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IL28 variation affects expression of tion affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy

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Background & Aims: Common genetic variation within the IL28 locus has been found to influence the effect of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. Expression of *IL28* in peripheral blood cells has been reported to be higher in patients with *IL28* SNP genotypes associated with favorable response.

Methods: We analyzed 52 liver and 114 blood samples obtained from patients with HCV genotype 1b. We used reverse transcription-real time polymerase chain reaction to analyze expression levels of *IL28* and several interferon stimulated genes (ISGs), including *MxA*, double stranded RNA dependent protein kinase (*PKR*), 2′–5′ oligo-nucleotide synthetase (*OAS1*), *ISG15*, and *SOCS1*.

Results: Interestingly, expression of *IL28* was significantly lower in patients with the response-favorable rs8099917 TT genotype compared to those with TG or GG genotypes ($p < 0.005$). In hepatic cells, expression of *MxA*, *PKR*, *OAS1*, and *ISG15* were also significantly lower in rs8099917 TT patients ($p < 0.001$, $p = 0.005$, $p = 0.001$, $p < 0.001$, respectively), whereas in peripheral blood mononuclear cells ISG expression levels did not differ significantly. Among patients treated with peg-interferon plus ribavirin therapy, liver mRNA levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly or marginally lower in responders who became negative for HCV RNA ($p = 0.001$, 0.004, 0.014, 0.051, and 0.015, respectively).

Conclusions: Expression levels of ISGs are differentially regulated in the liver and peripheral blood. The mechanism underlying the expression levels of IL28 and ISGs and the correlation with the effect of the therapy should be further investigated.

Keywords: IL28; Liver biopsy; ISG15; MxA; Single nucleotide polymorphism.
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Abbreviations: HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; IRRDR, interferon and ribavirin response determining region; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NVR, non-viral responder; OAS1, 2′–5′ oligoadenylate synthetase 1 ; PKR, double stranded RNA dependent protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Introduction

Chronic hepatitis C virus infection often results in the development of chronic hepatitis, which leads to cirrhosis and hepatocellular carcinoma [1,2]. Currently, patients with chronic HCV infection are treated with a combination of pegylated interferon and ribavirin [3,4]. The eradication rate of the virus has been reported to be about 50% in patients treated with the standard 48 week therapy [4–6]. Although the eradication rate of the virus has been slightly improved by extending the treatment period to 72 weeks, there are many patients who fail to eradicate the virus [7]. Furthermore, many patients fail to complete the therapy because of severe side effects.

Many predictive factors have been reported so far that affect response to combination therapy. Viral factors, such as substitutions at core amino acids 70 and 91 [8,9], or within the interferon sensitivity determining region (ISDR) [10,11] or the interferon and ribavirin response determining region (IRRDR) [12] have been reported.

Among host factors, many single nucleotide polymorphisms (SNPs) associated with outcome of therapy have been identified. They include SNPs in interferon-alpha pathway genes [13] and interferon induced genes [14], within the promoters of the *MxA* [15] and osteopontin [16] genes, and within an intron of *MAPK APK3* [17].

Recently, Ge et al. [18] identified SNPs located 5′ to the *IL28B* gene that affect response to combination therapy. Furthermore, two other research groups also independently reported that these SNPs are associated with the effectiveness of combination therapy [19,20]. More recently, Thomas et al. reported that the SNP allele related to favorable therapy response is also associated with spontaneous clearance of HCV [21]. They reported that the allele related to HCV clearance is the major allele in the majority of Asian and European countries.

IL28A, *IL28B*, and *IL29* gene products belong to the interferon lambda family [22,23]. These cytokines are interferons functionally, but have been reported to be structurally related to the IL-10 family

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[24]. IL29 has been reported to reduce the replication levels of the HCV replicon [25] as well as hepatitis B virus [26]. IL29 has also been reported to reduce the replication of HCV cooperatively with interferon alpha and gamma [27]. These observations suggest that higher expression levels of interferon lambda should be observed in the liver and should correspond with a favorable response to therapy. However, no report has analyzed the expression levels of these cytokines and levels of ISG expression in the liver. In this study, we investigated mRNA expression levels of *IL28*, IL28 receptor, and several ISGs using biopsy samples obtained from patients with chronic hepatitis C and analyzed the relationship between the *IL28* genotype and the effect of combination therapy.

Materials and methods

Patients

We analyzed liver specimens from 52 patients who underwent liver biopsies at Hiroshima University Hospital between December 2002 and November 2008 and who were treated with a peg-interferon plus ribavirin combination for chronic hepatitis C genotype 1b at the same or other hospitals. Clinical characteristics of patients are shown in Table 1. Patients received weekly injections of peg-interferon-alpha-2b for 48 weeks with the dosage adjusted by body weight (60 μg for 35–45 kg, 80 μg for 46–60 kg, 100 μg for 61–75 kg, 120 μg for 76–90 kg, and 150 μg for 91–120 kg). Ribavirin was administered orally with the dosage based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. The response to therapy categories are defined as follows: sustained viral responders (SVR) were negative for HCV RNA 24 weeks after cessation of therapy; relapsers were negative for HCV RNA only transiently during and after the therapy; and non-viral responders (NVR) never became negative for HCV RNA. Liver biopsy specimens, which were obtained in routine clinical practice in an amount beyond what was needed for pathological diagnosis, were kept frozen at −80 °C until analysis. Liver samples obtained by surgical operation from patients who received resection for hepatocellular carcinoma were also kept frozen. Fibrosis stage and activity were diagnosed according to the criteria of Desmet et al. [28].

Although we attempted to analyze blood samples from the same patients who provided liver specimens, more than half of these patients were not treated at Hiroshima University Hospital. Accordingly, we collected blood samples from 114 genotype 1b patients who visited Hiroshima University Hospital from November 2009 to March 2010 to analyze ISG mRNA levels. We excluded patients who were under treatment with therapies including interferon or immunosuppressants. Patients who had eliminated HCV with therapy were also excluded. Clinical characteristics of patients who contributed blood samples for ISG analysis are shown Table 1.

All patients provided written informed consent to participate in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the ethical committee of Hiroshima University and RIKEN.

Genotyping

We genotyped SNPs rs8099917 and rs12979860 from 52 patients using either the Invader assay or the Taqman assay. In the Invader assay, allele-specific oligonucleotide pairs and invasive probes were designed and supplied by Third Wave Technologies (WI). FRET probes were labeled with FAM or VIC corresponding to alleles. The 10 μl reaction volume consisted of 0.5 μl of signal buffer, 0.5 μl of FRET probes, 0.5 μl of structure-specific cleavage enzyme, 1 μl of allele-specific probe mix, and 2 μl of PCR product diluted 1:10. Samples were incubated at 95 °C for 5 min and then at 63 °C for 15 min in an ABI PRISM 7700 (Applied Biosystems), and then fluorescence data were collected. Signal intensity was calculated as the ratio of FAM or VIC to ROX, an internal reference. Genotypes were determined visually in the dye components view of the SDS software.

In the TaqMan assay, we carried out PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, CA), 1 ng DNA, 0.2 μM of each primer, and 40 nM of probe provided by Applied Biosystems in 3-μl reactions. Each 384-well plate contained 376 samples of an unknown genotype and 8 no-DNA control samples. Thermal cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 92 °C for 15 s, and 58 °C for 1 min. Thermal cycling was done on an ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems), and then fluorescence data were collected and the genotypes were determined using the SDS software [29,30].

We calculated linkage disequilibrium using the LD method in the genetics library in the R 2.11 statistics package (http://www.r-project.org) and found high linkage disequilibrium between rs8099917 and rs12979860 (r^2 = 0.99 and D' = 1).

Table 1. Characteristics of the two cohorts of patients analyzed for ISG expression levels. All patients were infected with HCV genotype 1b.

	Treated patients	WBC patients
Characteristic	(n = 52)	(n = 114)
Age [median (range)]	56 (31-77)	63 (30-88)
Sex (Male/Female)	29/23	63/51
ALT [median (range)] IU/L	47 (13-246)	62 (15-259)
γ-GTP [median (range)] IU/L	47 (15-708)	53 (10-469)
Fibrosis (F1/F2/F3/F4)	20/18/4/10	23/28/17/11
Activity (A1/A2/A3)	13/30/9	14/48/14
Virus titer [median (range)] kIU/L	850 (15-6500)	850 (0.5-8200)
Core 70[a] (Wild/Mutant/ND)	27/19/6	40/21/53
Core 91[a] (Wild/Mutant/ND)	24/22/6	34/27/53
ISDR[b] substitutions (0/1/>2/ND)	14/16/12/10	37/12/9/56
rs8099917 allele (TT/TG/GG)	30/17/5	88/33/1
Outcome of therapy (SVR/relapser/NVR)[c]	25/19/8	not applicable

Outcome of therapy	TT	TG	GG
SVR	20	5	0
Relapser	4	8	2
NVR	1	4	3
Total	42	24	6

[a]Hepatitis C virus core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants. ND, not determined.
[b]Interferon sensitivity determining region: the number of substitutions relative to the ISDR of the reference sequence [31].
[c]SVR, sustained viral responder; NVR, non-viral responder.

Quantitative analysis of mRNA of ISGs

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of each RNA sample was reverse transcribed with ReverseTra Ace (TOYOBO Co. Ltd., Japan) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the mRNA for *IL28*, *MxA*, 2′–5′ oligoadenylate synthetase1 (*OAS1*), double stranded RNA dependent protein kinase (*PKR*), *ISG15*, and *SOCS1* with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). As it was difficult to measure *IL28A* and *IL28B* mRNA separately, we measured *IL28A* plus *IL28B* mRNA and expressed *IL28* mRNA. Amplification and detection were performed using an ABI PRISM 7300 (Applied Biosystems). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of MxA protein in peripheral mononuclear cells

The MxA protein level in whole blood sample was measured using an ELISA system (MxA ELISA Kit, Kyowa Medex, Tokyo, Japan). Briefly, lysing solution was added to blood samples and the lysate was applied to ELISA plates coated with a MAb (KM1135, Kyowa Medex, Tokyo, Japan). After 2 h of incubation, the plates were washed, and a different peroxidase-labeled MAb (KM1124, Kyowa Medex)

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was added. After 1 h of incubation and washing, substrate was added. Chemiluminescence was detected using Multiskan MS (Labsystems Version 8.0, Helsinki, Finland). The sensitivity of MxA in this ELISA system was 3.2 ng/ml.

Analysis of amino acid sequences in the core and ISDR region

PCR amplification and nucleotide and amino acid sequence analysis of core and ISDR were performed as reported previously [31] with a slight modification. Briefly, 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 the 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., Otsu, Japan) 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 a 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 BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).

The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank Accession No.: D90208) [32]. Amino acids at positions 70 and 91 of the core region identical to the reference sequence (arginine and leucine, respectively), were considered as wild type. The number of amino acid substitutions in the ISDR was determined as in Enomoto et al. [11,12].

Statistical analysis

Statistical analysis was performed using R version 2.11 or PASW Statistics 18 (SPSS Inc., IL). Categorical data were analyzed using Fisher exact tests, and continuous data were analyzed using the non-parametric Mann–Whitney *U* test. Given the large number of possible predictors, we used multiple logistic regression with variable selection to identify a model with the most important predictors for virological response. To identify independent predictive factors, variables that were significant at the 0.05 level in univariate non-parametric tests were considered as candidate factors for multiple logistic regression analysis. Multicollinearity among predictor variables were examined using hierarchical clustering based on Spearman rank. The model was reduced using forward/backward stepwise selection using the stepAIC function in R, and then boostrap validation was performed using the rms library (formerly called the Design library). Partial residual plot and leverage plots were examined to identify outliers and assess model assumptions. The rms calibrate function was used to calculate R^2 shrinkage, and log odds were corrected for over-optimism using penalized maximum likelihood [33].

Table 2. Primers used in this study.

HCV core protein	
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'
HCV NS5A 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.

Results

IL28B SNP genotype and mRNA expression levels of ISGs in liver samples

We genotyped two SNPs (rs8099917 and rs12979860) in the *IL28B* locus, which have been reported to affect the outcome of the therapy, and compared them with mRNA expression levels in ISGs. Because of linkage disequilibrium, the results are the same for both SNPs, and so only results for rs8099917 are presented. Other SNPs in this locus for the association with therapy outcome were several orders of magnitude less significant (data not shown). Expression levels of *IL28* mRNA in blood cells have been reported to be significantly higher in the patients homozygous for the response favorable allele (rs8099917 TT or rs12979860 CC) in peripheral blood [19,20]. However, our results showed that expression levels of *IL28* mRNA in the liver were significantly lower in rs8099917 TT patients (Table 3). Furthermore, hepatic mRNA levels of each of the major anti-viral ISGs, i.e., *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly lower in rs8099917 TT patients (Table 3). In contrast, expression levels of *SOCS1*, which functions as a repressor of interferon signaling, did not differ significantly between the two groups of patients (Table 3).

IL28B SNP genotype and mRNA expression levels of ISGs in peripheral blood

We examined mRNA expression levels in blood cells. In contrast to liver expression levels, mRNA expression levels of *IL28* and other ISGs were not statistically different between the two groups of patients (Table 3). *IL28B* mRNA levels, as well as four of the five ISGs, were only slightly higher in rs8099917 TT patients (Table 3).

MxA protein levels in peripheral mononuclear cells

We examined the levels of MxA protein in the peripheral mononuclear cells of 43 patients with genotype 1b chronic hepatitis C who were treated with combination therapy. In this case, consistent with previous reports [19,20], the protein levels of MxA were marginally higher in patients homozygous for the major allele (Fig. 3). Furthermore, MxA protein levels in these patients were significantly higher two days after the beginning of therapy (Fig. 1).

IL28 locus genotypes and the effect of combination therapy

Fifty-two patients with chronic hepatitis C genotype 1b were treated with combination therapy. Numbers of SVR, relapser, and NVR patients were 25 (48%), 19 (37%) and 8 (15%), respectively. Responses to therapy by rs8099917 genotype are noted in Table 1. SVR was most frequent in rs8099917 TT patients.

Effect of the combination therapy and mRNA expression levels

As shown in Fig. 2, when patients were divided into VR (SVR + relapser) and NVR categories, expression levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly higher in NVR patients (Fig. 2). There was no significant difference in *SOCS1* mRNA expression between the two groups of patients. Similarly, when patients were classified as SVR and non-SVR (relapser and

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Table 3. Effect of rs8099917 genotype on ISG expression in hepatic and peripheral blood cells.

ISG		TT		GT/GG	p
Liver					
IL28	0.00044	(0.00012-0.005)	0.012	(3E-04-0.023)	0.00493
IL28RA	0.0013	(0.00084-0.0019)	0.0015	(0.0011-0.0019)	0.39
MxA	0.0034	(0.0011-0.0094)	0.02	(0.0084-0.06)	8.04E-05
PKR	0.25	(0.022-0.45)	0.77	(0.26-1.1)	0.00493
OAS1	0.18	(0.10-0.31)	0.54	(0.22-1.1)	0.00106
ISG15	0.29	(0.14-0.59)	2	(0.87-3.9)	8.65E-07
SOCS1	0.0016	(0.0011-0.0024)	0.0017	(0.0012-0.0030)	0.707
Peripheral blood					
IL28	0.00078	(0.00045-0.0010)	0.00062	(0.00032-0.001)	0.31
IL28RA	0.016	(0.011-0.023)	0.015	(0.011-0.02)	0.34
MxA	0.011	(0.0043-0.029)	0.011	(0.0036-0.053)	0.9
PKR	0.18	(0.12-0.3)	0.18	(0.10-0.27)	0.386
OAS1	1.9	(0.75-3.4)	1.3	(0.85-2.3)	0.242
ISG15	3	(1.2-7.7)	2.7	(1.7-4.9)	0.59
SOCS1	0.022	(0.014-0.032)	0.019	(0.014-0.027)	0.292

The median and interquartile range are shown for the TT and GT/TT genotypes for SNP rs8099917 in the hepatic cells (upper) and in peripheral blood mononuclear cells (lower). Results of Mann–Whitney *U* test for effect of genotype on ISG expression levels are shown.

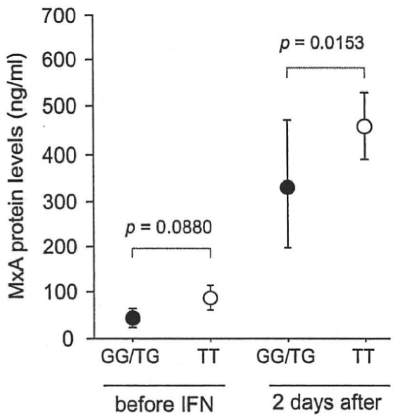

Fig. 1. MxA protein levels in peripheral white blood cells before and two days after the beginning of therapy. Points are classified by rs8099917 genotype (GG/TG vs. TT).

NVR), expression levels were also higher in non-SVR patients than SVR for all ISGs except *SOCS1*, although statistical significance was seen only for *Mx1* and *ISG15* ($p = 0.033$ and 0.031, respectively) (data not shown).

IL28 locus genotypes and amino acid sequences of core and ISDR

As amino acid mutations in the core protein and ISDR region have been reported to be associated with the effect of combination therapy, we examined the relationship between *IL28* genotype and amino acid substitutions within the ISDR and at core amino acids 70 and 91. mRNA expression of the genes examined tended to be higher in patients with core amino acid 70 and 91 mutants and ISDR mutants, and expression levels of *IL28* ($p = 0.035$), *MxA* ($p = 0.031$), and *SOCS1* ($p = 0.018$) were significantly higher in patients with amino acid 91 substitutions (Fig. 3).

Factors associated with the effect of combination therapy

We examined combinations of factors associated with the effect of combination therapy for patients with genotype 1b. Gene expression levels among ISGs were correlated (Fig. 4). To identify factors that contribute independently to virological response, we performed multiple logistic regression analysis using ISG expression levels as well as *IL28B* genotypes and the number of viral substitutions for patients with HCV genotype 1b (Table 4). Following forward/backward stepwise selection based on AIC score, only *ISG15*, *MxA*, *IL28*, and *OAS1* remained in the model, and only *MxA* was significant at the 0.05 level. Age, sex, and other patient and viral factors were not significant.

Discussion

The association of *IL28* locus polymorphisms and response to peg-interferon and ribavirin combination therapy has been reported independently by three groups of researchers [18–20]. Two of the three studies have reported that expression of IL28 in peripheral leukocytes was higher in patients homozygous for the favorable allele [19,20]. It seems reasonable that higher levels of IL28 combined with administration of peg-interferon and ribavirin is related to better response to the therapy. In fact, an additive effect of lambda interferon and alpha interferon has been reported [27]. Accordingly, we assumed that expression levels should be also higher in the liver in such patients.

Interestingly, however, the expression levels of *IL28* were significantly lower in rs8099917 TT patients (Table 3). Expression

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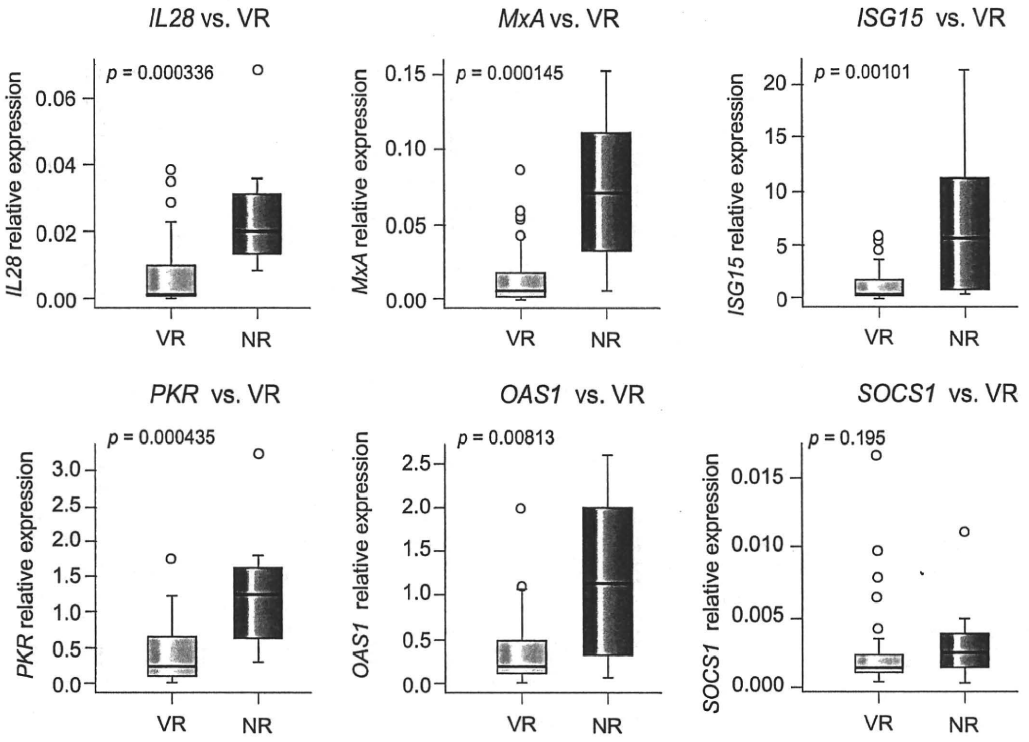

Fig. 2. Intrahepatic expression of *IL28* and interferon stimulated genes by response to therapy. Figures under each panel show the classification of patients with HCV genotype 1b by response to therapy: VR, sustained viral responder and relapser; NR, nonviral responder.

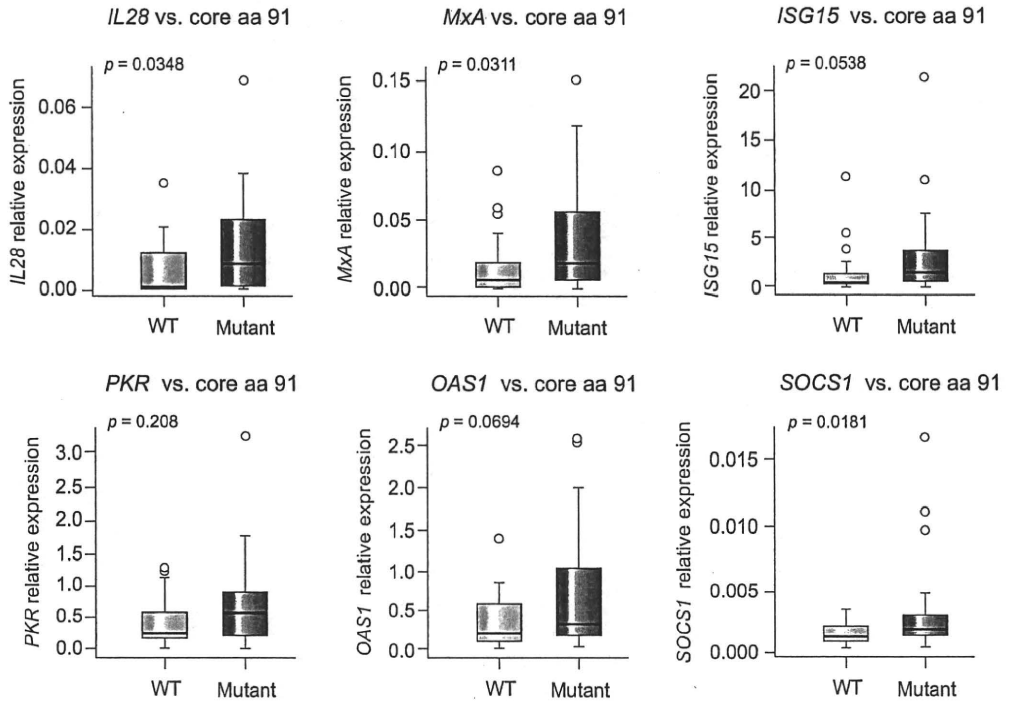

Fig. 3. Association between viral factors and ISG expression. Patients with a substitution at HCV core protein amino acid (aa) 91, which is associated with poorer response to treatment, showed significantly or marginally significantly increased expression of several ISGs involved in establishment of antiviral state as well as decreased expression of one ISG involved in suppression of interferon signaling.

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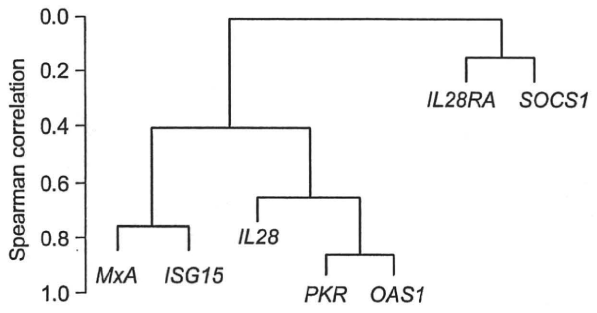

Fig. 4. Spearman correlation among predictor variables. Hierarchical clustering identified groups of genes with similar expression patterns.

levels of each of the ISGs involved in establishment of anti-viral defense (*MxA*, *PKR*, *OAS* and *ISG15*) were also lower in rs8099917 TT patients. We infer from these results that expression levels of *IL28* and other ISGs are regulated differently in the liver compared to peripheral blood cells. The finding that ISG expression levels were lower in patients homozygous for the major allele associated with a favorable response is consistent with Sarasin-Filipowicz et al. [34], who showed that lower ISG expression levels in the liver are associated with positive response to therapy, and vice versa. Feedback mechanisms that down-regulate the response to interferon administered during therapy might negatively affect the response to therapy in HCV infected liver cells. Our result showed that only the expression level of *SOCS1* did not differ among patients with rs8099917 genotype, which implies that the expression level of *SOCS1* relative to ISGs is higher in rs8099917 TT patients. Such relatively higher expression levels of inhibitory genes may contribute to the poor response to the therapy.

The relationship between *IL28B* polymorphisms and *IL28B* expression level remains unknown. Another SNP in strong linkage disequilibrium with the SNPs analyzed in this study resides in a possible promoter region of the *IL28B* gene [18], which might, therefore, affect mRNA expression levels, but different expression levels of ISG mRNA between liver and peripheral mononuclear cells cannot be explained simply by a single SNP in the promoter region. Further study is necessary to address this issue.

As it has been reported that amino acid substitutions in the core protein and the ISDR are associated with different responses to therapy [8–11], we attempted to uncover a relationship between core aa70 and 91 substitutions and expression levels of ISGs. Previously we found that core aa70 wild type viruses accumulated in rs8099917 TT patients, and several studies have reported poor response to therapy in the case of aa70 and aa91 substitutions [8]. Consistent with these results, in this study we found an association between elevated ISG expression and core aa91 substitutions, both of which are associated with poor response to therapy.

Multivariate analysis in this study reflected the tiered relationships among the predictors. The *IL28* rs8099917 genotype term was highly significant when analyzed alone, but it was

Table 4. Factors associated with virological response in patients with HCV genotype 1b (sustained viral response or transient/relapse response).

Variable	Univariate tests			Multiple logistic regression			
	n	OR	*p*	n	OR	(95% CI)	*p*
Age	52	1.13	0.4573				
Sex	52	0.604	0.2777				
rs8099917 (TT vs TG/GG)	52	3.68	0.0072				
Fibrosis stage	52	1.67	0.5672				
Activity	52	0.495	0.5788				
ALT	47	0.597	0.1845				
Gamma-GTP	47	0.539	0.0881				
Core aa70 (WT vs mutant)	46	1.24	0.7002				
Core aa91 (WT vs mutant)	46	1.43	0.4513				
ISDR (0 vs ≥1)	42	1.12	1.0000				
Titer	44	1.2	0.6377	52	0.297	(0.0794-1.11)	0.0706
IL28	52	0.273	0.0003				
IL28RA	51	0.792	0.3381	52	0.186	(0.047-0.736)	0.0165
MxA	52	0.255	0.0001	52	0.38	(0.124-1.16)	0.0892
ISG15	52	0.44	0.0010				
PKR	52	0.186	0.0004				
OAS1	52	0.372	0.0081	52	9.14	(0.974-85.7)	0.0528
SOCS1	52	0.87	0.1954	-	-	-	-

Univariate tests (Fisher exact and Mann–Whitney *U* tests) and multiple logistic regression analysis were used to examine the association between viral response and *IL28B* rs8099917 genotype, ISG gene expression, age and sex. Following multiple logistic regression *IL28*, *MxA*, and *OAS1* expression remained significant at the 0.05 level. Odds ratios for multiple logistic regression were adjusted using penalized maximum likelihood.

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not significant when *IL28* and ISG mRNA expression levels were included in the model, suggesting that whatever the mechanism of action reflected by this polymorphism, it may directly or indirectly affect expression of the *IL28B* gene and downstream ISGs. Similarly, *MxA* and *ISG15* clustered together by Spearman rank correlation (Fig. 4), making it unlikely that both would remain significant in a multivariate model, and in this case the ISG with the stronger univariate effect (*MxA*) was selected.

Conclusions

In summary we found that the expression levels of ISGs in hepatic cells are inversely related with *IL28* SNP genotype relative to peripheral mononuclear cells. Analysis of the mechanism underlying different expression levels among *IL28* genotypes, especially differential regulation of anti-viral ISGs and *SOCS1*, should be important in understanding the mechanism behind variations in response to therapy and give us an insight into ways to develop more effective therapeutic regimens.

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Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both *in vitro* and *in vivo*

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Background & Aims: ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B, attenuates liver disease in several animal models of acute and chronic liver injury. ME3738 is thought to inhibit replication of hepatitis C virus (HCV) by enhancing interferon (IFN)-β production, as determined using the HCV full-length binary expression system. We examined the effect of ME3738 combined with IFN-α on HCV replication using the genotype 1b subgenomic replicon system and an *in vivo* mouse HCV model.

Methods: HCV replicon cells (ORN/3-5B/KE cells and Con1 cells) were incubated with ME3738 and/or IFN-α, and then intracellular IFN-stimulated genes (ISGs) and HCV RNA replication were analyzed by reverse-transcription-real time polymerase chain reaction and luciferase reporter assay. HCV-infected human hepatocyte chimeric mice were also treated with ME3738 and/or IFN-α for 4 weeks. Mouse serum HCV RNA titer, HCV core antigen, and ISGs expression in the liver were measured.

Results: ME3738 induced gene expression of oligoadenylate synthetase 1 and inhibited HCV replication in both HCV replicon cells. The drug enhanced the effect of IFN to significantly increase ISG expression levels, inhibit HCV replication in replicon cells, and reduce mouse serum HCV RNA and core antigen levels in mouse livers. The combination treatment was not hepatotoxic as evident histologically and did not reduce human serum albumin in mice.

Conclusions: ME3738 inhibited HCV replication, enhancing the effect of IFN-α to increase ISG expression both *in vitro* and *in vivo*, suggesting that the combination of ME3738 and IFN might be useful therapeutically for patients with chronic hepatitis C.

Keywords: Human hepatocyte chimeric mouse; Interferon-stimulated genes.
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Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL, interleukin; ISG, interferon stimulated gene; MxA, myxovirus resistance protein A; OAS, oligoadenylate synthetase; PKR, double stranded RNA-dependent protein kinase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator; USP18, ubiquitin specific peptidase 18.

Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. To date, the most effective therapy for viral clearance is a 48- or 72-week combination therapy of pegylated interferon (IFN)-α and ribavirin. However, successful eradication of the virus is achieved in only about 50% of treated patients [4–6]. Moreover, therapy induces significant adverse effects, such as fever, fatigue, and anemia [4], resulting in poor tolerability. More effective and less toxic treatment is, therefore, desired.

ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B [7], attenuates liver disease in several animal models of acute and chronic liver injury induced by concanavalin A, ethanol, lithocholate, and bile duct ligation [8–12]. ME3738 induces interleukin (IL)-6 expression, and serum amyloid A and α1-acid glycoprotein act as downstream targets of the IL-6 signal to protect against concanavalin A-induced liver injury [8–10]. The drug also prevents the progression of hepatic fibrosis in rats with bile duct ligation through suppression of activation and collagen synthesis of hepatic stellate cells [12].

Recently, Hiasa et al. reported that ME3738 inhibited HCV replication by enhancing IFN-β production using the HCV full-length binary expression system that uses full-length genotype 1a HCV complementary DNA plasmid with a T7 promoter sequence and an adenoviral vector expressing T7 polymerase [13]. However, it is not clear if the production of IFN-β and subsequent expression of IFN-stimulated genes (ISGs) was induced by the transcribed HCV genomes through detection by innate

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immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS*) *1*, myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN-α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCAC CCTATCA-3′. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000*g*. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β-actin (Sigma, Tokyo, Japana) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into $uPA^{+/+}/SCID^{+/+}$ mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN-α. IFN-α-treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN-α. Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000*g* for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean ± SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann–Whitney *U*-test. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN-α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

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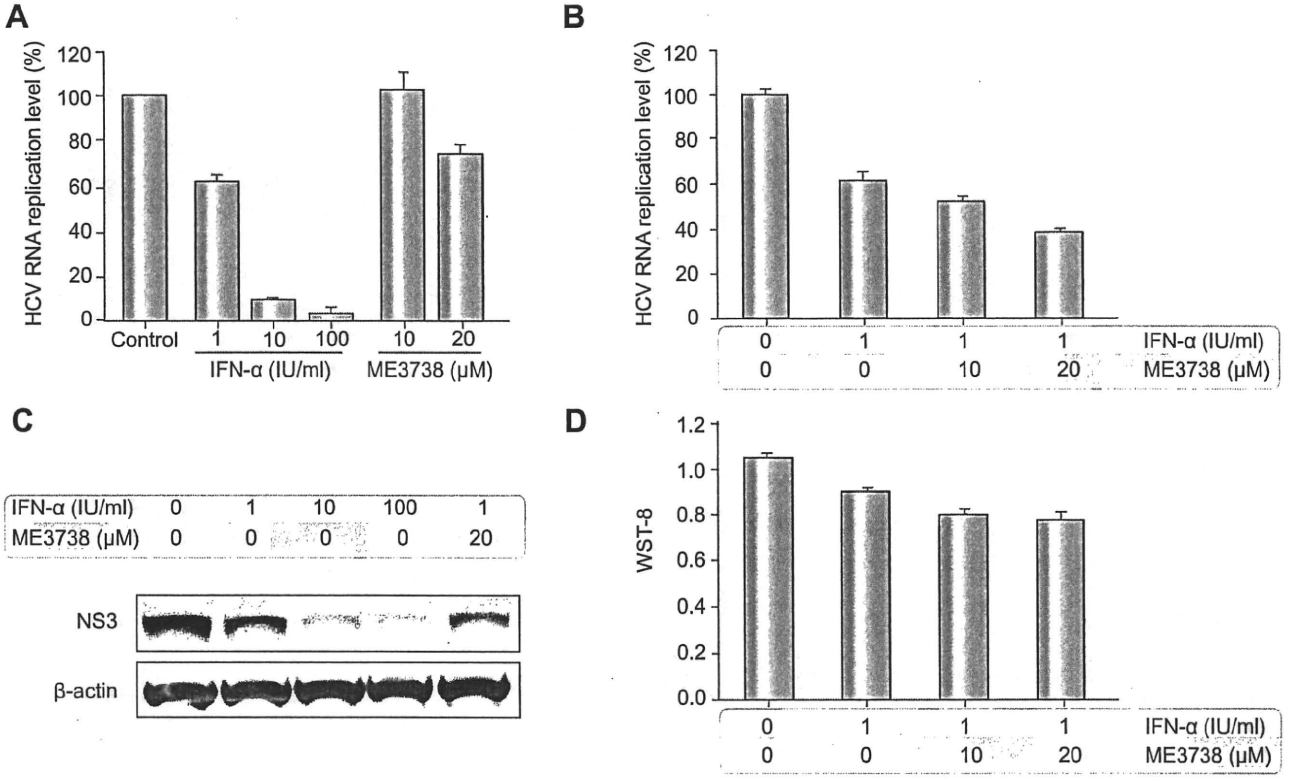

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μM of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN-α. IFN-α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN-α-treatment and was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN-α/ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN-α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN-α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN-α/ME3738 combination treatment was lower but was not significant with IFN-α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN-α.

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN-α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OA1S*, *MxA* and *ISG15* to levels higher than IFN-α alone in both cells. These results indicate that ME3738 enhances the effect of IFN-α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6–10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN-α alone, or ME3738/IFN-α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN-α-treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN-α-treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN-α-treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN-α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that

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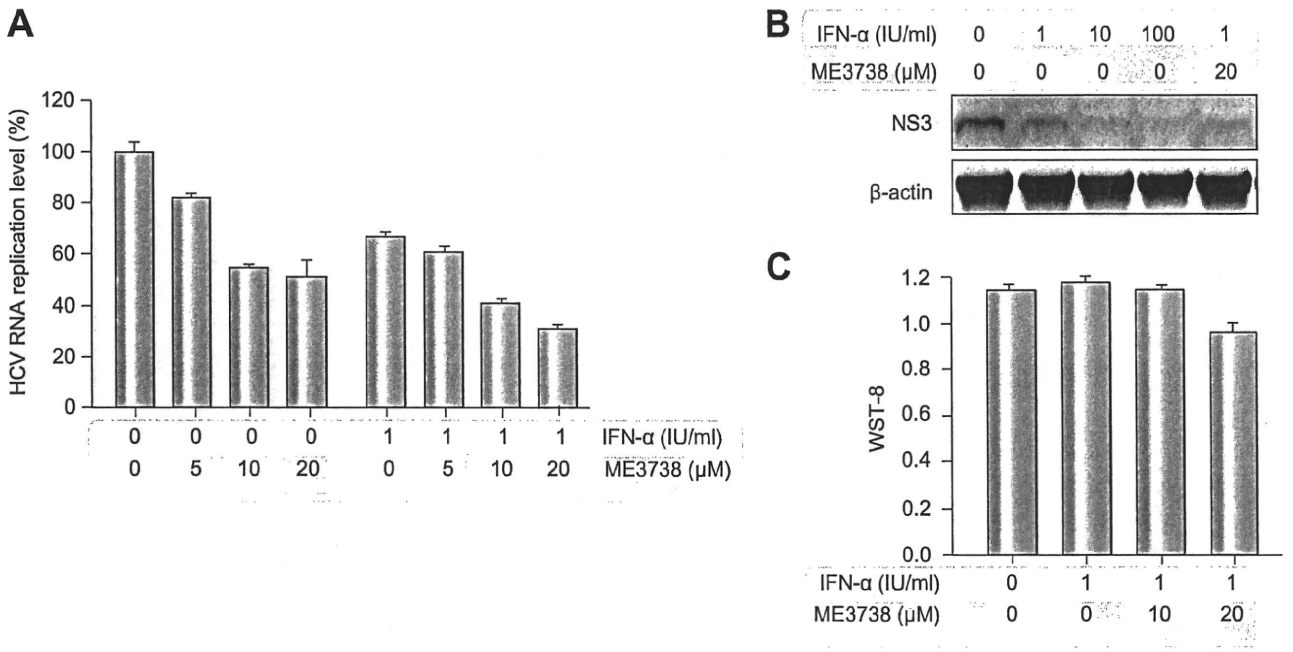

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (C) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

the reduction of HCV in chimeric mice was not due to toxicity of the drugs (Fig. 4A). This was also supported by histopathological findings, including lack of cytotoxic changes in the livers of all four groups of mice (Fig. 4C). The effect of ME3738 to increase ISG expression was assessed in mouse liver following treatment with a high concentration of ME3738 for 1 week and a single injection of IFN-α. ME3738 alone showed no increase in the expression of ISGs in mouse livers (Fig. 5). IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OAS1*, *PKR* and *USP18* mRNA levels in mouse livers to levels higher than IFN-α alone. These results indicate that ME3738 inhibits HCV replication, enhancing the effect of IFN-α to increase ISG expression *in vivo*.

Discussion

Although the treatment outcome of chronic HCV infection has improved with the advent of pegylated IFN-α and ribavirin, the eradication rate of HCV is only about 50%. Many patients are unable to receive this therapy because of the harmful side effects or the financial costs. Development of effective, safe and inexpensive therapies should be encouraged.

ME3738 is reported to attenuate various liver pathologies in animals [8–12]. Furthermore, Hiasa et al. reported recently that ME3738 induces IFN-β mRNA expression and inhibits the replication of HCV [13]. We thus attempted in this study to evaluate the effect of ME3738, especially in combination with IFN-α, on HCV.

The results of the present study show that ME3738 induced the gene expression of *OAS* (Fig. 2) and inhibited HCV replication (Fig. 1A). Hiasa et al. reported that ME3738 enhanced the expression of *IFN-β* mRNA and that the enhanced production of IFN-β resulted in the increased expression of ISGs [13]. They showed also that the effect of ME3738 on HCV was abolished following the inhibition of IFN-β expression with siRNA or antibody. Our results are consistent with their findings. The extent of the increase in ISG expression was smaller in Hiasa et al. [13] than in our results. This is probably because they used the T7-genotype 1a-cDNA transient transfection-infection system to produce HCV in HepG2 or Huh7 cells [13,24,25] and assessed the effect of ME3738 by utilizing naturally produced IFN-β. The amount of IFN is likely to be very small in their system compared to that used in our study. We also tried to detect *IFN-β* mRNA in our replicon system but were unable to detect it in our replicon cells (Huh7 based ORN/3-5B/KE cells and Con1 cells). This is probably due to a defect of the innate immune system in producing IFN-β in those cells. This is consistent with their finding that ME3738 had an inferior effect in Huh7 cells than in HepG2 cells to produce ISG products [13].

As we showed in this study, ME3738 enhances the effect of IFN against HCV replication both *in vitro* (Figs. 1B and 2A) and *in vivo* (Figs. 4A and 4B). ME3738 enhanced the effect of IFN-α by increasing the expression levels of ISGs both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). How ME3738 enhances the transcription of ISGs is unknown at this stage. ME3738 was reported initially to protect liver cells against injury through induction of IL-6 [8,9]. IL-6 is reported to provide protection to certain cells [26–28] by preventing apoptosis. In the present study, we tried to detect IL-6 protein in the serum and mRNA in the liver of ME3738-treated mice. However, the levels of both were too low to measure. Further studies should be conducted to elucidate the mechanism by which ME3738 enhances immunity against viral infections.

Our results showed that ME3738 did not reduce cell viability. We also showed that the drug is not hepatotoxic, as inferred by HSA level and liver histology. Since ME3738 is reported to

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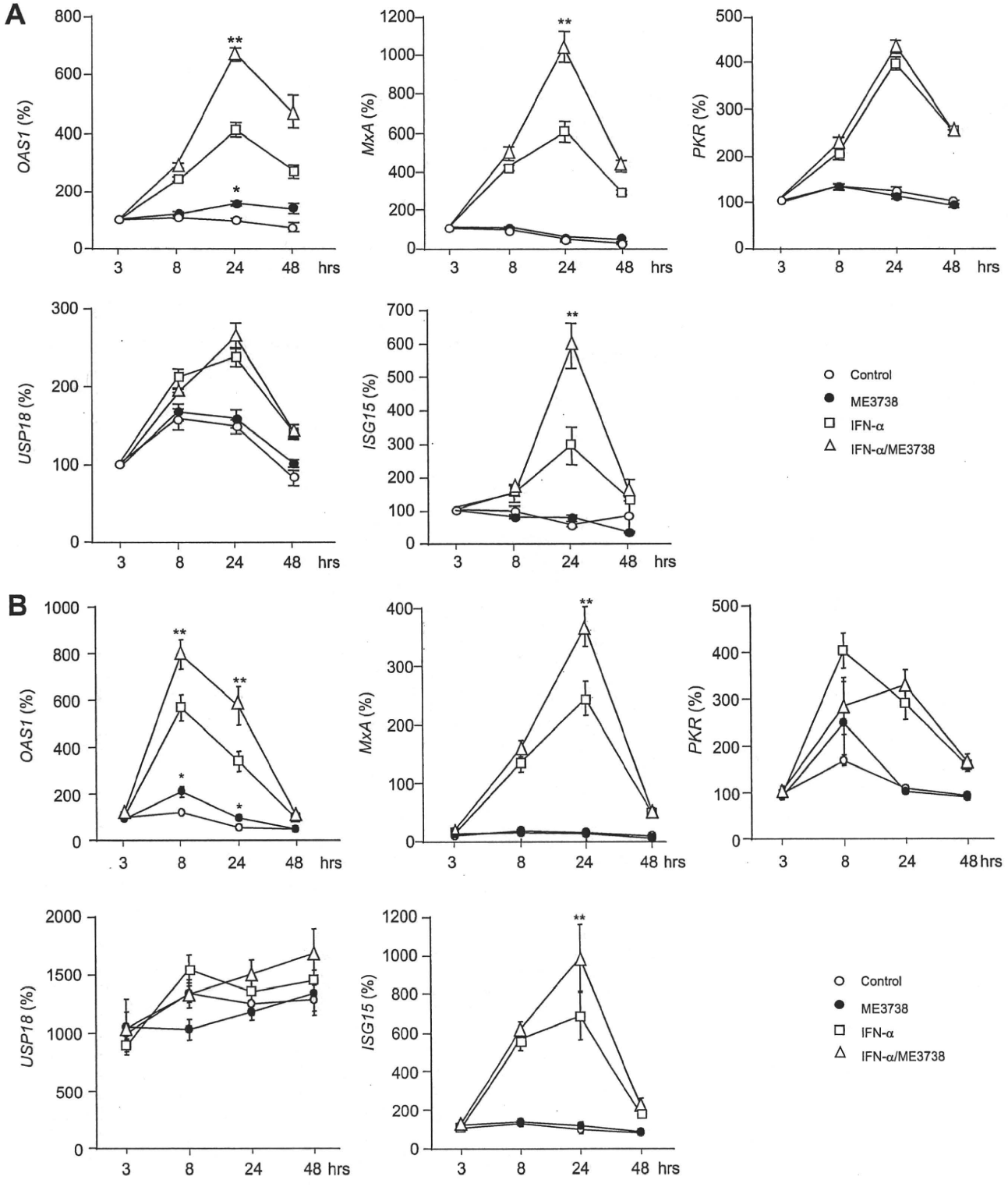

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (*OAS*), myxovirus resistance protein A (*MxA*), double stranded RNA-activated protein kinase (*PKR*), *USP-18* and interferon-stimulated gene (*ISG*) *15* were measured at the indicated times. RNA levels were expressed relative to *GAPDH* mRNA. Data are shown as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α. (*p <0.05 compared with Control, **p <0.05 compared with IFN-α treatment).

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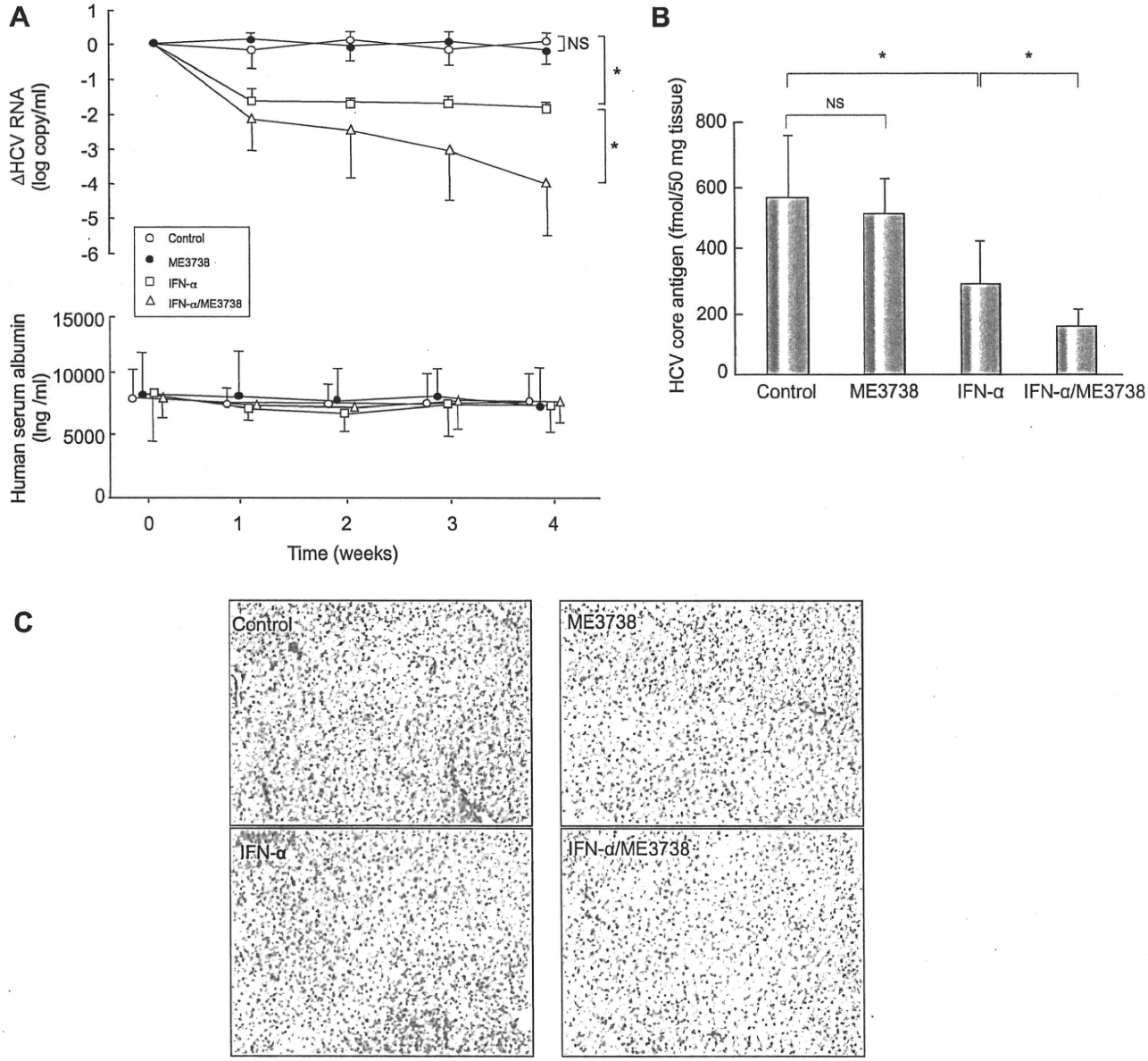

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. HCV core antigen was measured in the mouse livers after 4 weeks of treatment (B). Data are mean ± SD of 6 mice. (*p <0.05; **p <0.01; NS, not significant). (C) Liver samples obtained from mice were stained with hematoxylin-eosin (Original magnification, 100×). Note the lack of specific changes in the mice of each group. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

Table 1. Concentrations of ME3738 in mouse serum samples.

	Control	ME3738	IFN-α	ME3738/ IFN-α
ME3738 (μM)	<0.01	4.02 ± 0.90	<0.01	2.44 ± 0.21

Concentrations of ME3738 in serum samples obtained from mice after 4 weeks of treatment were measured by liquid chromatography/mass spectrometry/mass spectrometry. Data are shown as mean ± SD of three mice. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

attenuate liver disease in several animal models of acute and chronic liver injury [8–12], the drug could be suitable for treatment of patients with chronic hepatitis C. In the current regimen of PEG-IFN and ribavirin combination therapy, IFN reduces the replication rate of the virus by inducing expression of ISGs in liver cells. Ribavirin enhances the effect of IFN synergistically through an unknown mechanism. ME3738 also enhances the effect of IFN similarly to ribavirin and may protect liver cells from apoptosis. Combination therapy using these three drugs might yield excellent anti-viral and anti-inflammatory effects. Alternatively, ME3738 could be used instead of ribavirin if the drug shows a superior effect in combination with IFN. Further animal and human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

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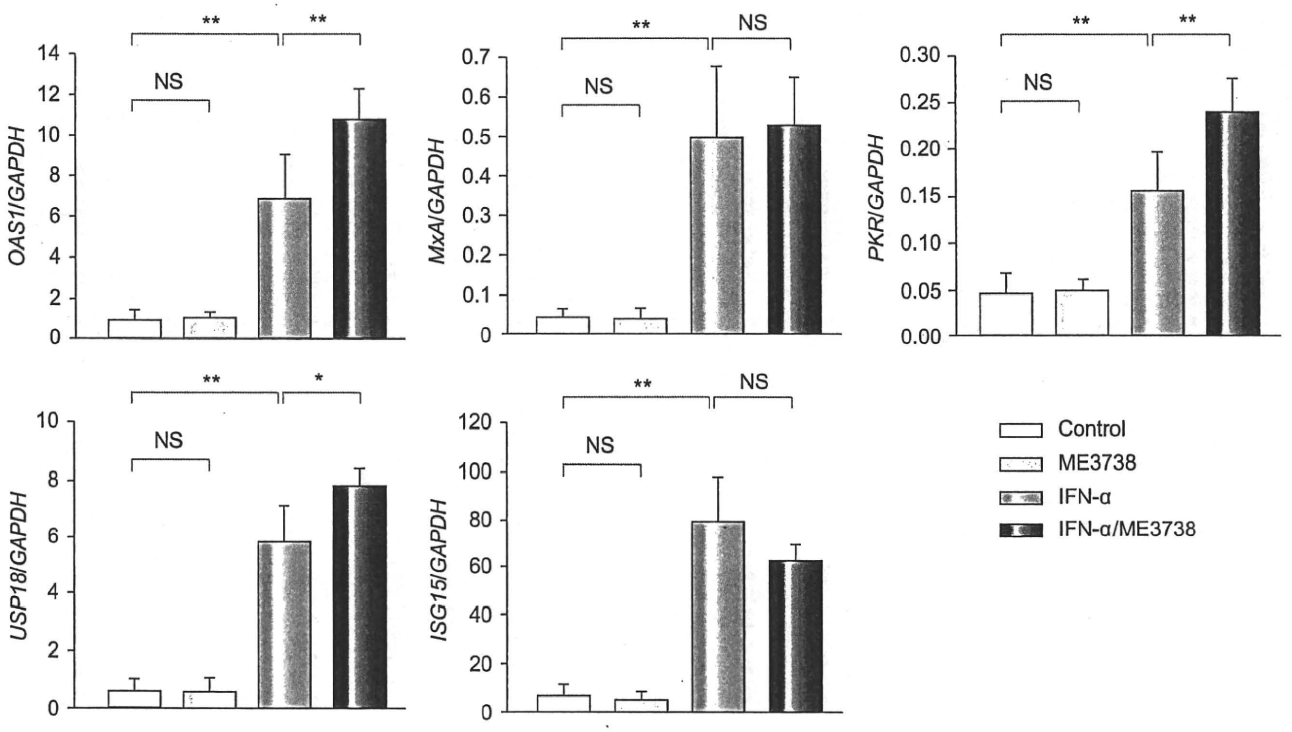

Fig. 5. Interferon-stimulated genes expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers was measured. RNA levels are expressed relative to *GAPDH* mRNA. Data are presented as mean ± SD of six mice. Control: Mice treated with neither ME3738 nor IFN-α. (*p <0.05; **p <0.01; NS, not significant).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

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.

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.

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).

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)] × $10^4/mm^3$	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			p value[a]	OR
		11	12	22		(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		Multiple	
	Estimate	p	Estimate	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

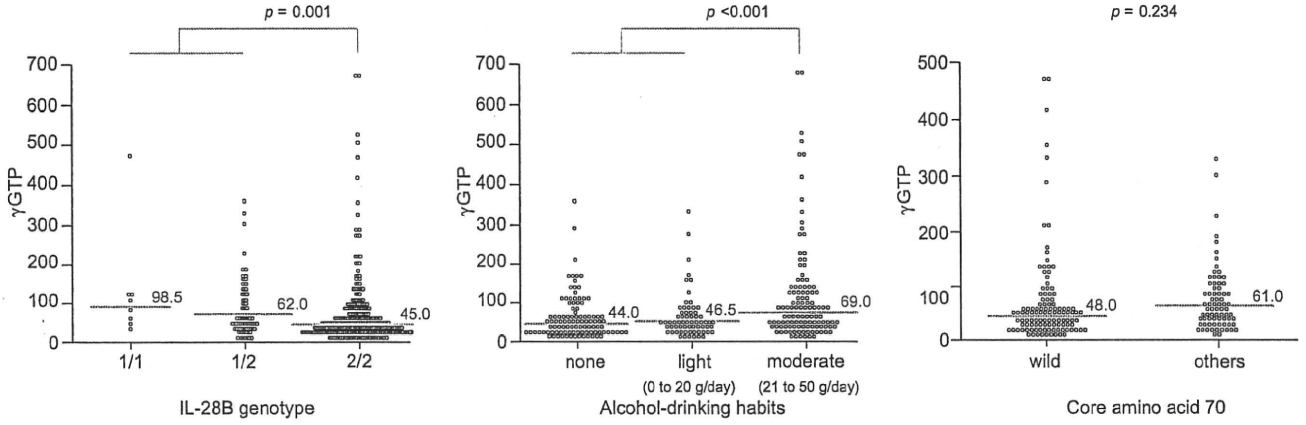

Fig. 1. gamma-GTP levels and IL28 genotype, alcohol intake, and core amino acid substitutions. gamma-GTP levels according to (A) IL28 genotypes, (B) alcohol consumption, and (C) core amino acid 70 substitutions are shown. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP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