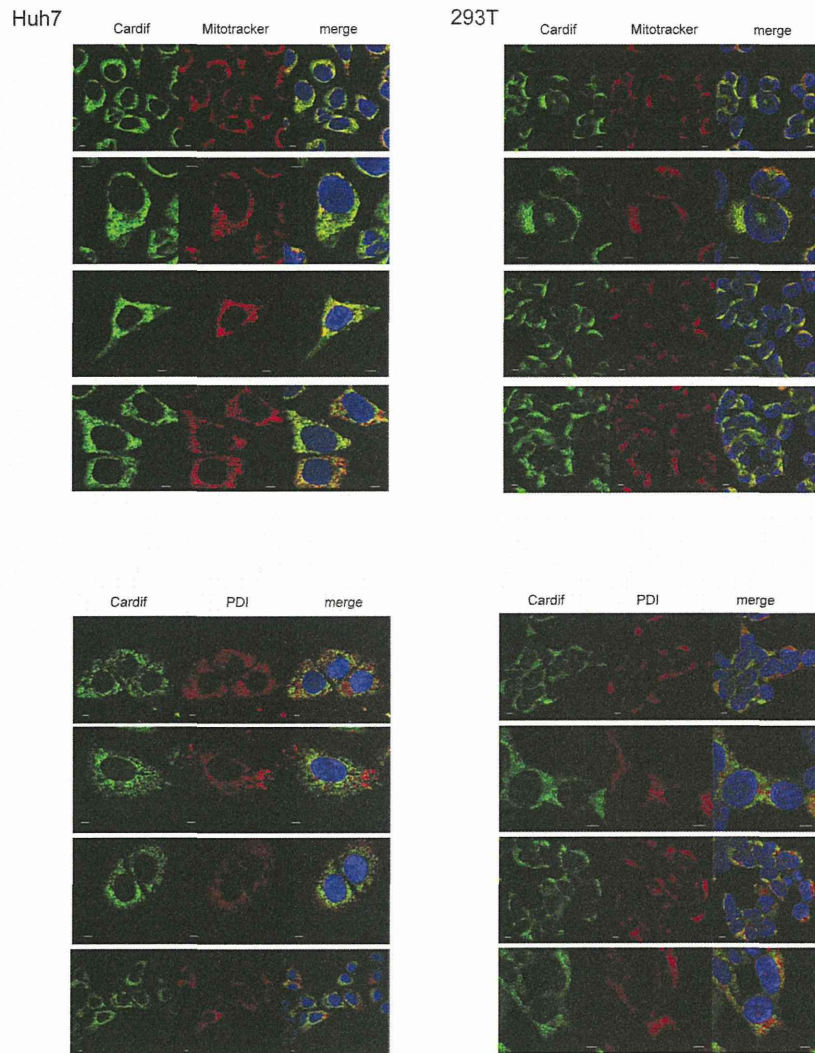


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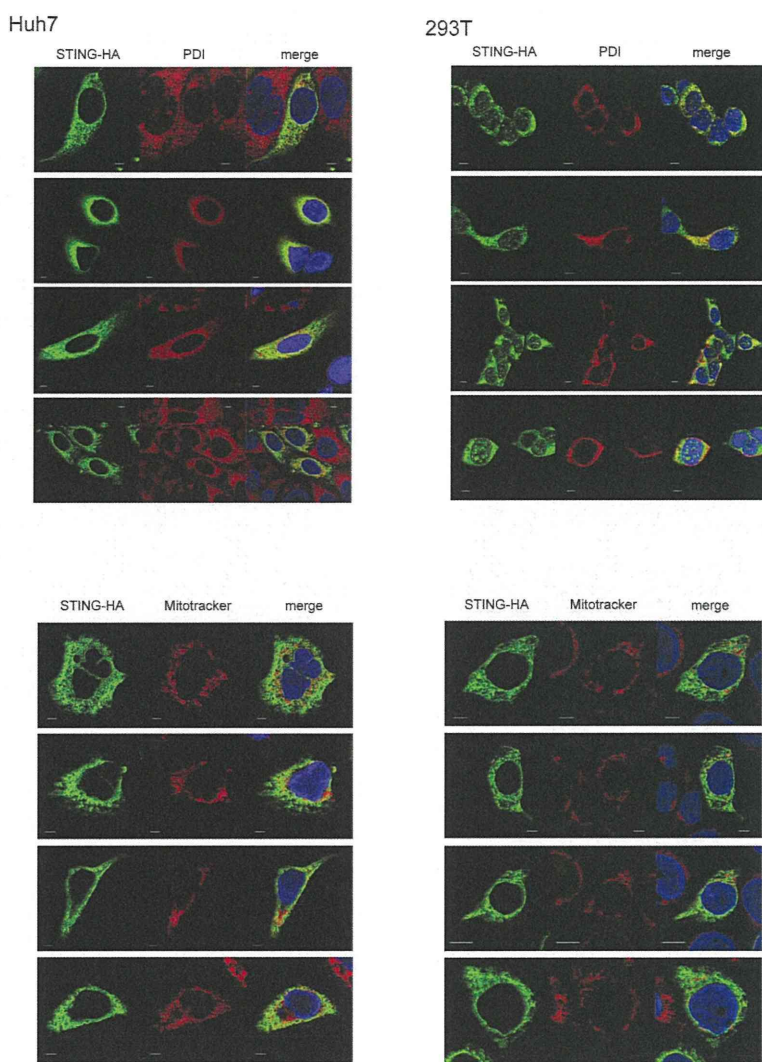
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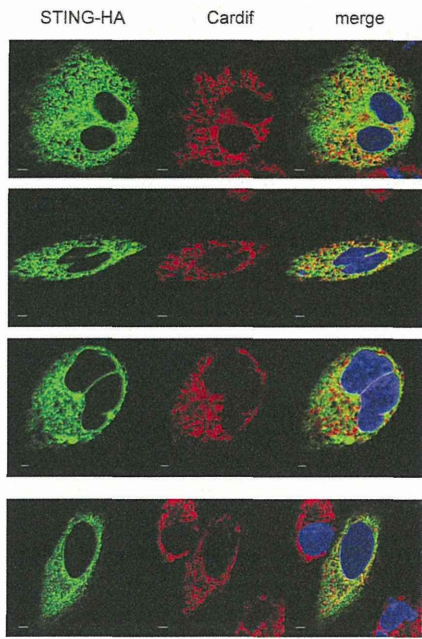


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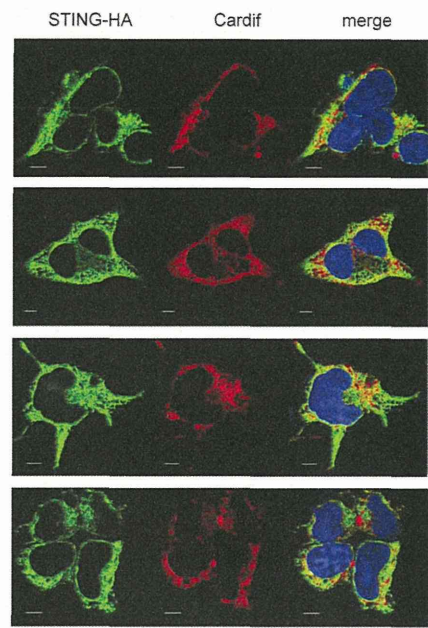
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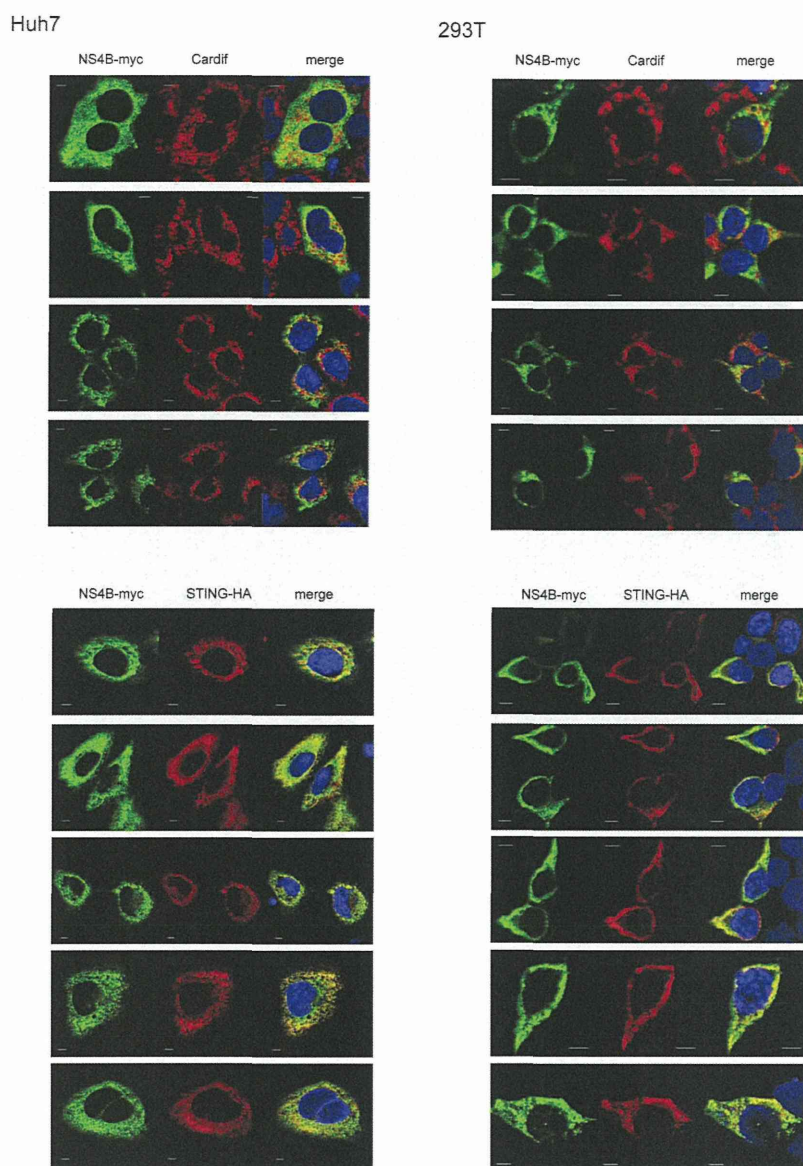
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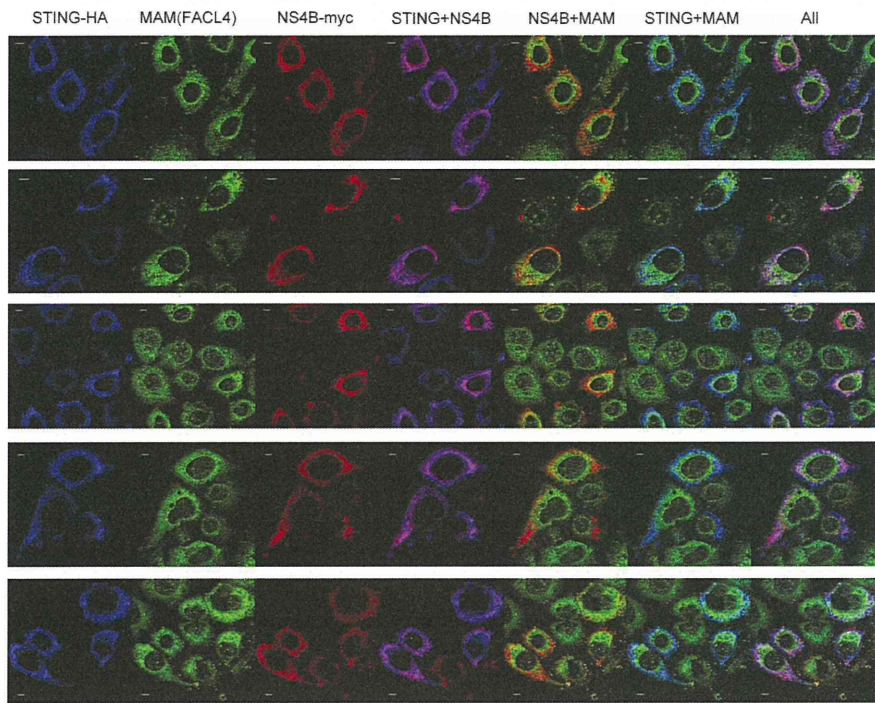


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Add-on Therapy of Pitavastatin and Eicosapentaenoic Acid Improves Outcome of Peginterferon Plus Ribavirin Treatment for Chronic Hepatitis C

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Despite the use of pegylated-interferon (peg-IFN) plus ribavirin combination therapy, many patients infected with hepatitis C virus (HCV)-1b remain HCV-positive. To determine whether addition of pitavastatin and eicosapentaenoic acid (EPA) is beneficial, the “add-on” therapy option (add-on group) was compared retrospectively with unmodified peg-IFN/ribavirin therapy (standard group). Association of host- or virus-related factors with sustained virological response was assessed. In HCV replicon cells, the effects of pitavastatin and/or EPA on HCV replication and expression of innate-immunity- and lipid-metabolism-associated genes were investigated. In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than standard group. In both groups, sustained virological response rates were significantly higher in patients with genotype TT of IL-28B (rs8099917) than in those with non-TT genotype. Among the patients with non-TT genotype, sustained virological response rates were markedly higher in the add-on than standard group. By multivariate analysis, genome variation of IL28B but not add-on therapy remained as a predictive factor of sustained virological response. In replicon cells, pitavastatin and EPA suppressed HCV replication. Activation of innate immunity was obvious in pitavastatin-treated cells and EPA suppressed the expression of sterol regulatory element binding protein-1c and low-density lipoprotein

receptor. Addition of pitavastatin and EPA to peg-IFN/ribavirin treatment improved sustained virological response in patients infected with HCV-1b. Genotype variation of IL-28B is a strong predictive factor in add-on therapy.

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KEY WORDS: cholesterol; hepatitis C virus; IL28B; replicon system

Abbreviations: EPA, eicosapentaenoic acid; HCV, hepatitis C virus; HMGCR, HMG-CoA reductase; IRF3, IFN regulatory factor 3; ISG15, IFN-stimulated gene 15; ITPA, inosine triphosphatase; LDLR, low-density lipoprotein receptor; MAVS, mitochondrial antiviral signaling; NPC1L1, Niemann-Pick C1 like 1; OR, odds ratio; PCR, polymerase chain reaction; peg-IFN, pegylated-interferon; PUFA, polyunsaturated fatty acid; RIG-I, retinoic acid inducible gene I; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; TRAF6, TNF receptor associated factor 6.

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INTRODUCTION

Nearly, 170 million people are infected with hepatitis C virus (HCV) worldwide and natural history studies show that 5–20% of patients develop cirrhosis after approximately 20 years of infection [Alter, 2005]. Currently, pegylated-interferon (peg-IFN) plus ribavirin combination therapy has become the standard care for chronic hepatitis C because it achieves high rates of sustained virological response [Aghemo et al., 2009]. However, in patients infected with genotype 1b HCV (HCV-1b), at most, 50% of individuals achieve a sustained virological response following combination therapy, and HCV-1b in high viral loads (>5.0 log IU/ml) accounts for $>70\%$ of patients with HCV infection in Japan [Kumada et al., 2006]. The response to IFN-based treatment is influenced by virus-related factors including viral load and genotypes; host-related factors, such as sex, age, insulin resistance, staging of the disease and responses to previous antiviral therapies; as well as therapeutic factors, such as dose and duration of treatment [Shiffman, 2002; Backus et al., 2007; Kanwal et al., 2007; Bortoletto et al., 2010]. In addition, as a critical genetic factor for governing the outcomes of peg-IFN plus ribavirin combination therapy, genome variation of IL28B and inosine triphosphatase (ITPA) have been identified recently. At the spot of rs8099917 in the IL28B region, patients infected with HCV-1b with the major variation type (TT) show markedly higher sustained virological response rates than those with the minor variation type (TG + GG) [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. Single nucleotide polymorphism (SNP) variation of the ITPA gene at rs1127354 is associated with anemia as an adverse effect during peg-IFN plus ribavirin combination therapy [Fellay et al., 2010; Azakami et al., 2011; Suzuki et al., 2011; Thompson et al., 2011]. In patients who have rs1127354 genotype CC (major type), ribavirin-induced anemia is more frequent and forces a reduction in dose of ribavirin, which worsens the therapeutic outcome. Alternatively, viral amino acid substitutions at core 70 and 91 are significant predictors of treatment outcome. In particular, a point mutation of core 70 from Arg to Gln is significantly associated with non-sustained virological response in patients infected with HCV-1b [Akuta et al., 2005, 2007; El-Shamy et al., 2012].

Investigation of patients treated by peg-IFN plus ribavirin combination therapy has indicated that serum cholesterol and statin use predict virological response to therapy [Harrison et al., 2010]. Recent studies have shown that virological response is improved by addition of fluvastatin or pitavastatin to peg-IFN and ribavirin treatment [Bader et al., 2008; Sezaki et al., 2009; Shimada et al., 2012]. Statins were associated with a reduced risk of hepatocellular carcinoma in a large cohort of patients with diabetes [El-Serag et al., 2009]. In other studies, it has been demonstrated that polyunsaturated fatty acids (PUFAs) inhibit HCV

replication by a mechanism that is independent of their roles in regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007]. Takaki et al. [2007] have reported that eicosapentaenoic acid (EPA), a type of n-3 PUFA, allows maintenance of the original ribavirin dose in chronic hepatitis C patients during peg-IFN plus ribavirin combination therapy. However, the effects of these lipid modulators on chronic hepatitis C patients with intractable IL-28B allele remain unknown.

As a result of this experimental and therapeutic evidence, a new antiviral strategy to improve treatment outcome for chronic hepatitis C was designed, that is, addition of pitavastatin and EPA to peg-IFN plus ribavirin combination therapy (add-on therapy). The validity of the add-on therapy was evaluated by comparing its effect on the final outcome (i.e., sustained virological response) with that of unmodified peg-IFN plus ribavirin combination therapy (standard therapy), and pretreatment predictors of virological response were investigated. Additionally, the antiviral effect of pitavastatin and/or EPA was estimated in HCV replicon cells.

MATERIALS AND METHODS

Study Patients

In Kyushu Medical Center, a standard protocol in Japan (subcutaneous peg-IFN α 2a [180 μ g] or peg-IFN α 2b [median dose of 1.5 μ g/kg, range 1.3–1.7] weekly, along with oral ribavirin daily for 48 weeks) was adopted for chronic hepatitis C patients from 2005 to 2008. The dose of ribavirin was adjusted according to body weight: 600 mg for patients weighing <60 kg, 800 mg for those weighing 60–80 kg, and 800 mg for those weighing >80 kg. From 2008, oral pitavastatin (2 mg/day) and ethyl eicosapentate (1,800 mg/day) have been added to the standard protocol (add-on protocol). It has been shown that statins contribute to improving the virological response [Bader et al., 2008; Sezaki et al., 2009]. The add-on protocol was expected to improve treatment, and was applied to all patients after 2008 in Kyushu Medical Center, but a randomized study could not be designed. In these protocols, 48- and 24-week regimens were applied to patients infected with HCV-1b and HCV-2, respectively. Patients who experienced previous therapy using peg-IFN were excluded. Patients with cirrhosis were not included. Because of the possibility that vitamin E and bile acids including ursodeoxycholic acid promote HCV replication [Chang and George, 2007; Yano et al., 2007; Scholtes et al., 2008; Nakamura et al., 2010], treatment with these agents was withdrawn at least 1 month before the initiation of antiviral treatment. The study protocol was approved by the Ethics Committee of the National Hospital Organization, and written informed consent was obtained from all patients. Finally, 238 patients (genotype 1b/2 = 176/62) who were treated with the standard protocol (standard group) and 162 patients (genotype 1b/2 = 101/61) who were treated with the add-on protocol

TABLE I. Profile and Baseline Characteristics of Patients Infected With HCV-1b

Number of patients	Standard group	Add-on group	P
Gender: M/F	91/85	46/55	NS
Age (years)	59.5 ± 10.2	57.2 ± 12.5	NS
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	147/21/8	77/18/6	NS
HCV RNA (log IU/ml)	5.73 ± 0.16	6.08 ± 0.64	0.001
IL-28B (rs8099917): TT/TG + GG/ND	39/18/119	69/29/3	NS
ITPA (rs1127354): CC/CA + AA/ND	43/14/119	70/27/4	NS
Staging: F ₀₋₁ /F ₂₋₃ /ND	15/47/114	27/53/21	NS
ALT (IU/l)	74.5 ± 58.3	62.4 ± 45.2	NS
GGT (IU/l)	55.8 ± 46.8	51.9 ± 45.4	NS
WBC (/μl)	4,859 ± 1,239	4,870 ± 1,395	NS
Hemoglobin (g/dl)	13.9 ± 1.3	13.7 ± 1.5	NS
Platelet (/μl)	16.3 ± 5.7	19.1 ± 6.5	0.006
% of patients treated with enough total doses of Peg-IFN ^a	61.1	75.7	NS
% of patients treated with enough total doses of RBV ^b	76.4	77.1	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ -glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.

^aEnough total doses: >80% of planned doses.

^bEnough total doses: >60% of planned doses.

(add-on group) were enrolled and retrospectively analyzed. The profile and baseline characteristics of patients infected with HCV-1b are shown in Table I. In all patients infected with HCV-1b or HCV-2, baseline HCV RNA levels in serum were ≥ 5.0 log IU/ml.

Laboratory Data

Hematological, biochemical and virological parameters were determined by the clinical laboratory at Kyushu Medical Center. Serum HCV RNA concentrations were determined by the COBAS TaqMan PCR HCV test (Roche Diagnostics, Tokyo, Japan). Sustained virological response was defined as undetectable HCV RNA at week 24 after completion of therapy. Genotyping for the IL28B (rs8099917) and ITPA (rs1127354) polymorphisms was performed by TaqMan[®] SNP Genotyping Assays (Applied Biosystems, Branchburg, NJ) that apply a polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay. To determine amino acid polymorphism in HCV core protein, the PCR method with primers specific for polymorphism at core 70 was performed as described previously [Nakamoto et al., 2009].

Cell Lines and Treatment

The human-hepatoma-derived cell line, Huh7/Rep-Feo-1b, which stably expresses the HCV Rep-Feo replicon, was a kind gift from the Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University. The HCV subgenomic replicon plasmids, which contained NS3, NS4, NS5A, and NS5B, were derived from the HCV-N strain (genotype 1b), and the construct expressed a chimeric reporter protein of luciferase and neomycin phosphotransferase that allowed selection of cells and rapid measurement of the replication levels in stable replicon-expressing cells [Yokota et al., 2003; Tanabe et al., 2004; Toyoda et al., 2011]. Cells were maintained in Dulbecco's

modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin in a humidified 37°C/5% CO₂ incubator. Pitavastatin (donated by Kowa Pharmaceutical Co, Tokyo, Japan) and EPA (Otsuka Pharmaceutical Co, Tokyo, Japan) were dissolved in 10% carboxyl methylcellulose and chloroform, respectively, and stored in stock solutions at a concentration of 10 and 20 M, respectively. According to previous reports and our pretests for inhibition rates of HCV replication and cytotoxicity [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006], Huh7/Rep-Feo-1b cells were treated with 20 μ M EPA, 10 μ M pitavastatin, or 20 μ M EPA plus 10 μ M pitavastatin for 48 hr. The concentrations of EPA and pitavastatin may have been reasonable because they were lower than the reported maximum blood concentration of EPA or pitavastatin in healthy adult men with usual daily doses. For control cells, the same volume of 10% carboxyl methylcellulose and chloroform used for treated cells was added to medium and incubated for 48 hr.

Cell Proliferation/Viability and Luciferase Assays

The proliferation and viability of cultured cells were checked by Cell Viability and Proliferation Assay Kit (Funakoshi, Tokyo, Japan). Luciferase activity assay was performed using the Bright-Glo Luciferase Assay System (Promega, Tokyo, Japan). According to the manufacturer's protocol, luciferase was extracted from control and treated cells, and luciferase activity was quantified by use of a luminometer.

Real-Time PCR

mRNA expression levels in Huh7/Rep-Feo-1b cells under EPA and/or pitavastatin treatment were

analyzed using real-time RT-PCR and compared with untreated Huh7/Rep-Feo-1b cells. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1.0 µg RNA using GeneAmp™ RNA PCR (Applied Biosystems) with random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 µl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM MgCl₂, 0.5 µM upstream and downstream PCR primers, and 2 µl first-strand cDNA as a template. To control for reaction variations, all PCR data were normalized against the expression of retinoblastoma binding protein 6 [Nakamura et al., 2011]. The real-time RT-PCR primer sets in this study are listed in Table II.

Statistical Analysis

Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Differences between categorical variables were analyzed using Fisher's exact test or χ^2 test. Mann-Whitney *U* test was used for continuous variables. Multivariate analysis was used to identify factors independently associated with the achievement of sustained virological response. The odds ratio (OR) and 95% confidence intervals were also calculated. *P* < 0.05 was considered to be statistically significant.

RESULTS

Sustained Virological Response Rates in Patients Infected With HCV-1b and HCV-2

Peg-IFN and/or ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin levels, neutrophil counts or platelet counts, or the development of other adverse effects. Therefore, to evaluate therapeutic effects properly, sustained virological response rates were examined by intention to treat analysis. Within the enrolled patients, 62 and 61 patients infected with HCV-2 were included in

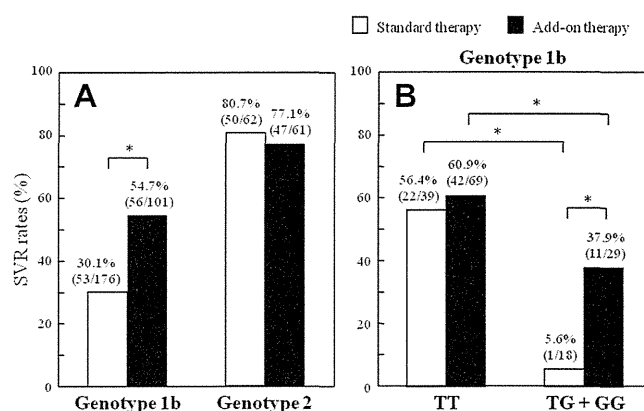


Fig. 1. Sustained virological response rates in chronic hepatitis C patients: comparison between standard and add-on therapy. **A:** Results for HCV genotype 1b and 2. **B:** Results for genome variation of IL28B (rs8099917); genotype TT and non-TT (TG + GG). Data for HCV-1b patients are shown. **P* < 0.01.

the standard and add-on therapy groups, respectively. In these patients, no significant difference was found in sustained virological response rates between the standard and add-on therapy groups; 80.7% and 77.1%, respectively (Fig. 1A). Hence, all subsequent examinations were conducted on patients infected with HCV-1b.

In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than in the standard therapy group (54.7% vs. 30.1%, *P* < 0.0001; Fig. 1A), although background HCV RNA levels were significantly higher in the add-on therapy group (Table I). Platelet counts were higher in the add-on therapy group but those in the standard therapy group were still sufficient for IFN-based therapy. Of note, no significant difference was found between the standard and add-on therapy groups for the rate of patients in whom sufficient total doses of peg-IFN (>80% of planned doses) and ribavirin (>60% of planned doses) were administered (Table I).

TABLE II. Sequences of Primers Used for Real-Time PCR

Genes	Forward (5' → 3')	Reverse (5' → 3')
RIG-I	GGCCCACTGCCCCAGGTCAT	TCCCAACACCAACCGAGGC
MAVS	CCCTCTGGCATCTCTTCAATACC	TTCGTCCGCGAGATCAACTA
IRF3	CCAGCTTGGACAATCCCACCTC	GAAGGCTGTACCTCGAACTC
TRAF6	GAGGTCTCCACCCGCTTTGA	TTGAGCAAGTGAGGGCAAGCTA
IFNβ1	GCGACACTGTTTCGTGTTGTCA	CCAAGCAAGTTGTAGCTCATGGA
ISG15	GGGCTGGGACCTGACGGTGA	GGACAGCCAGACGCTGCTGG
HMGR	GCCTGGCTCGAAACATCTGAA	CTGACCTGGACTGGAAACGGATA
SREBP-1	GCTGTCCACAAAAGCAAATCTCT	GTCAGTGTGTCCTCCACCTCAGT
LDLR	CAACGGCTCAGACGAGCAAG	AGTCACAGACGAACTGCCGAGA
RBBP6	GCGACCTGCAGATCACCAA	TGCCATCGCTGGTTCAGTTC

RIG-I, retinoic acid inducible gene I; MAVS, mitochondrial antiviral signaling; IRF, interferon regulatory factor; TRAF, TNF receptor associated factor; IFN, interferon; ISG, interferon-stimulated gene; HMGR, HMG-CoA reductase; SREBP, sterol regulatory element binding protein; LDLR, LDL receptor; RBBP, retinoblastoma binding protein.

Effect of IL28B and ITPA Genotypes on Viral Response

According to genetic variation of IL28B gene (rs8099917), sustained virological response rates in patients infected with HCV-1b were determined (Fig. 1B). In both the standard and add-on therapy groups, sustained virological response rates were significantly higher in patients with the major type genome variation (TT) than in those with the minor type (TG + GG). In the latter, sustained virological response rates were markedly higher in the add-on than in the standard therapy group (37.9% vs. 5.6%, $P = 0.007$). In patients with the major type genome variation, addition of pitavastatin and EPA induced higher sustained virological response rates although no significant difference was found between the two treatment groups. In comparison between the major (CC) and minor (non-CC) types of ITPA (rs1127354), sustained virological response rates were comparable between the standard and add-on therapy groups (Fig. 2A). However, in the add-on group, the percentage of patients infected with HCV-1b who completed therapy without dose reduction of ribavirin was significantly higher among those with the minor type of ITPA than the major type (45.8% vs. 21.5%, $P = 0.004$; Fig. 2B).

Viral Kinetics With Add-on Therapy

Viral kinetics in patients infected with HCV-1b were examined in the add-on therapy group according to genome variation of the IL28B (rs8099917), and compared between the sustained virological response and non-sustained virological response groups. In patients with major variation type (TT), viral decline was significantly greater at all times (days 3–84) in

the sustained virological response than in the non-sustained virological response group (Fig. 3A). However, in patients with minor variation type (TG + GG), viral kinetics were similar within the first 2 weeks of treatment in the sustained virological response and non-sustained virological response groups (Fig. 3B). Accordingly, sustained virological response was affected by the depth of early phase viral decline in patients with major variation but not in patients with minor variation. Viral kinetics in patients with minor type variation (TG ± GG) of IL-28B were compared between the standard therapy and add-on therapy

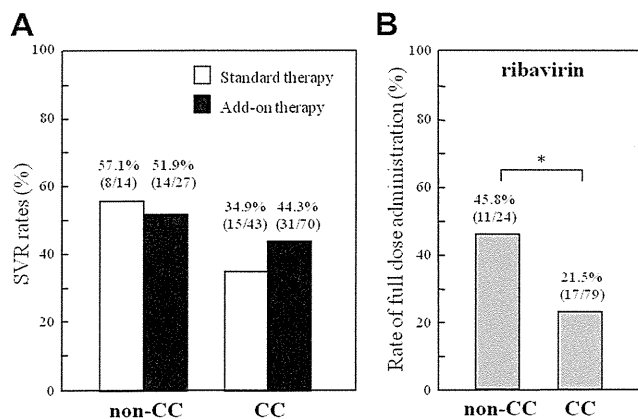


Fig. 2. Clinical data of patients infected with HCV-1b: comparison between genome variations of ITPA. **A:** Sustained virological response rates were compared between standard and add-on therapy. Results are presented for each genome variation of ITPA (rs1127354); genotype CC and non-CC. **B:** Numbers of patients in whom planned ribavirin doses were completed. Results in patients infected with HCV-1b treated with add-on therapy are shown in each genome variation of ITPA (rs1127354); genotype CC and non-CC. * $P < 0.05$.

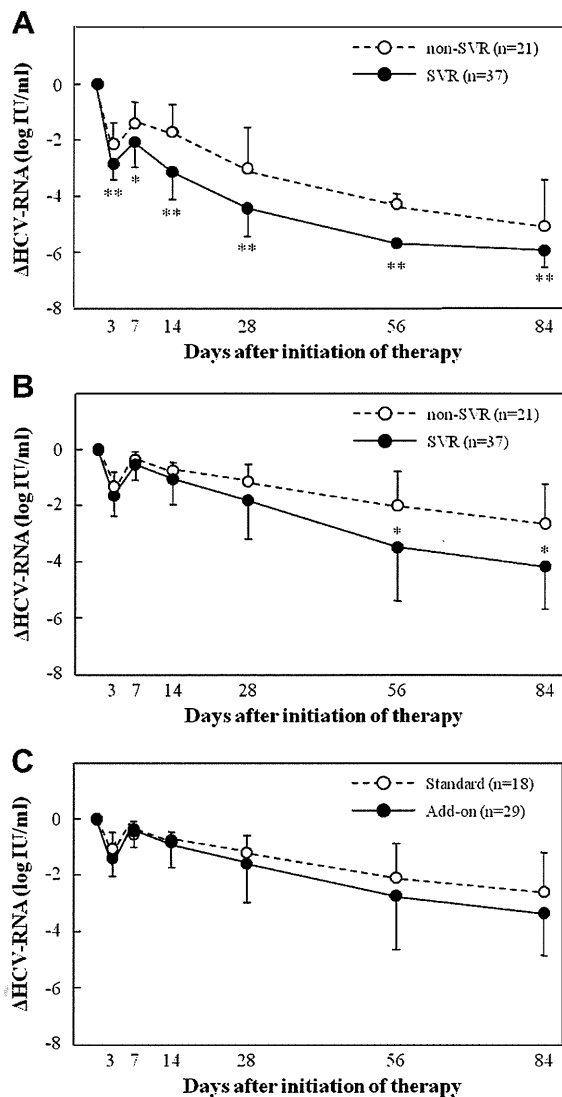


Fig. 3. Viral kinetics in patients infected with HCV-1b. **A:** Results in patients with major type variation (TT) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. **B:** Results in patients with minor type variation (TG + GG) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. **C:** Results in patients with minor type variation (TG + GG) of IL-28B: comparison between standard therapy and add-on therapy groups. * $P < 0.05$, ** $P < 0.01$ (sustained virological response vs. non-sustained virological response).

groups (Fig. 3C). As a result, viral decline was somewhat greater after day 28 in the add-on therapy group but the difference was not significant.

Effect of Amino Acid Substitutions of HCV Core 70 on Viral Response

Add-on therapy was significantly more effective in patients with IL28B minor variation (TG + GG) compared with standard therapy, therefore, we investigated the association between HCV core 70 amino acid mutation and therapeutic outcome. In 27 patients infected with HCV-1b, who had minor variation of IL28B (TG + GG) and were treated with add-on therapy, core 70 amino acid mutation was determined. Sustained virological response was achieved in 10 patients and core 70 mutation (Gln) was found in 6 of the 10 patients (60%). Within the 17 non-sustained virological response patients, the mutation was identified in eight patients (47.1%). Accordingly, within these patients, the core 70 amino acid substitutions did not affect sustained virological response in the add-on therapy.

Predictive Factors Associated With Sustained Virological Response

Among the factors listed in Table III, predictive factors associated with sustained virological response were examined in patients infected with HCV-1b. Univariate analysis identified six parameters that correlated significantly with sustained virological response; age ($P = 0.0038$), fibrotic staging ($P = 0.0012$), γ -glutamyl transpeptidase ($P = 0.0009$), platelet count ($P = 0.0132$), genetic variation of IL28B ($P < 0.0001$) and add-on therapy ($P < 0.0001$; Table III). In multivariate analysis, significant contribution factors for sustained virological response were age (<60 years; OR 3.06, $P = 0.0221$), IL28B (genotype TT; OR 6.69, $P = 0.0019$) and staging (F_{0-1} ; OR 5.71, $P = 0.0035$;

TABLE IV. Multivariate Analysis for Predictive Factors Associated With Sustained Virological Response

Factors	Category	95% confidence intervals	<i>P</i>
Age (years)	1. ≥ 60 : 1.0	1.20–8.24	0.0221
	2. <60: 3.06		
IL-28B (rs8099917)	1. TG + GG: 1.0	2.17–24.66	0.0019
	2. TT: 6.69		
Staging	1. F_{2-3} : 1.0	1.91–20.51	0.0035
	2. F_{0-1} : 5.71		

Table IV). When IL28B was excluded from the factors in multivariate analysis, addition of pitavastatin and EPA (add-on therapy) was also selected as a significant contribution factor for sustained virological response (OR 2.13, $P = 0.0395$).

Subgenomic HCV Replicon System

Suppression of HCV RNA replication by pitavastatin and/or EPA was examined in Huh7/Rep-Feo-1b cells by luciferase assay. The concentrations of pitavastatin and EPA for the following experiments were determined according to previous studies [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006] and our pilot study for cytotoxicity and luciferase assay (data not shown). Huh7/Rep-Feo-1b cells were incubated with or without 10 μ M pitavastatin and/or 20 μ M EPA for 48 hr. As a precondition, the proliferative activity and viability of pitavastatin- and/or EPA-treated cells were comparable with those of control cells (data not shown). As a result, luciferase activity was significantly suppressed in EPA- and/or pitavastatin-treated cells compared with the control cells (Fig. 4A). At these concentrations, the suppressive effect was more marked in pitavastatin-treated than EPA-treated cells.

TABLE III. Univariate Analysis Between Non-Sustained Virological Response and Sustained Virological Response Groups

Factors	Non-SVR	SVR	<i>P</i>
Gender (M/F)	82/86	55/54	NS
Age (years)	60.5 \pm 10.6	55.7 \pm 12.0	0.0038
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	136/24/8	88/15/6	NS
HCV RNA (log IU/ml)	6.03 \pm 0.16	5.91 \pm 0.55	NS
IL-28B (rs8099917) TT/TG + GG/ND	35/44/89	12/64/33	<0.0001
ITPA (rs1127354) CC/CA + AA/ND	59/20/89	54/21/34	NS
Staging (F_{0-1} / F_{2-3} /ND)	11/59/98	31/41/37	0.0012
Treatment add-on/standard	45/123	56/53	<0.0001
ALT (IU/l)	72.3 \pm 57.7	63.9 \pm 45.2	NS
GGT (IU/l)	65.3 \pm 56.0	41.1 \pm 27.1	0.0009
WBC (μ l)	4,935 \pm 1,392	4,791 \pm 1,254	NS
Hemoglobin (g/dl)	13.8 \pm 1.4	13.8 \pm 1.4	NS
Platelet (μ l)	16.7 \pm 5.7	19.1 \pm 6.6	0.0132
% of patients treated with enough total doses of Peg-IFN ^a	60.9	74.4	NS
% of patients treated with enough total doses of ribavirin ^b	71.9	80.8	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ -glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.

^aEnough total dose: >80% of planned doses.

^bEnough total doses: >60% of planned doses.

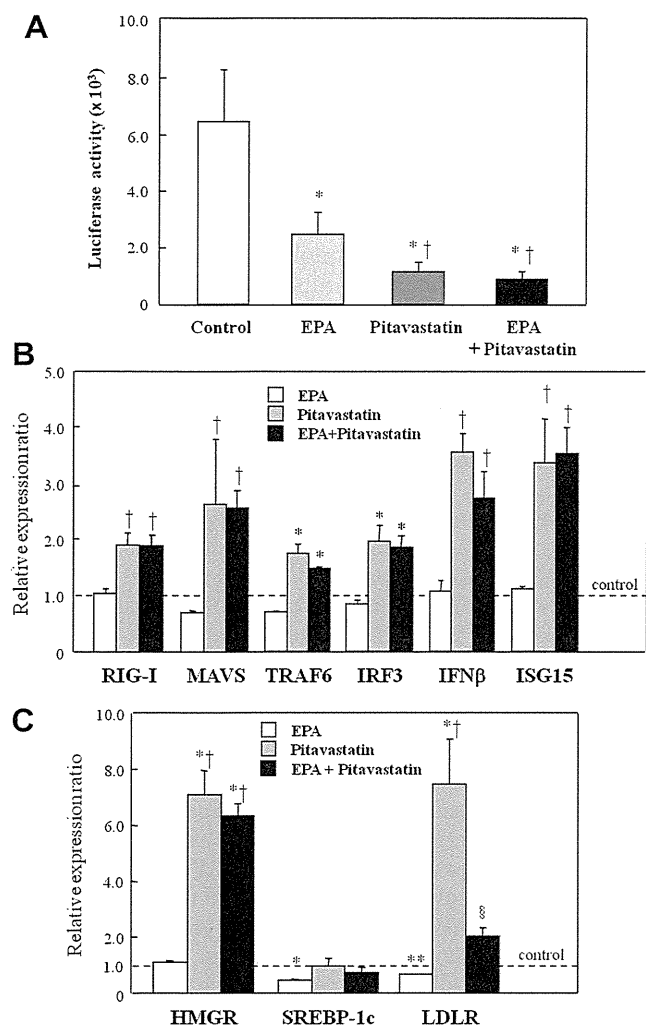


Fig. 4. Treatment of pitavastatin and/or EPA in HCV replicon cells. **A:** HCV replication was estimated by luciferase assay. Huh7/Rep-Feo-1b cells were treated with pitavastatin (10 μ M) and/or EPA (20 μ M) for 48 hr. * P < 0.01 versus control, † P < 0.01 versus EPA. **B:** Expression levels of RIG-I, MAVS, TRAF6, IRF3, IFN β , and ISG15 genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μ M) and/or EPA (20 μ M) for 48 hr. * P < 0.01 versus control and EPA, † P < 0.05 versus control and EPA. **C:** Expression levels of HMGR, SREBP-1c and LDLR genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μ M) and/or EPA (20 μ M) for 48 hr. * P < 0.01 versus control, ** P < 0.05 versus control, † P < 0.01 versus EPA, § P < 0.01 versus pitavastatin.

In Huh7/Rep-Feo-1b cells, the expression levels of innate-immunity-associated genes were examined after 48 hr treatment with 10 μ M pitavastatin and/or 20 μ M EPA. As shown in Figure 4B, retinoic acid inducible gene I (RIG-I), mitochondrial antiviral signaling (MAVS), TNF receptor associated factor 6 (TRAF6), IFN regulatory factor 3 (IRF3), IFN β and IFN-stimulated gene 15 (ISG15) showed similar trend in expression. Accordingly, their expression was significantly increased by pitavastatin but not by EPA, and EPA did not show an additive effect with pitavastatin. With the same treatments, expression of

lipid-metabolism-associated genes was analyzed (Fig. 4C). HMG-CoA reductase (HMGR) expression was significantly enhanced by pitavastatin but not by EPA. The sterol regulatory element binding protein 1c (SREBP-1c) expression was significantly suppressed by EPA but not by pitavastatin. Low-density lipoprotein receptor (LDLR) expression was significantly suppressed by EPA, whereas the expression was activated by pitavastatin, but the activation was lost in the presence of EPA.

DISCUSSION

For ethical reasons, standard therapy could not be selected after 2008; therefore, the present study was unable to eliminate some methodological issues that limit the interpretation and drawing of firm conclusions. For example, the percentage of patients receiving sufficient total dose of peg-IFN was lower in the historical standard group although the difference was not significant and, in order to prevent dose reduction, additional means might have been performed on the add-on group after 2008. However, in univariate and multivariate analyses, total dose of peg-IFN was not detected as a significant factor for sustained virological response. Nevertheless under these limitations, the presented clinical and in vitro studies indicate some sufficient trends in treatment response.

Previous studies on hepatic lipid metabolism have shown that, in the liver of patients with HCV infection, synthesis of cholesterol and fatty acids is still activated, regardless of overaccumulation of lipids [Kohjima et al., 2009; Nakamuta et al., 2009, 2011; Fujino et al., 2010]. This means that addition of pitavastatin and EPA to standard therapy is pathophysiologically reasonable for patients with chronic hepatitis C. Sustained virological response rates in patients infected with HCV-2 were sufficiently high and comparable between the standard and add-on therapy groups (Fig. 1A). Therefore, this study was focused on patients infected with HCV-1b with high virus load. This investigation of sustained virological response in patients treated with add-on or standard therapy had two clinically important findings.

First, add-on therapy led to significantly higher sustained virological response rates than did standard therapy (Fig. 1A). Although overall sustained virological response rate in this study was lower compared with the results from some other institutions, it may be because intention to treat analysis was used in this study and the ratio of IL28B minor (TG + GG) patients was higher in the standard and add-on groups. When sustained virological response rates were compared only in patients with IL28B major or in those with IL28B minor, the sustained virological response rates were not lower compared with those in other reports (data not shown). The suppressive effect against HCV replication by statins and EPA, and their synergistic action with IFN, has already been demonstrated in some HCV replicon systems [Ye

et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007; Ikeda and Kato, 2007]. In our investigation using the luciferase assay in Huh7/Rep-Feo-1b cells, a similar suppressive effect was seen with both pitavastatin and EPA treatments (Fig. 4A). It has been reported that the statins impede HCV replication through inhibition of host protein geranylgeranylation and FBL2 has been identified as a geranylgeranylated cellular protein required for HCV RNA replication [Wang et al., 2005; Nakamuta et al., 2011]. PUFAs, including EPA, inhibit HCV replication, although the precise mechanism is still unclear but may be independent of the route regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005]. The synergistic and additive effect of EPA with pitavastatin was not significant in our luciferase assay; therefore, statins and EPA may act against cognate targets.

Second, the add-on therapy improved sustained virological response rates especially in patients with the minor type variation (TG + GG) of the IL28B gene (rs8099917), in whom sustained virological response is expected to be poor after standard therapy (Fig. 1B). Recent studies have revealed that SNPs within or adjacent to IL28B region provide a strong predictive value for the outcome of IFN-based therapy in patients infected with HCV-1b [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. With add-on therapy, sustained virological response rates were significantly higher in patients with major type (TT) than minor type (TG + GG) variations, meaning that genome variation of IL28B (rs8099917) still governs the outcome even in add-on therapy. However, in the patients with minor type variation, the sustained virological response rate (37.9%) in the add-on group was markedly higher compared with that in the standard therapy group (Fig. 1B). This sustained virological response rate may be sufficiently high for clinical use of add-on therapy in patients infected with HCV-1b with minor type variation. From this point of view, the add-on therapy is a clinically valuable strategy for chronic hepatitis C. In the analysis of viral dynamics in patients with minor type variation of IL28B, no significant difference was found in viral decline within 84 days between the standard and add-on groups (Fig. 3C). Although there is still no evidence, in the add-on therapy, late phase viral decline (3 months after treatment initiation) may be more important for the achievement of sustained virological response in patients with minor type variation.

It has been emphasized that mutation of amino acids 70 and 91 in the core region of HCV-1b as a virus-related factor, as well as genome variation of IL28B gene as a host-related factor, greatly influences the outcome of IFN-based antiviral treatments. According to recent clinical studies in patients infected with HCV-1b, substitution of core 70 is assessed as a more influential factor affecting the outcome of peg-IFN plus ribavirin combination therapy,

rather than that of core 91 [Akuta et al., 2007; Hayes et al., 2011; El-Shamy et al., 2012]. Even in the latest triple therapy with peg-IFN, ribavirin and a NS3/4A protease inhibitor, telaprevir, patients infected with HCV-1b with core 70 mutation were reported to be severely resistant to the therapy [Akuta et al., 2010]. In our assessment of patients with minor type IL28B variation at rs8099917 (TG + GG), mutation at core 70 was likely not to diminish the outcome of add-on therapy, although the number of patients examined was small (Fig. 4). Therefore, the lipid modulators, pitavastatin and EPA, may be expected to be more effective for patients infected with HCV-1b with core 70 mutation, compared with an NS3/4A protease inhibitor. However, for reliable assessment, further clinical data are needed from patients treated with add-on therapy.

As part of its pathogenic strategy, HCV interferes with the innate immune response of its host; mainly in the RIG-I/MAVS pathway [Breiman et al., 2005; Tasaka et al., 2007; Baril et al., 2009; Jouan et al., 2010; Lemon, 2010; Liu and Gale, 2010; Ekisioglu et al., 2011]. RIG-I undergoes a conformational change upon HCV RNA binding and interacts with MAVS, resulting in phosphorylation and nuclear translocation of IRF3, which leads to transcriptional activation and synthesis of IFN β . IFN β activates the Jak-STAT (Janus kinase-signal transducer and activator of transcription) signaling pathway and acts through the expression of ISGs. TRAF6 is recruited to the MAVS complex and is required for activation of nuclear factor- κ B, which forms an enhanceosome on the IFN β promoter in coordination with IRF3. In HCV-infected cells, NS3/4A protease cleaves MAVS, and the RIG-I/MAVS pathway is impeded. In the present study, the HCV replicon system was used to examine how pitavastatin and EPA influence the RIG-I/MAVS pathway, which plays an important role in the innate antiviral host response to HCV infection. The expression profile of innate-immunity-associated genes in pitavastatin- and/or EPA-treated Huh7/Rep-Feo-1b cells showed that only pitavastatin activated expression of the tested genes, RIG-I, MAVS, IRF3, TRAF6, IFN β , and ISG15, similarly (Fig. 4B). EPA treatment did not increase expression levels of these genes. It is unclear whether the activation of these innate-immunity-associated factors directly contributes to elimination of HCV or whether inhibition of HCV replication by pitavastatin treatment directly leads to the activation of innate immunity through lowering NS3/4A protease expression.

Cholesterol, fatty acids, and lipid rafts have been demonstrated to be critical for efficient replication, infection and secretion of HCV [Simons and Ehehalt, 2002; Kushner et al., 2003]. For example, HCV replication was suppressed by inhibition of the liver X receptor α -SREBP-1c pathway [Kapadia and Chisari, 2005]. Therefore, negative modulation of lipid synthesis may be an antiviral step of statins and EPA. In pitavastatin treatment of HCV replicon cells, HMGR

and LDLR expression was enhanced in response to inhibition of cholesterol synthesis, whereas EPA decreased the expression of SREBP-1c and LDLR, by which fatty acid synthesis and cholesterol uptake might be lowered (Fig. 4C). Of note, although pitavastatin alone enhanced the expression of LDLR, the enhancement was abolished by addition of EPA (Fig. 4C). This effect of EPA indicates the clinical significance of the add-on therapy because LDLR is known to be an important cellular factor that is required for cell entry/infection of HCV. EPA addition is expected to accelerate the antiviral effect of peg-IFN, ribavirin and pitavastatin through repression of HCV entry/infection as well as HCV replication. It has recently been reported that HCV particles are enriched in cholesterol and virion cholesterol is involved in HCV cell entry, depending on Niemann-Pick C1-like 1 (NPC1L1), which is an HCV cell entry factor as well as a cellular cholesterol uptake receptor [Yamamoto et al., 2011; Sainz et al., 2012]. The NPC1L1 may be amenable to therapeutic intervention.

The analysis of viral dynamics during add-on therapy indicated that early phase viral decline within the first 2 weeks influenced the achievement of sustained virological response in patients with major type variation (TT) but not in those with minor type variation (TG + GG; Fig. 3A,B). It cannot be explained clearly why high sustained virological response rates were obtained in patients infected with HCV-1b with minor type variation, regardless of poorer viral decline with add-on therapy. Although there is still no evidence, in patients with minor type variation, statins and EPA may show their effect in a later phase, and the EPA effect of impeding HCV entry/infection through suppression of LDLR expression may contribute partly to the achievement of sustained virological response.

In univariate analysis, addition of pitavastatin and EPA, as well as genotype TT of IL28B at rs8099917, was positively associated with sustained virological response in peg-IFN plus ribavirin combination therapy (Table III). However, this association disappeared in a multivariate analysis, and IL28B variation remained as an independent factor. One of the major reasons may be that, compared with the addition of pitavastatin and EPA, TT variation of IL28B in the profile of individuals has overwhelming weight for governing the effect of peg-IFN plus ribavirin combination therapy. In our study, SNP variation of ITPA (rs1127354) did not influence treatment outcome but the planned dose of ribavirin was maintained well in patients with minor type variation (non-CC), as reported in previous studies (Fig. 2).

In conclusion, the lipid modulators, pitavastatin and EPA, could enhance the efficacy of peg-IFN plus ribavirin combination therapy through their synergistic antiviral effect, particularly in patients infected with HCV-1b with an intractable IL-28B allele. Although the research is still in the preliminary stages, there is a possibility that addition of pitavastatin and EPA may be effective for HCV-1b with core 70

mutation, and may increase sustained virological response rates in patients treated with triple therapy of peg-IFN, ribavirin, and telaprevir.

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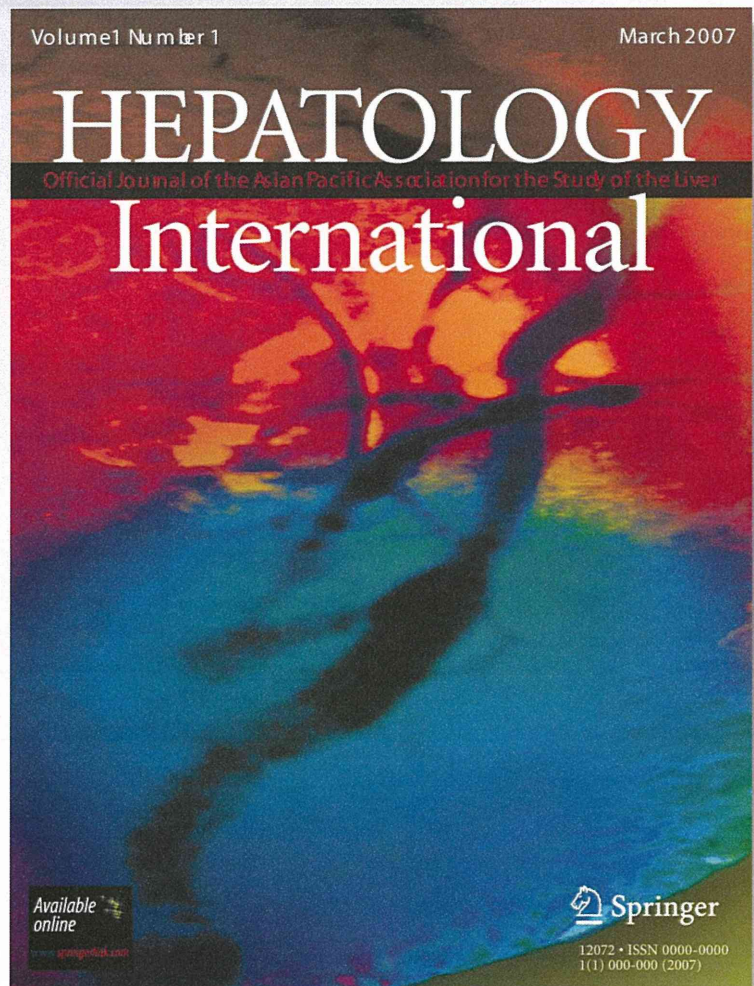
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Association of *ITPA* gene variation and serum ribavirin concentration with a decline in blood cell concentrations during pegylated interferon-alpha plus ribavirin therapy for chronic hepatitis C

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Abstract

Background Genetic variation leading to inosine triphosphatase (*ITPA*) deficiency protects chronic hepatitis C patients receiving ribavirin against hemolytic anemia. The relationship between *ITPA* gene variation and serum ribavirin concentration was analyzed in association with a reduction in blood cells and dose reduction of pegylated interferon (PEG-IFN) or ribavirin.

Patients and methods A total of 300 hepatitis C patients treated with PEG-IFN plus ribavirin were analyzed. Genetic polymorphisms were determined in *ITPA* and the quantitative reduction in blood cells from the baseline was analyzed every 4 weeks for the duration of treatment and after the end of therapy. The decline in hemoglobin (Hb) or platelet (PLT) level at week 4 compared to baseline was also assessed according to ribavirin concentrations.

Results Patients with the *ITPA-CA/AA* genotypes showed a lower degree of Hb reduction throughout therapy than those with the *ITPA-CC* genotype and a marked difference in mean Hb reduction was found at week 4 (*CA/AA* −1.0 vs. *CC* −2.8, $p < 0.001$). The *ITPA-CC* genotype had significantly less reduction in the mean platelet count than the *ITPA-CA/AA* genotypes early during treatment ($p < 0.001$ for weeks 4 and 8). Patients with the *ITPA-CA/AA* genotypes were less likely to develop anemia, regardless of the concentration of ribavirin. Patients with baseline PLT counts below $130 \times 10^3/\mu\text{l}$ had a significantly lower tendency to achieve sustained virological response (SVR), especially those with the *ITPA-CA/AA* genotypes. *ITPA* gene variation was not extracted by multivariable analysis as an important predictor of SVR.

Conclusions Despite the fact that *ITPA* variants were less likely to develop anemia, patients with low baseline PLT counts were difficult to treat, especially those with the *ITPA-CA/AA* genotype. These results may give a valuable pharmacogenetic diagnostic tool for the tailoring of dosing to minimize drug-induced adverse events.

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