AGC CGA GTA GTG TT-3′
Inner reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
ISDR[a]	
Outer forward	5′-TTC CAC TAC GTG ACG GGC AT-3′
Outer reverse	5′-CCC GTC CAT GTG TAG GAC AT-3′
Inner forward	5′-GGG TCA CAG CTC CCA TGT GAG CC-3′
Inner reverse	5′-GAG GGT TGT AAT CCG GGC GTG C-3′

[a] Interferon sensitivity determining region.

Table 3. Amino acid substitutions in the core region of HCV and IL28 genotype.

SNP	Allele (1/2)	Genotype 11	12	22	p value[a]	OR (95% CI)[b]
rs8099917 T/G						
	Core aa70					
	Wild	2	17	101	1.21E-04	0.30
	Non-wild	3	28	46		(0.14–0.55)
	Core aa91					
	Wild	3	18	86	0.034	0.50
	Non-wild	2	27	59		(0.26–0.95)
	ISDR					
	0–1	2	37	89	0.120	1.90
	>2	2	7	39		(0.84–4.3)
	HCV genotype					
	1	6	63	190	0.443	0.81
	2	1	25	58		(0.47–1.4)

[a] p value by χ^2 test for the minor allele dominant model.
[b] Odds ratio for the minor allele in a dominant model.

Table 4. Factors associated with higher gamma-GTP levels.

Variable	Simple Estimate	Simple p	Multiple Estimate	Multiple p
Age	−0.00004	0.899436		
Sex (male vs. female)	0.04647	5E-09	0.033	0.0003
BMI	−0.00257	0.044003		
Activity (A2–4 vs. A0–1)	−0.02518	0.004103	−0.015	0.1415
Fibrosis (F2–4 vs.F0–1)	−0.03	0.000382	−0.021	0.0348
Alcohol consumption	−0.03962	6.81E-06	−0.029	0.0013
IL28 genotype (2/2 vs. 1/2, 1/1)	0.02641	0.003522	0.03	0.001
HCV genotype (1 vs. 2)	−0.0068	0.471293		
Log virus titer (Log IU/ml)	0.00032	0.748826		
Core aa70 (wild vs. others)	−0.01589	0.117424		
Core aa91 (wild vs. others)	−0.01422	0.162341		
ISDR (0–1 vs. ⩾2)	0.00253	0.824685		

Simple and multiple regression analyses were used to examine the association between serum gamma-GTP and the values of other markers. All of the statistical analyses were two sided, and p <0.05 was considered significant.

and substitutions in the HCV core protein amino acids 70 and 91, as well as the HCV genotype and the number of amino acid substitutions in the ISDR. As shown in Table 3, there are significant associations between amino acid substitutions in the core region and the genotype of the rs8099917 SNP at the IL28B locus. In particular, patients homozygous for the major IL28 allele were significantly associated with wild type core amino acid 70 (OR = 0.30; p = 1.21E-04). A similar trend is seen with core amino acid 91 substitutions (OR = 0.50; p = 0.034). Patients with more than one amino acid substitution in the ISDR region also tended to occur in patients homozygous for the major allele, although the difference was not statistically significant (Table 3). There was no correlation between the HCV genotype and the IL28 allele.

We further examined the relationship between IL28 and biochemical markers such as ALT, gamma-GTP, total cholesterol, HDL cholesterol, serum iron, and HCV RNA levels. Only the gamma-GTP level was significantly associated with the IL28 genotype. As shown in Fig. 1A, the gamma-GTP levels were lowest in the IL28 major allele homozygotes and highest in minor allele homozygotes. As drinking alcohol is known to elevate gamma-GTP levels, we examined the effect of alcohol intake in our cohort. As shown in Fig. 1B, there was an association between alcohol and gamma-GTP levels. As we found that the gamma-GTP level is higher in patients with core amino acid 70 substitutions (Fig. 1C), we performed multivariate analysis to examine what factors contribute to higher levels of gamma-GTP. As shown in Table 4, a simple regression analysis revealed that serum gamma-GTP levels were associated with sex, BMI, inflammation activity, liver fibrosis, alcohol consumption, and IL28 genotype, whereas in multiple regression analysis, sex, liver fibrosis, alcohol consumption, and IL28 genotype remained positively associated with serum gamma-GTP levels.

Histological findings and polymorphism in the IL28 locus

We then analyzed the relationship between the IL28 locus polymorphisms and histological findings. We divided patients into mild fibrosis (F0 and F1) and severe fibrosis (F2–4) as well as lower activity (A0 and A1) and higher activity (A2 and A3) and compared these factors against IL28 genotypes. As shown in Table 5, both inflammatory activity and fibrosis were significantly associated with IL28 genotype. Inflammation was more active (A2–3) in patients homozygous for IL28 major alleles

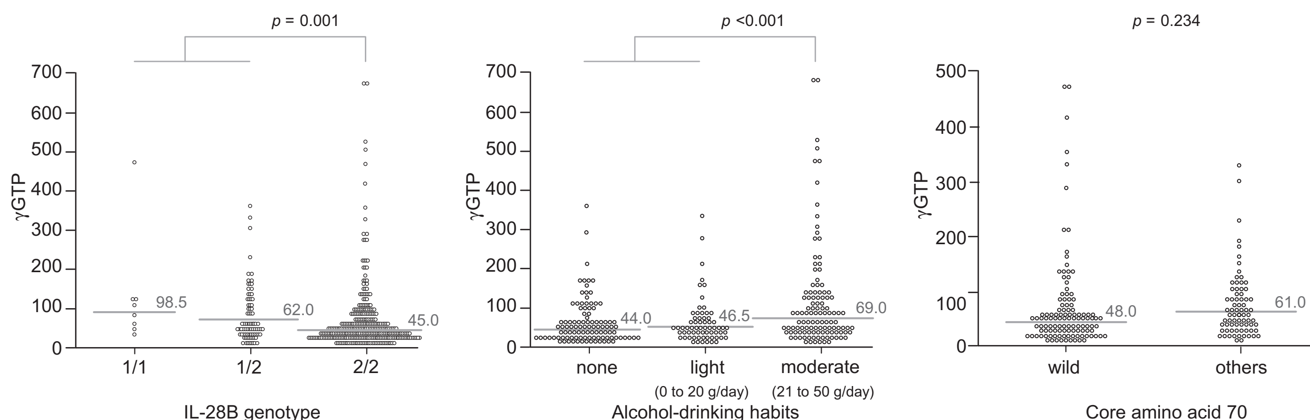

Fig. 1. gamma-GTP levels and IL28 genotype, alcohol intake, and core amino acid substitutions. gamma-GTP levels according to (A) IL28 genotypes, (B) alcohol consumption, and (C) core amino acid 70 substitutions are shown. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP levels.

Table 5. Histological findings and IL28 genotypes.

SNP	Allele (1/2)		Genotype			p value[a]	OR
			11	12	22		(95% CI)[b]
rs8099917	T/G						
		Fibrosis					
		F0–1	3	38	79	0.036	1.66
		F2–4	5	53	186		(1.03–2.69)
		Activity					
		A0–1	2	35	68	0.025	1.75
		A2–3	6	56	199		(1.07–2.86)

[a] p value by χ^2 test for the minor allele dominant model.
[b] Odds ratio for the minor allele in a dominant model.

(OR = 1.75; p = 0.025). Similarly, fibrosis was more severe in patients homozygous for IL28 major alleles (OR = 1.66; p = 0.036). We also performed analysis of the association of IL28 alleles and histological findings after adjusting for other factors that might influence the activity and fibrosis of the liver, such as age, gender, and alcoholic consumption. The IL28 allele was associated with F and A factors independently with adjustment for these predictive factors related to severity of liver fibrosis and inflammation (data not shown).

Relationship between histological activity, the IL28 allele, and gamma-GTP

As we described above, histological activity is more active in patients homozygous for IL28 major alleles. However, it seems contradictory that IL28 major allele homozygosity was associated with low levels of gamma-GTP, but severe activity was associated with high gamma-GTP. As shown in Fig. 2, however, when we compare the allele and activity the frequency of patients with higher activity (A2 and A3) were statistically more frequent in patients homozygous for major alleles (Fig. 2 and Table 5). When we compare gamma-GTP levels of A2 and A3 patients between patients homozygous for major alleles against the others, the levels were significantly lower in the major allele homozygous patients (Fig. 2). A0 and A1 patients showed similar results (Fig. 2). gamma-GTP levels are also significantly higher in patients with higher activities (A2 and A3) than in patients with lower activities (A0 and A1) (Fig. 2).

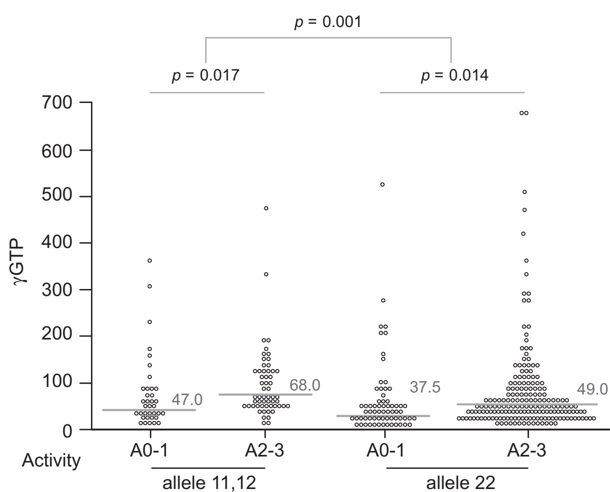

Fig. 2. Relationship between gamma-GTP levels, histological activity and IL28 genotype. gamma-GTP levels are plotted according to IL28 alleles, and histological activity. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP levels.

Discussion

Polymorphism at the IL28 locus has been reported to be associated with the effectiveness of interferon and ribavirin combination therapy [15–17]. We have also found that the polymorphism is associated with the effect of interferon monotherapy on genotype 1b infected patients as well as genotype 2a infection in Japanese as well as Taiwanese patients (Chayama K, personal communication). The polymorphism has also been reported to be associated with spontaneous eradication of the hepatitis C virus [19]. As levels of IL28 gene transcripts have been reported to be higher in patients homozygous for the interferon response allele [16,17], we hypothesized that the polymorphism is also associated with inflammation and progression of chronic hepatitis. As expected, there were significant associations between IL28 genotypes and histological inflammatory activity as well as the degree of fibrosis in chronically HCV infected patients (Table 5). It seems reasonable that the inflammation is stronger in patients with elevated IL28 production because this molecule induces expression of interferon stimulated genes, including some inflammatory cytokines. As the polymorphism is associated with the effect of interferon therapy, the interferon therapy performed before biopsy might alter the results. In fact, a part of patients in this study were treated with peg-interferon and ribavirin combination therapy and the treatment outcome was associated with IL28 genotypes and core amino acid substitutions (data not shown). However, when we analyzed the relation between the IL28 allele and histological findings or core amino acid substitutions in only treatment-naïve patients, the results were unchanged, suggesting that the results obtained in this study are applicable without regard to history of interferon therapy.

Interestingly, the IL28 genotype was also associated with gamma-GTP levels and core amino acid substitutions, both of which are known to be predictive of response to interferon and ribavirin combination therapy [7,8,24]. The levels of liver enzymes such as ALT, AST, and gamma-GTP are usually higher in patients with high inflammatory activity. However, we observed that the levels were actually lower in patients with the favorable allele at the IL28 locus (the major allele in the Japanese population) (Figs. 1A and 2). A lower level of gamma-GTP has been reported to be associated with positive response to combination therapy. Further studies are needed to clarify the mechanism underlying the relation between gamma-GTP levels and therapy effectiveness. It would also be interesting to study the relationship between the IL28 allele and steatosis in the liver because gamma-GTP tends to be elevated in patients with steatosis, and steatosis caused by HCV core protein has been reported [25].

Similarly, viral wild type core amino acids 70 and 91 (i.e., core 70R and 91L), which were already known to be associated with positive response to combination therapy [7,8], were also found to be associated with the favorable human IL28 alleles (Table 3). If viruses with wild type core amino acids 70 and 91 are more

susceptible to interferon therapy, such strains should be less frequent in patients with higher cytokine levels. Viruses with wild type core 70 and 91 amino acids must therefore have some survival advantage in order to replicate in cells in which the level of IL28 production is high. Searching for a target molecule in the signaling cascade from sensing of the virus to production of IL28 might help resolve this question.

We also observed an association between high gamma-GTP levels and core amino acid 70 and 91 substitutions (Fig. 1C), although in multivariate analysis only IL28 genotype, liver fibrosis, sex, and alcohol consumption were significant predictors of gamma-GTP. It seems likely that these factors mutually interact in the presence of the virus and cytokines. Understanding these relationships will reveal the mechanism underlying the effective response to combination therapy and may suggest new strategies to cope with the hepatitis C virus.

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