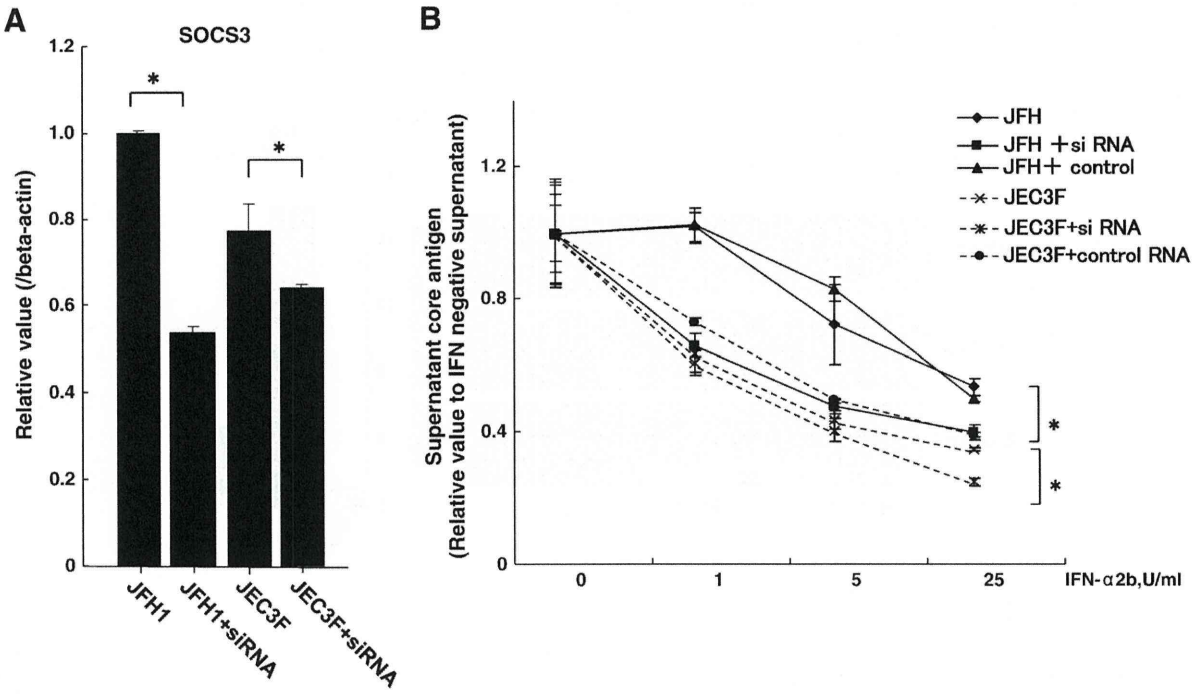

Fig. 5. Differences in sensitivities to IFN between SOCS3-knock down, HCV transfected cells. JFH1 or JEC3F 10 μg RNA, and 80 pmol siRNA SOCS3-HSS113312 or MOCK were electroporated into 5×10^6 uninfected Huh7.5.1 cells. A. Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of SOCS3 were determined by real time PCR. Values are shown as relative to those of JFH1 infected Huh 751 cells. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05. B. Dose-dependent suppression of HCV replication by IFN in SOCS3-knock-down, HCV-infected cells. The above siRNA and HCV RNA-transfected cells were divided into 12 wells. Forty eight hours after transfection, the cells were treated with 0, 1, 5 and 25 U/ml of IFN-alpha 2b. Seventy two hours after treatment, quantification of HCV core antigen in culture fluids was carried out. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

chronic hepatitis C patients (Malaguarnera et al., 1997). Consistent with those reports, we found that IL-6 strongly induced SOCS3 expression in Huh7.5.1 cells (Fig. 6C). More importantly, cellular IL6 expression levels were in the order of uninfected<JEC3F<< JFH1-infected cells, which correlated well with SOCS3 expression (Fig. 4) and with cellular responses to IFN (Fig. 2). In addition, the IFN-resistant JcoreC3F, in which the core region of JEC3F had been re-substituted by the JFH1-core, induced comparatively higher levels of IL-6 and SOCS3 mRNA to JFH1 (Fig. 7). Taken together, our results indicate that the amino acid sequence of the core protein determines IL-6 and SOCS3 expression levels and, as a consequence, resistance to IFNs.

It remains to be clarified what are the inducers of IL-6. There are reports that HCV core protein activates toll-like receptor (TLR)-2 in Huh7 cells and in adult human hepatocytes (Hoffmann et al., 2009; Mozer-Lisewska et al., 2005). TLRs are known to activate downward NF-kappaB signaling that upregulates IL-6 expression. Alternatively, IL-6 may be secreted in response to cellular steatosis and insulin resistance. HCV patients with obesity or insulin resistance are refractory to IFN treatments. Such patients have higher levels of hepatic SOCS3 expression than those without obesity or insulin resistance (Miyaaki et al., 2009; Walsh et al., 2006). More recently, Sabio, *et al* have reported that fatty acid-induced secretion of IL-6 from adipocytes upregulates hepatic SOCS3, leading to insulin-resistance (Sabio et al., 2008).

In conclusion, our study demonstrates that HCV intragenotypic and inter-strain differences in IFN sensitivity can be, in most part, attributable to the amino acid sequence of the HCV core protein and that such IFN sensitivities are determined by cellular expression levels of SOCS3 and IL-6. Therapeutic targeting of IL-6 potentially may be a key to targeting IFN-resistance and improving antiviral chemotherapeutics against HCV.

Materials and Methods

Reagents and antibodies

Recombinant human interferon alpha-2b was from Schering-Plough (Kenilworth, NJ). Anti-CD 81 antibody (JS-81)was from BD Biosciences (Franklin Lakes, NJ) (Morikawa et al., 2007), anti-IL6 receptor antibody was from Chugai pharmaceutical Co (Tokyo, Japan), anti-SOCS3 was from Cell Signaling (Beverly, MA), and anti-IL6 antibody was from R&D Systems (Minneapolis, MN).

Cloning of HCV cDNA from patient serum

A serum sample was obtained from a 32- year-old male who developed acute hepatitis after intravenous drug injection. Serum was obtained one week after the onset of symptoms. Total RNA was extracted from 150 μl of serum using ISOGEN (Nippon Gene, Osaka, Japan). cDNA was synthesized using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase. PCR primers, based on a genotype 2b prototype sequence, HC-J8 (accession number: D10988), were used to amplify 14 fragments of HCV cDNA covering nt. 13-9478 (nucleotide numbers corresponded to HC-J8) by PCR. All amplicons were purified and cloned into the pGEM-T EASY vector (Promega, Madison, WI) and nucleotide sequences were determined using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems). The consensus sequence of five clones was adopted for each region. Each consensus sequence segment of HCV was assembled into pJFH1-full (Wakita et al., 2005) by substituting the insert sequence of pJFH1-full.

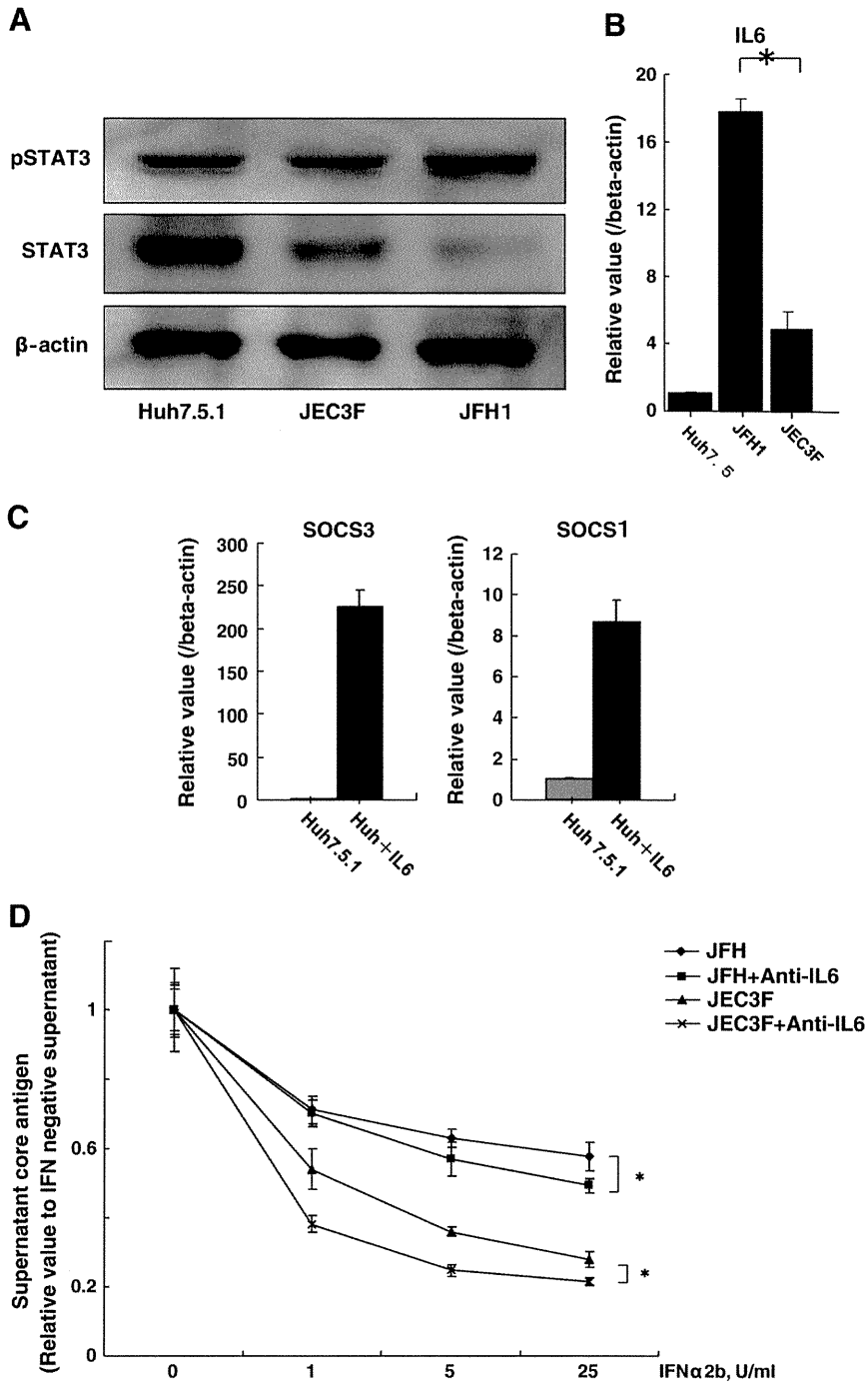

Fig. 6. IL-6 expression in HCV infected cells and change in IFN sensitivity by treatment with anti-IL6 antibody. A. Expression of cytoplasmic phospho-STAT3 in uninfected and HCV-infected Huh7 cells. JEC3F, JFH1 10 μg RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours total cellular protein was isolated. Ten μg of extracted protein were used for analysis of phosphorylated STAT3, STAT protein and β-actin as controls. B. Expression of Interleukin-6 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of IL6 were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean ± sd. C. IL-6 induces SOCS3 strongly in uninfected Huh7.5.1 cells. Uninfected Huh7.5.1 cells were treated with 10 ng/ml recombinant human IL6 (PEPRO TEC EC, London, England). Fifteen minuets after treatment, total RNA was isolated. Relative gene expression levels of SOCS1 and SOCS3 were determined by real time PCR. Uninfected Huh7.5.1 cells that were not treated with IL6 were used as a control. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown is mean ± sd. D. Dose-dependent suppression of HCV replication by IFN in HCV-infected cells pre-treated with anti-IL-6 antibody. Immediately after electroporation, HCV RNA-transfected cells were divided into 12 wells and pretreated with 1 μg/ml anti-IL6 antibody. Forty eight hours after transfection, the cells were washed with PBS and treated with 0, 1, 5 and 25 U/ml of IFN-alpha 2b. Seventy two hours after treatment, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean ± sd. E. Core protein secretion levels following treatment of HCV-transfected cells with anti-IL-6 antibody. After treatment with anti-IL-6 antibody, HCV RNA-transfected cells were divided into 12 wells. Five days after transfection, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean ± sd. F. Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative SOCS3 gene to beta-actin gene expression were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

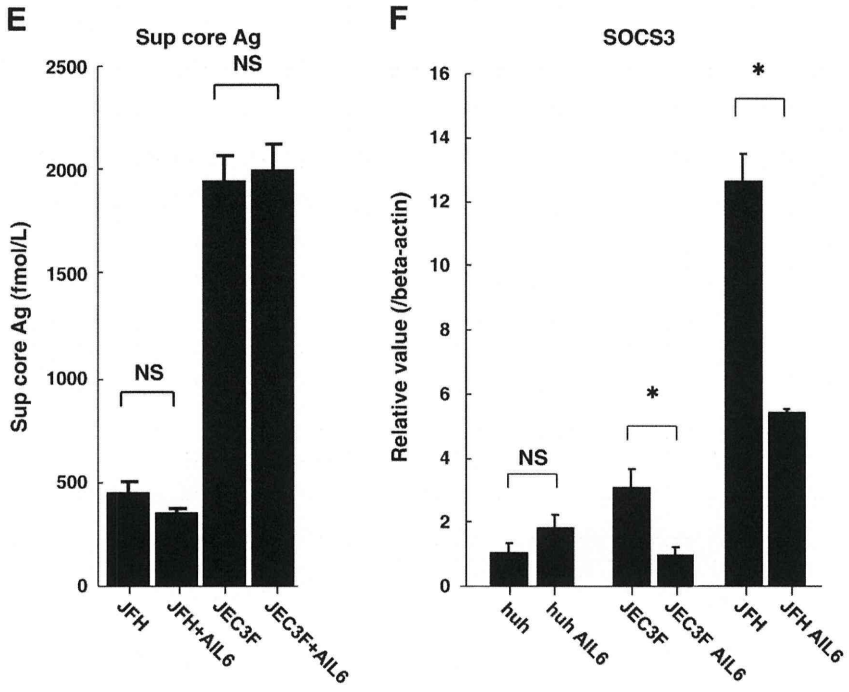

Fig. 6 (*continued*).

Construction of 2b/JFH-1 based intragenotypic chimeras and transfection

Chimeric HCV constructs of HCV-2b and JFH1 were shown in Figs. 1A and 7A. To construct 2b/JFH1-based intragenotypic chimera, JE31F, the 2b sequence of core through E2 (nt. 342- 2541) was fused to the EcoRI-JFH1- 5′-untranslated region (UTR) DNA by fusion PCR. The fused 5′UTR-E2 fragment and JFH1-E2-NS3 (nt2541 through 5324) were assembled by fusion PCR and cloned into pGEM-T EASY. The product was digested by EcoRI and AfeI and insert into pJFH1. Plasmids pJE39F, pJEC3F, pJcoreC3F and p2bcore JFH1 were constructed using a similar procedure. Plasmids pJEC3F and pJE39F were joined between NS2 and NS3, and within NS2 at nt. 2867, respectively. Plasmid pJcoreC3F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The plasmid p2bcoreJFH1 was made by substitution of the core region of JFH1 with that of 2b/JFH1.

Cells and cell culture

Huh7.5.1 cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2.

HCV cell culture system

Full-length HCV expression plasmids were as follows: pJFH1-full (Wakita et al., 2005), pJE31F, pJE39F, pJEC3F, pJcoreC3F, p2bcoreJFH1, and pFL-H77/JFH1, pFL-J6/JFH1 (Lindenbach et al., 2005). These plasmids were linearized at their 3′ ends and used as templates for HCV RNA synthesis using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNase I (RQ-1, RNase-free DNase, Promega) treatment, the HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7.5.1 cells were washed twice with PBS, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4 mm electroporation cuvette and finally subjected to an electric pulse (1,050 μF and 270 V) using the Easy Jet system (EquiBio, Middlesex, UK). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a cell culture dish.

Quantification of HCV core antigen in culture supernatants

Culture supernatants of HCV RNA transfected Huh7.5.1 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at -80 °C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Re-infection analyses

Titer-adjusted supernatants (including 0.03 fmol HCV core antigen) from HCV RNA-transfected cells were inoculated onto naïve Huh7.5.1 cells plated on a 6 cm plate at a density of 3×10^5 cells per plate. Forty-eight hours after inoculation, anti-core immunostaining was carried out with mouse anti-HCV core protein monoclonal antibody and the numbers of infected cells were counted. HCV core antigen in culture supernatants was measured at 24 hours, 48 hours, 72 hours and 144 hours after inoculation.

Real-time RT-PCR analysis

For the detection of HCV RNA in culture supernatant, supernatant was passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at -80 °C until use. Protocol and primers for the realtime RT-PCR analysis of HCV-RNA has been described previously (Sekine-Osajima et al., 2008). For the detection of endogenous mRNAs, total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate cDNA from each sample using SuperScript II. Expression of mRNA was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City CA). Some primers have been described (Sekine-Osajima et al., 2008). SOCS3; forward, 5′-CAC ATG GCA CAA GCA CAA GAA G-3′ and reverse,

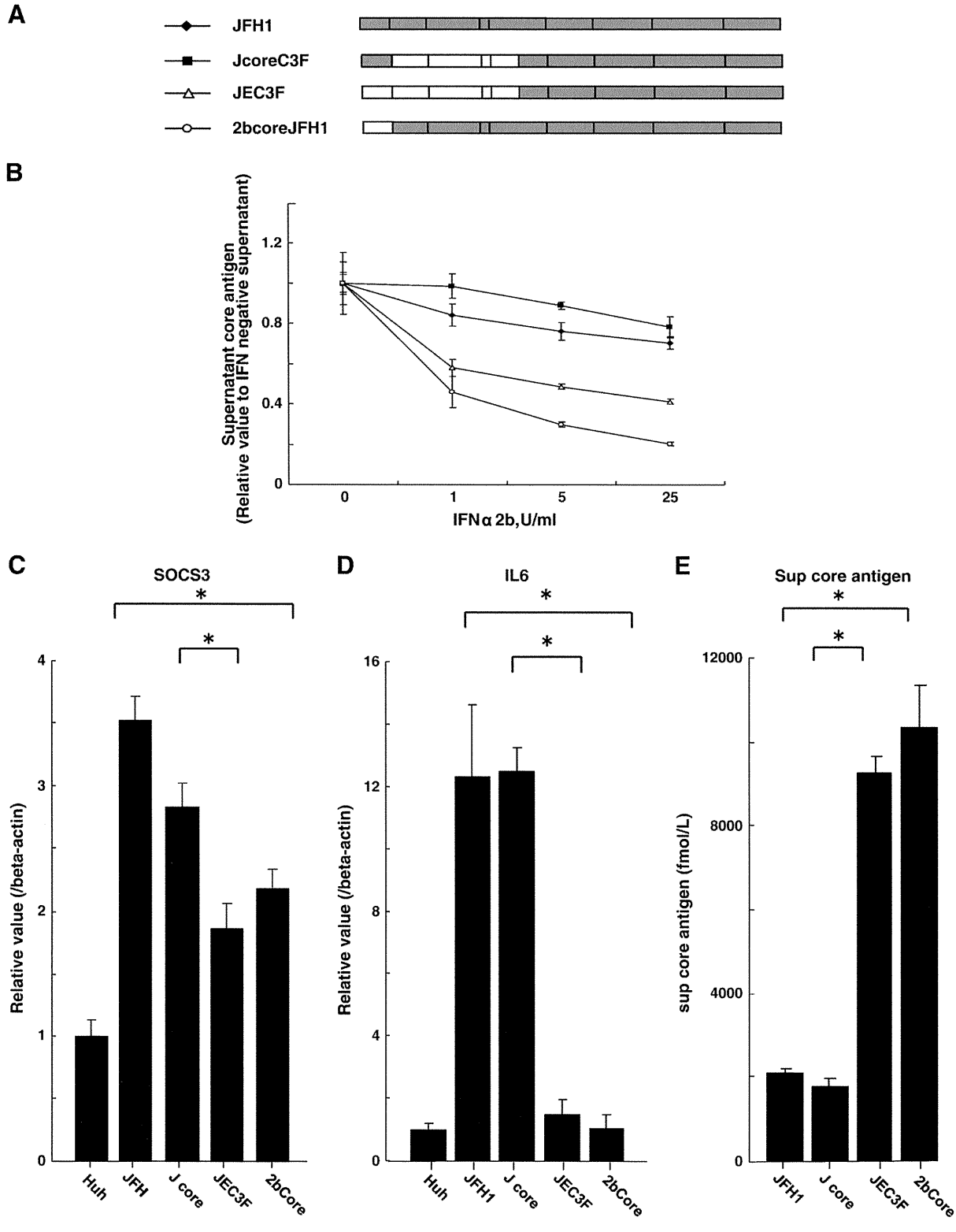

Fig. 7. Replacement of the HCV-2b-core region with JFH1-core causes upregulation of SOCS3 and IL-6 and restores resistance to IFN. A. Genome maps of JFH-1, JEC3F , J core C3F, 2b core JFH1 recombinant cDNA. J core C3 F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The 2b core JFH was made by substitution of the core region of JFH1 with that of 2b/JFH1. B. Comparison of IFN-alpha sensitivity among JFH1 and JEC3F and core region substitution chimeric viruses. Ten μg of J core C3F, 2b core JFH1, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and were divided into 12 wells. Forty eight hours after transfection, the cells were treated with 0, 1, 5 and 25 U/ml of IFN-alpha 2b. Seventy two hours after treatment, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05. C, D. Core substitution leads to SOCS3 and IL-6 mRNA over-expression. Forty eight hours after transfection into cells, total RNA was isolated. Relative gene expression level SOCS3 (panel C) and IL6 (panel D) were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05. E. Change of secretion of core protein following core protein substitution. HCV RNA-transfected cells were divided into 12 wells. Five days after transfection, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

5′-GGA GAA GCT GGA GAC TCA GGT G-3′, SOCS1; forward, 5′-CAC TTC CGC ACA TTC CGT TCG-3′ and reverse, 5′-GAG GCC ATC TTC ACG CTA AGG-3′, IL6; forward, 5′-GGT ACA TCC TCG ACG GCA TCT-3′ and reverse, 5′-GTG CCT CTT TGC TGC TTT CAC-3′, 25OAS; forward, 5′- CCA CCT TGG AAA GTG CCG ACA ATG CAG ACA-3′ and reverse, 5′-CGA GTC TTT AAA AGC GAT TGC CAG ATG ATC -3′, MxA; forward, 5′-GCC AGC AGC TTC AGA AGG CCA TGC TGC AGC -3′ and reverse, 5′-GGG CAA GCC GGC GCC GAG CCT GCG TCA GCC -3′.

The siRNAs

The siRNAs directed against SOCS3 were designed as follows: SOCS3-HSS113312 stealth (sequence 5′- CCC AGA AGA GCC UAU UAC AUC UAC U-3 ′and 5′-AGU AGA UGU AAU AGG CUC UUC UGG G-3′, Invitrogen) was used. 10 μg in vitro-synthesized HCV-RNA and 80 pmol siRNA SOCS3-HSS113312 or MOCK or control siRNA (negative universal control Med #2, Invitrogen) were electroporated into 5×10 naïve Huh7.5.1 cells using the protocol described in *HCV cell culture system*. Forty-eight hours after transfection, expression levels of SOCS3 mRNA were measured by real-time PCR. The difference in IFN sensitivity between SOCS3 knock down HCV infected cells and control HCV infected cells was determined by measuring supernatants HCV core antigen 72 hours after addition of IFN.

Immunohistochemistry for HCV core

HCV-JFH1 transfected or infected Huh7.5.1 cells were cultured on 22 mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 hour at 37 °C, and with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Western blot analysis

Western blotting was performed as described (Tanabe et al., 2004). Briefly, 10 μg of total cell lysate was separated by SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and was visualized by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

Statistical analyses

Statistical analyses were performed using Student's *t*-test; p-values of less than 0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary Fig. 1. Infectivity of the full-length 2b HCV RNA and 2b/JFH1 chimeric virus, JEC3F. A. Challenge of human liver-engrafted albumin-uPA/SCID mice with culture fluid from JFH1 and JEC3F cells. Cell culture fluids from the JFH1 clone and JEC3F were injected intravenously into human liver engrafted albumin-uPA/SCID mice. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined. B. Fig. 1B Challenge of human liver-engrafted albumin-uPA/SCID mice by intrahepatic injection of in vitro synthesized, full-length 2b HCV RNA. Five hundred μl of RNA solution containing 30 μg of in vitro synthesized full-length 2b HCV RNA was injected into the livers of anesthetized chimeric mice through a small abdominal incision. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined.

Supplementary Fig. 2. Comparisons of replication efficiency of JFH1and J6/JFH1, 2b/JFH1 chimeras after transfection into Huh7.5.1-cells. A. Structures of theJ6/JFH1 and 2b/JFH1 genomes. J6 is joined between NS2 and NS3 with JFH1. 2b-HCV is joined with JFH1within NS2 at nt. 2867. B Measurements of core protein in cell culture fluids. Ten μg of JFH1, J6/JFH1, 2b/JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and the cells were cultured in 100 mm-diameter plates. The culture fluids from JFH1, J6/JFH1, H77/JFH1or 2b/JFH1-transfected Huh7.5.1 cells were collected separately on the days indicated and the levels of core antigen were measured. These experiments were done three times with similar results independently. *Panel B* shows representative date.

Supplementary Fig. 3. Inhibition of infection by blocking CD81. Huh 7.5.1 cells were plated into a 6 well plate at 1.4×10^5 cells per well. After 48 hours, the cells were incubated with anti-CD81 or isotypematched control antibody at the concentration indicated for 1 hour. Subsequently, cells were infected with 1 ml of JEC3F stock cell culture fluids at day 2 for 4 hours and washed with PBS. 48 hours after inoculation, anti-core immunostaining was performed with mouse anti-HCV core protein monoclonal antibody (Panels B and C). Quantification of HCV core antigen was carried out in culture fluids at 48 hours after infection (Panel A).

Supplementary Fig. 4. Comparison between 2b and JFH-1 core amino acid sequence.

Note: Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.07.041.

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Original Article

ITPA gene variant protects against anemia induced by pegylated interferon-α and ribavirin therapy for Japanese patients with chronic hepatitis C

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Aim: Host genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in chronic hepatitis C patients receiving ribavirin. In this study, we evaluated the clinical significance of *ITPA* variants in Japanese hepatitis C patients who were treated with pegylated interferon plus ribavirin.

Methods: In this multicenter retrospective cross-sectional study, 474 hepatitis C patients were enrolled who were treated with pegylated interferon plus ribavirin in four geographically different hospitals in Japan. Patients were grouped according to hemoglobin decline of more than 3 g/dL at week 4. Two single nucleotide polymorphisms (SNP) within or adjacent to the *ITPA* gene (rs6051702, rs1127354) were genotyped.

Results: A functional SNP, rs1127354, within the *ITPA* exon was strongly associated with protection against anemia with only one (0.8%) in 129 patients with the *ITPA* minor variant A developing severe anemia ($P = 5.9 \times 10^{-20}$). For rs6051702, which had significant association in European-Americans, significant but weak association with severe hemoglobin reduction was found in Japanese ($P = 0.009$). In patients excluding genotype 1b and high viral load, those with the *ITPA* minor variant A achieved significantly higher sustained viral response rate than those with the major variant (CC) (96% vs 70%, respectively, $P = 0.0066$).

Conclusion: *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of ribavirin-induced anemia in Japanese patients. Patients with the *ITPA* minor variant A (~27%) have an advantage in pegylated interferon plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate.

Key words: *c20orf194*, hemolytic anemia, hepatitis C virus, *ITPA* (inosine triphosphatase), pegylated interferon plus ribavirin therapy

INTRODUCTION

APPROXIMATELY 3% OF the worldwide population is infected with the hepatitis C virus (HCV), which represents 170 million people, with 3–4 million individuals newly infected each year. Chronic hepatitis C (CHC) has a variable course; although 20–25% of CHC patients maintain persistently normal serum aminotransferases and experience relatively slow histological progression, other patients present a more active biochemical course.[1–3] Overall, 30% of the CHC patients progress to cirrhosis in their lifetime,[3] and 3–8% of cirrhosis patients develop hepatocellular carcinoma (HCC) every year.[4–6] Among various factors, older age and hepatic steatosis are significant factors accelerating the rate of progression in CHC.[3,7–9]

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Antiviral treatment has been shown to improve liver histology and decrease incidence of HCC in CHC.[6,10] Current therapy for CHC consists of treatment with pegylated interferon (PEG IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism.[11,12] However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or a cure of the infection.[11,13] Older patients have showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities.[14–16] In particular, hematological abnormalities and RBV-induced hemolytic anemia often necessitate dose reduction and premature withdrawal from therapy in 10–14% of patients.[11,17–20] New drugs and therapeutic approaches for CHC are actively developed and several candidates are in the early trial phase.[21,22] Given these backgrounds, effective pre-treatment screening for predictor biomarkers with the aim to evaluate possible risks over benefits from currently available treatment would allow avoiding these side-effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of the treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies have demonstrated remarkable associations between single nucleotide polymorphisms (SNP) near or within the region of the *IL28B* gene, which codes for IFN-λ3.[23–28] Another recent study indicated that genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in CHC patients receiving RBV.[29] The results obtained in one GWAS study need to be evaluated and confirmed in the context of different geographical and racial populations, and independent cohorts. Here, we describe clinical evaluation of two SNP within or adjacent to the *ITPA* gene (6051702 and rs1127354), that was recently highlighted by the GWAS of HCV treatment-induced anemia.[29]

METHODS

Patients

IN THIS RETROSPECTIVE cross-sectional case–control study, 474 patients with chronic HCV infection treated at Tokyo Medical and Dental University Hospital, Nagoya City University Hospital, Yamanashi University Hospital, Nagasaki Medical Center and Hyogo University of Health Science Hospital in Japan were enrolled from April 2007 to April 2009. Each patient was treated with PEG IFN-α-2b (1.5 μg/kg s.c. once a week) or PEG IFN-α-2a (180 μg/kg once a week) plus RBV (600–1000 mg daily depending on bodyweight). The treatment duration was set at a standard 48 weeks for genotype 1b high viral load (≥5 log copies/mL) patients and 24 weeks for genotype 1 low viral load (≤5 log copies/mL) and genotypes 2 and 3 patients. On-treatment dose reduction and discontinuation of PEG IFN or RBV were decided based on the recommendations of package inserts or clinical situations in individual patients to avoid possible side-effects. The rates of PEG IFN and RBV administration achieved were calculated as percentages of actual total dose administrated of a standard total dose of 24 weeks, according to bodyweight before therapy. Hepatitis B surface antigen (HBsAg) positive and/or anti-HIV positive individuals were excluded from this study. Hemoglobin (Hb) values were measured at baseline and every week until 8 weeks. We considered Hb decline at week 4 to be a clinically important time point, as previously reported.[29] The threshold of Hb reduction of more than 3 g/dL was chosen as a clinically significant Hb decline according to the previous reports.[29–31]

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, sex, previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pre-treatment biochemical parameters, such as white blood cells, neutrophils, Hb, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/mL). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0, showing no activity; A1, showing mild activity; A2, showing moderate activity; and A3, showing severe activity. Fibrosis was staged on a scale of 0–4: F0, showing no fibrosis; F1, showing moderate fibrosis; F2, showing moderate fibrosis with

few septa; F3, showing severe fibrosis with numerous septa without cirrhosis; and F4, showing cirrhosis.

SNP genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphisms, rs1127354 in *ITPA*, rs6051702 in *C20orf194*, and rs8099917 around the *IL28B* gene were determined by real-time detection polymerase chain reaction with a TaqMan probe or DigiTag2 assay typing one tag SNP located within each locus.[23] Another functional SNP, rs727010 within the *ITPA* gene, was excluded due to no variants in the Asian genetic population as reported in the International HapMap Project database. Our preliminary genotyping of a 100-patient population did not find variants in that SNP.

Outcomes

The primary end-point was Hb decline and dose reduction of PEG IFN or RBV in week 4, the secondary end-point was SVR. An SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. A transient viral response (TVR) meant that HCV RNA became undetectable during treatment but reappeared at the end of follow up. A null response (NR) was defined as persistently positive HCV RNA throughout the treatment. Adverse events and drug adherence were recorded.

Statistical analyses

The association between individual ITPA SNP and the incidence of significant Hb decline was tested by a basic allelic test and calculated using the χ^2-test. Multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of less than 0.05 as the criteria for model inclusion. These statistical analyses were conducted by using SPSS software package ver. 18J (Chicago, IL, USA) or Microsoft Excel Mac 2008 (Redmond, WA, USA). Discrete variables were evaluated by Fisher's exact probability test. The *P*-values were calculated by two-tailed Student's *t*-tests for continuous data and χ^2-test for categorical data, and those of less than 0.05 were considered statistically significant.

RESULTS

THE CLINICAL CHARACTERISTICS of the 474 patients are summarized in Table 1. First, we compared baseline clinical and host genetic characteristics of patient groups according to the SNP within the *ITPA* gene, rs1127354, between major homozygote (CC) and a group of heterozygote (CA) and minor homozygote (AA) (Table 2). There were no significant differences in age, sex, blood cell counts, ALT levels, serum viral loads, frequencies of core 70/91 mutations[32,33] and the numbers of NS5A interferon sensitivity determining region (ISDR) mutations[34,35] between the two groups. The SNP in the *ITPA* gene did not show significant linkage between the SNP around the *IL28B* gene, rs8099917, which is strongly associated with IFN treatment responses.[23,25] In contrast, the SNP in the *ITPA* gene showed significant linkage with the SNP in *C20orf194*, rs6051702 ($P = 7.1 \times 10^{-14}$).[29]

Next, we analyzed two SNP, rs6051702 in *C20orf194* and rs1127354 in *ITPA* loci, respectively, for their association with significant Hb decline at 4 weeks of PEG IFN plus RBV treatment using a basic allelic model that compares frequencies of alleles in cases versus controls. The SNP, rs6051702, which showed the strongest association in the European-American population,[29] was

Table 1 Baseline characteristics of participating patients

Total number	474
Age (years)	57.2 ± 10.0
Sex (male/female)	264/210
Bodyweight (kg)	61.1 ± 10.8
HCV genotypes	
1b/2a/2b/3a	416/31/26/1
1b, high viral load/others	387/87
NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2)	285/49
Core mutations (genotype 1b, $n = 379$)	
C70 (wild/mutant)	240/139
C91 (wild/mutant)	234/145
Histology at biopsy ($n = 278$)	
Grade of inflammation (A0/1/2/3)	4/97/154/23
Stage of fibrosis (F0/1/2/3/4)	8/102/74/74/20
White blood cells (/μL)†	5707 ± 1495
Neutrophils (/μL)†	2568 ± 1013
Hemoglobin (g/dL)†	14.2 ± 1.4
Platelet count ($\times 10^{-3}$/μL)†	158 ± 58
ALT (IU/L)†	89 ± 66
Serum HCV RNA (log [IU/mL])‡	6.0 ± 0.9
PEG IFN (PEG IFN-α-2a/PEG IFN-α-2b)	40/434
Hb decline at week 4 (g/dl)	2.4 ± 1.4
Severe anemia, Hb <10 g/dL at week 4	56/474 (11.8%)

†Data are expressed as mean ± standard deviation.
‡Data are shown as median (range) values.
High viral load: HCV RNA ≥ 5 log IU/mL.
ALT, alanine transaminase; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; PEG, pegylated.

Table 2 Clinical and host genetic characteristics of patients according to *IPTA* gene variants

	ITPA SNP, rs1127354		
	CC ($n = 345$)	CA + AA ($n = 129$)	*P*-value
Age (years)†	57.2 ± 10.0	57.1 ± 10.1	0.87
Sex (male/female)	192/153	72/57	0.97
White blood cells (/μL)‡	5312 ± 1537	4995 ± 1388	0.92
Neutrophils (/μL)‡	1696 ± 1415	1803 ± 1516	0.49
Hemoglobin (g/dL)‡	14.2 ± 1.4	14.1 ± 1.4	0.52
Platelet count ($\times 10^{-3}$/μL)‡	157 ± 54	159 ± 70	0.81
ALT (IU/mL)‡	91 ± 70	83 ± 55	0.46
Serum HCV RNA (log copies /mL)†	6.1 ± 0.7	5.8 ± 1.1	0.093
NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2)	213/31	72/18	0.095
Core mutations (genotype 1b, $n = 389$)			
aa. 70 (wild/mutant)	179/96	61/43	0.25
aa. 91 (wild/mutant)	172/103	62/42	0.60
IL28B, rs8099917 (TT/TG/GG)	233/94/3	96/29/1	0.23
C20orf194, rs6051702 (AA/AC/CC)	254/85/6	47/72/10	**7.1×10^{-14}***

*P-values were calculated by student's t-test or by χ^2 analysis.
†Data are show as median (range) values.
‡Data are expressed as mean ± standard deviation.
IL28B SNP, major allele-T and minor allele-G.
C20orf194 SNP, major allele-A and minor allele-C.
ALT, alanine transaminase; HCV, hepatitis C virus; SNP, single nucleotide polymorphisms.

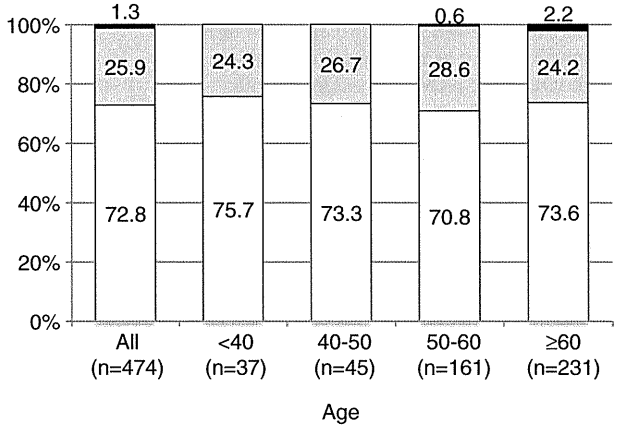

Figure 1 *ITPA* variant, rs1127354, known to be responsible for inosine triphosphatase deficiency and its age-related differences. *ITPA*, inosine triphosphatase. The numbers in parentheses denote numbers of patients.

associated with the Hb decline significantly but with smaller effect size (odds ratio [OR] = 1.40, $P = 9.0 \times 10^{-3}$, Table 3). Notably, another SNP in the *ITPA* gene, rs1127354, showed overwhelming association with the Hb decline (OR = 62.8, $P = 5.9 \times 10^{-20}$). The prevalence of *ITPA* variants is shown in Figure 1. Percentages of *IPTA*, rs1127354, major homozygote (CC), heterozygote (CA) and minor homozygote (AA) were 72.8%, 25.9% and 1.3%, respectively. There was no difference in the frequency of the *ITPA* variants throughout ages and sexes (Fig. 1).

To asses the clinical relevance of these SNP, we analyzed the proportion of patients suffering clinically significant anemia, which we defined as a decline in Hb levels of more than 3 g/dL or Hb levels of less than 10 g/dL, which is the threshold at which RBV dose reduction is recommended. As depicted in Figure 2, in *ITPA*-CC patients, Hb loss of more than 3 g/dL devel-

Table 3 Association of *C20orf194* and *IPTA* gene variants with treatment-induced Hb decline

Gene	SNP	Allele (major/minor)	MAF (%)	OR	*P*-value*
C20orf194	rs6051702	A/C	19.9	1.40	9.0×10^{-3}
ITPA	rs1127354	C/A	14.2	62.8	5.9×10^{-20}

*The SNP-phenotype associations were analyzed using a basic allelic test.
P-values were calculated by χ^2 analysis.
Hb, hemoglobin; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphisms.

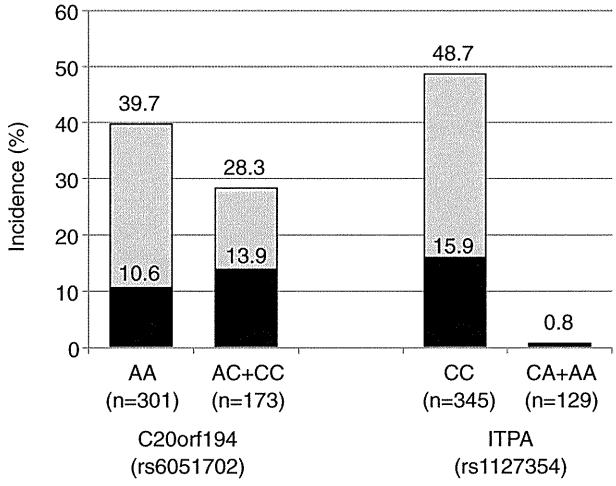

Figure 2 Effects of *c20orf194* and *ITPA* single nucleotide polymorphisms (SNP) on clinically significant anemia induced by pegylated interferon plus ribavirin treatment. Percentages of patients with hemoglobin (Hb) decline of >3 g/dL or Hb levels of >10 g/dL at week 4 of treatment are shown for each SNP in two genes, *c20orf94* (rs6051702) and *ITPA* (rs1127354).

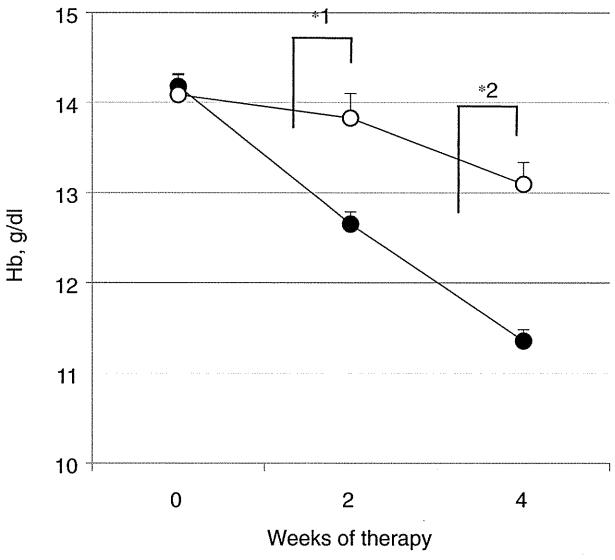

Figure 3 Time-dependent hemoglobin (Hb) decline in *ITPA* major and minor variants. Error bars indicate mean + standard error. Asterisks 1 and 2 indicate statistical significance of $P = 6.6 \times 10^{-13}$ and $P = 3.0 \times 10^{-29}$, respectively.

oped in 48.7% at week 4, and 15.9% of patients achieved Hb levels of less than 10 g/dL. In contrast, only one patient (0.8%) with *ITPA*-CA/AA developed anemia. These differences in the incidence of the treatment-induced Hb decline were consistent throughout ages. The time-dependent Hb decline in patients with *ITPA*-CC and *ITPA*-CA/AA is shown in Figure 3. In patients with *ITPA*-CC, mean Hb drop was 2.9 ± 1.3 g/dL, which was significantly higher than that of patients with *ITPA*-CA/AA (1.1 ± 0.7 g/dL). These results demonstrate that the *ITPA* minor variant A has a protective phenotype for the treatment-induced anemia. The positive predictive value of the *ITPA*-major (CC) for the development of severe anemia was 48.7%, while the negative predictive value of *ITPA*-hetero/minor (CA/AA) was 99.2%. In accordance with the incidence of anemia, there was significant difference in the incidence of RBV dose reduction. At week 4 of treatment, RBV doses were reduced in 27.9% of *ITPA*-CC patients while in only 14.4% of *ITPA*-CA/AA patients ($P = 0.012$, Fig. 4). Similarly to RBV, PEG IFN dose reduction was apparently higher in *ITPA*-CC patients though it did not reach statistical significance.

Knowing that significantly less frequent drug reduction occurred in patients with the *ITPA*-minor variant A, we next investigated if the *ITPA* gene variants affected final treatment outcomes. The treatment outcomes were available in 339 patients with genotype 1b and high viral load

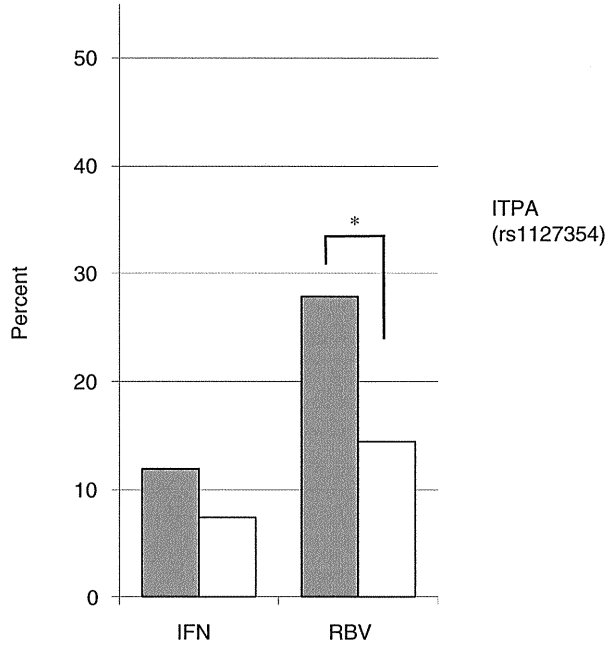

Figure 4 Percentages of patients requiring pegylated interferon (IFN) or ribavirin (RBV) dose reduction at week 4 in *ITPA* major and minor variants. *Y*-axis indicates percents of patients who required dose reduction. *$P = 0.012$.

Table 4 Sustained viral response rates of each group according to *IPTA* gene variants

IPTA SNP, rs1127354	Genotype 1b, high viral load		Others	
	CC	CA + AA	CC	CA + AA
SVR	92 (37.1%)	39 (42.9%)	41 (70%)	25 (96%)
TVR	90 (36.3%)	34 (37.3%)	15 (25%)	1 (4%)
NR	66 (26.6%)	18 (19.8%)	3 (5%)	0 (0%)
Total	248	91	59	26
P-value	0.33		**0.0066**	

High viral load; serum HCV RNA ≥ 5 logIU/mL.
"Others" include genotypes genotype 1b, serum HCV RNA <5 logIU/ml, genotypes 2a, 2b and 3a.
P-values were calculated by χ^2-test analyses of SVR versus TVR plus NR.
HCV, hepatitis C virus; SVR, sustained viral response; TVR, transient viral response; NR, null response.

(HCV RNA ≥5.0 log IU/ml) and 85 others, which included genotype 1b, low viral load and genotype 2a, 2b and 3a patients (Table 4). In patients with genotype 1b and high viral load, there was no significant difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA patients (37.1% and 42.9%, respectively). In contrast, there was a striking difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA in the other IFN-sensitive group (non-1b or low viral load); the SVR rate was 70% in *ITPA*-CC patients, while 96% of *ITPA*-CA/AA patients achieved SVR ($P = 0.0066$). These results indicate that the *ITPA* minor variant A is significantly associated with SVR in the IFN-sensitive group excluding genotype 1b and high viral load. Using those subpopulations of patients, we conducted a statistical analysis for association of several host and viral parameters with SVR. As shown in Table 5, univariate analysis identified four significant parameters including age, platelet count, stages of fibrosis and the ITPA SNP, rs1123354. Multivariate logistic regression analysis identified that only age and the ITPA SNP were significantly associated with SVR.

DISCUSSION

RECENT GWAS ON HCV infection have identified two important host genetic polymorphisms. One is the SNP in the *IL28B* gene, which is strongly associated with response to therapy of chronic genotype 1 HCV infection,[23–28] and another is the SNP in the *ITPA* gene, which precisely predicts RBV treatment-associated anemia in the European-American population.[29] In our present study, a functional SNP in the *ITPA* locus, rs1127354, is strongly associated with protection against anemia among 474 Japanese patients ($P = 5.9 \times 10^{-20}$, Table 3). Only one of 129 patients (0.8%) who carry the rs1127354 minor allele A had severe anemia (Figs 2,3). These data are consistent with the previous study in the US population[29] as well as a recent Japanese study by

Table 5 Univariate and multivariate logistic regression analyses of host and viral characteristics of patients excluding genotype 1b high virus load based on therapeutic responses ($n = 85$)

Variable	*P*-value (univariate)	*P*-value (multivariate)	OR	95% CI
Age	**0.017**	**0.047**	0.916	0.840–0.999
Sex (male vs female)	0.19	–		
Baseline Hb level	0.17	–		
Baseline platelet count	**0.019**	0.307	1.092	0.923–1.292
Stage of fibrosis (F0–2 vs 3–4)	**0.0020**	0.083	4.221	0.827–21.531
PEG IFN adherence (≥80% vs <80%)	0.67	–		
RBV adherence (≥80% vs <80%)	0.30	–		
ITPA SNP rs1127354 (CC vs CA + AA)	**0.0066**	**0.023**	12.680	1.386–116.042
IL28B SNP rs8099917 (TT vs TG + GG)	0.29	–		

CI, confidence interval; Hb, hemoglobin; IFN, interferon; ITPA, inosine triphosphatase; OR, odds ratio; PEG, pegylated; RBV, ribavirin; SNP, single nucleotide polymorphisms.

Ochi *et al.*[31] Our data were similar to these two reports; rs1127354 was the most significant SNP that was associated with RBV-induced anemia in Asian genetic populations. Additionally, we have demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected (Fig. 4) and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (70% vs 96%, $P = 0.0066$, Table 4). Taken together, our results demonstrate that the *ITPA* minor variant A is not only a protective allele of PEG IFN and RBV treatment-associated anemia in the Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN.

An SNP in *C20orf194*, rs6051702, which showed significant association with Hb reduction in European-Americans ($P = 1.1 \times 10^{-45}$),[29] was also significant in our study of a Japanese population, but with smaller effect size on Hb reduction ($P = 0.014$). The discrepancy may be due to the low levels of the linkage disequilibrium (LD) with the functional SNP in the *ITPA* gene in the Japanese/Asian population as compared with the high LD in white subjects. Indeed, it is reported that the predictive values of the *C20orf194* SNP varied between different races including African-Americans ($P = 0.19$) and Hispanics ($P = 9.5 \times 10^{-3}$).[29]

Ochi *et al.* sequenced the Japanese patient genome including ITPA and DDRGK1 loci, which are located adjacently on chromosome 20. They identified 83 SNP with major allele frequency of more than 0.05, of which four SNP including rs1127354 were significantly associated with RBV-induced anemia and which were in almost absolute LD with each other.[31] Their report indicates that the ITPA SNP, rs1127354, which we genotyped in the present study, represent a dominant variant of ITPA deficiency that protects against RBV-induced anemia in Japanese/Asian genetic populations. In our study, however, 51.3% of the ITPA-major (CC) patients did not develop significant Hb decline (Fig. 2). This finding suggests that there are other low-frequency ITPA variants or SNP in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

The response to PEG IFN plus RBV treatment is affected by several viral and host factors such as age, sex,[36,37] NS5A-ISDR[38] and core region.[32,33] To maintain good adherence to drugs, especially RBV, it is important to achieve good treatment responses. Increased RBV exposure during the treatment phase was associated with an increased likelihood of SVR in the US[39] and Japanese studies.[40] Because patients with *ITPA* minor variant A are refractory to RBV-induced anemia, they are advantaged in maintaining good adherence to RBV and may be given even higher doses of RBV, resulting in a higher SVR rate. However, a study by Fellay *et al.* and a very recent replication study by Thompsom *et al.*[30] did not observe any significant association between the *ITPA* minor variants and early or late anti-HCV treatment outcomes.[29] A possible explanation for the discrepancy is that older and histologically more advanced patients were predominant in our study. Mean age in the US study was 47.5 years while 57.2 years in our present study. The percentage of advanced fibrosis (F3 or F4) was 12.0% in the US study while 34% in our study. It is well known that the incidence of drug dose reduction or discontinuation could increase according to old age as well as advanced stages, that may compromise final treatment outcomes.[14,15] Importantly, we have additionally demonstrated that in patients with other than genotype 1b HCV, ITPA minor variant A was significantly associated with better SVR rates in univariate and multivariate analyses. Because the typical PEG IFN plus RBV treatment period is shorter (24 weeks) in genotype 1 low viral load and genotype 2 patients than in genotype 1 high viral load (48 weeks), early dose reduction of RBV may be more critical to the final treatment outcome.

Ribavirin is a synthetic guanosine analog, and has actions *in vitro* against a wide range of RNA and DNA viruses.[41] Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1,[42] anti-proliferative effect by inhibition of cellular GTP synthesis,[41] and direct inhibition of virus replication.[43] Although monotherapy with RBV clinically showed minimal effect on the viral load and almost no effect on the viral clearance,[44–47] combinatory use of RBV with IFN elicits strong synergistic effects against HCV *in vitro*[48] and *in vivo*.[49,50]

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose-related.[49,50] RBV is incorporated into erythrocytes where it undergoes phosphorylation to its pharmacologically active forms through adenosine kinase. The RBV-phosphate conjugates are unable to cross the erythrocyte cell membrane and are thus accumulated intracellularly and cleared slowly from red cells with a half-life of approximately 40 days.[51] Inosine triphosphatase (ITP) deficiency or low activity variants, in turn, lead to an accumulation of ITP in red blood cells and may compete with RBV triphosphate, and may protect from RBV-induced hemolysis.[52,53]

There are several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) being tested for

clinical efficacy against hepatitis C.[21,22] Most experts believe that when new drugs are approved to treat hepatitis C they will be used in combination with PEG IFN and RBV. Moreover, recent clinical trials including NS3 protease inhibitors have shown that PEG IFN plus RBV would be necessary to achieve optimal treatment responses.[18,19,54] Our present results may give a valuable pharmacogenetic diagnostic tool for the tailoring of RBV dosage to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV chemotherapeutics.

ACKNOWLEDGMENTS

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シクロフィリン阻害剤

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索引用語：シクロスポリンA，シクロフィリン，PPIase活性，
シクロフィリン阻害剤，DEBIO-025（alisporivir）

1 はじめに

タンパク質の高次構造形成や転写制御，ウイルス感染などに重要な役割を担っていることで知られるイムノフィリンの一つであるシクロフィリンは，ヒト後天性免疫不全ウイルス(HIV-1)のGag蛋白質と結合し，HIV-1ウイルス粒子に取り込まれることが報告され[1]注目を集めたが，その後もシクロフィリンがウイルス感染や増殖に関与することが相次いで報告されている[2~5]．

C型肝炎ウイルス(HCV)の研究においてシクロフィリンに注目が集まる契機となったのは，シクロフィリンを細胞内受容体とする免疫抑制剤シクロスポリンA（CsA)が持つ特異的なHCV抑制作用が，臨床[6]および基礎研究[7~10]から相次いで報告されてからであるが，シクロフィリンを新たなターゲットとした治療戦略が期待されている．

本稿では新規治療薬として期待されるシクロフィリン阻害剤について概略を述べるとともに，現在欧米を中心に臨床試験が行われているシクロフィリン阻害剤のひとつDEBIO(alisporivir)の最近の知見についても概説したいと思う．

2 シクロスポリンAとシクロフィリン

CsAは11個の疎水性アミノ酸からなる環状ペプチドである．1970年代初頭にサンド社(現ノバルティスファーマ)によって抗菌物質として発見されたが，1976年に*Borel*らによりT細胞の活性化を抑制することが報告され[11]，CsAは臓器移植後の拒絶反応抑制のための免疫抑制剤として広く用いられるようになった[12]．現在では，各種の臓器移植後の拒絶反応の抑制や自己免疫疾患，アレルギー性疾患などの治療薬として広く用いられている．

CsAは非常に疎水性の高い化合物であるため，CsAが薬理作用を示すためには細胞内受容体であるシクロフィリンと結合する必要が

Mina NAKAGAWA et al: Cyclophilin inhibitors
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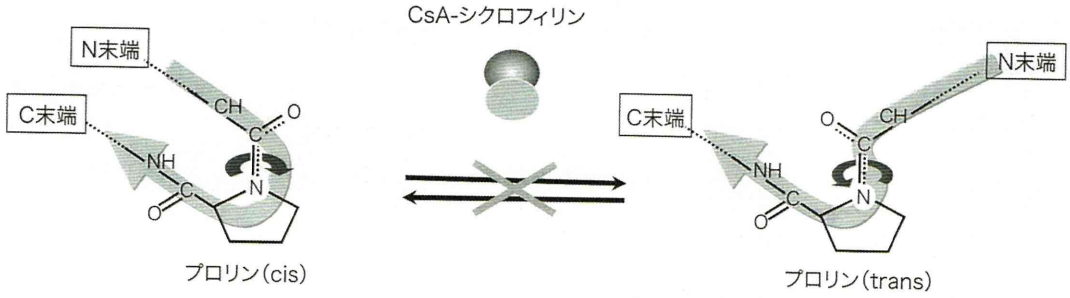

図1　シクロフィリンのもつPPIase活性はCsAと複合体を形成することにより阻害される
シクロスポリンA（CsA）の受容体蛋白であるシクロフィリンは，カルシニューリン抑制作用の他に，ペプチドを構成するプロリンのシスートランス結合を変換させるPPIase活性を示し，タンパク質を正しい構造に折れたたむ分子シャペロンの補助として機能することが知られているが，このPPIase活性はCsAと複合体を形成することにより阻害される．

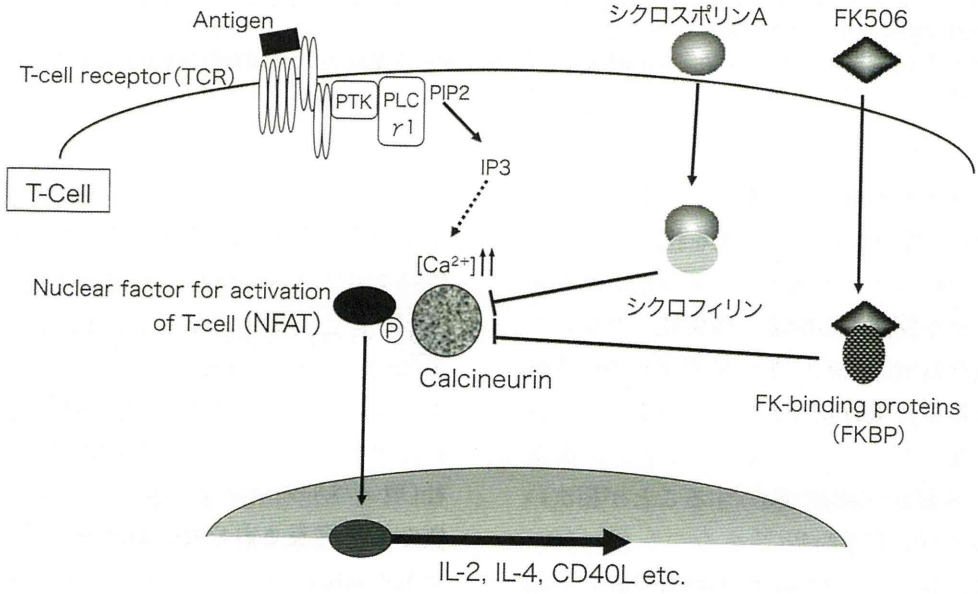

図2　CsA, FK506の免疫抑制機構
T細胞の受容体が抗原刺激を受けると，細胞内のカルシニューリン(CN)が活性化，CNは転写因子NFATを脱リン酸化し，種々のサイトカイン遺伝子発現を誘導する．CsAおよびFK506はそれぞれ，細胞内受容体蛋白であるシクロフィリン，FK binding protein (FKBP)と結合し複合体を形成し，CNの活性化を阻害することより免疫抑制作用を示す．

あることが知られている．シクロフィリンは1984年に膜ではなく細胞質に存在し，CsAに高い親和性を有する蛋白として初めて報告された[13]．シクロフィリンはT細胞だけではなく，すべての細胞に存在し，細胞の総蛋白質量の0.1～0.4％をしめる存在量の多いタンパク質である．当初シクロフィリンはCsAに結合すること以外にどのような作用をもっているか不明であったが，1989年シクロフィリンのもつPPIase（=Peptidyl-prolyl cis-trans isomerase)活性が明らかとなり，細胞内機能を制御する非常に重要な蛋白の一つとして多

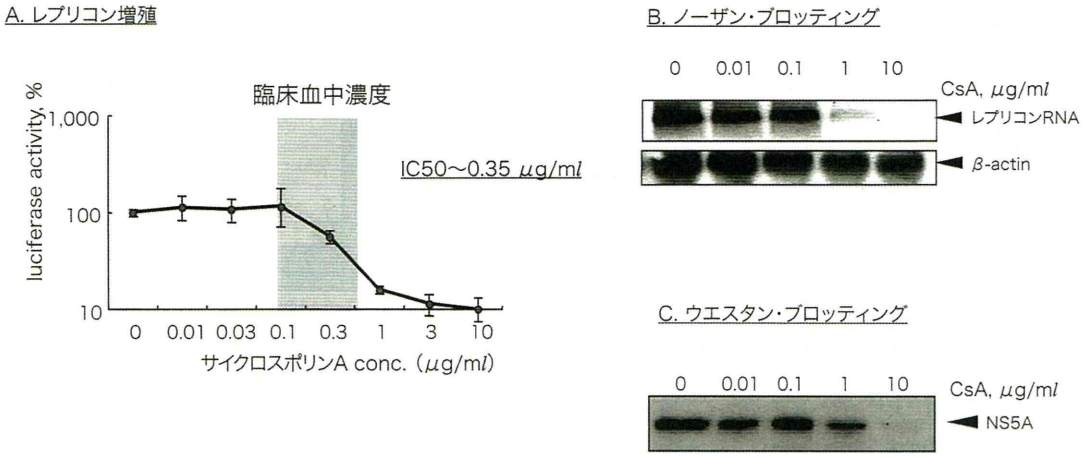

図3　CsAの抗HCV効果

HCVレプリコン増殖はCsAの濃度依存性に抑制され，その50％抑制濃度は～0.35 μg/mlであり，臨床血中濃度域にてその効果がみられることがわかった(A)．ノーザンブロッティング(B)およびウエスタンブロッティング(C)でも，レプリコンRNAおよびHCV蛋白の発現が濃度依存性に抑制されることが確認された．

いに注目を集めるようになった[14,15]．

PPIase活性とは一言でいえば，タンパク質のなかのペプチド結合のシス型とトランス型の変換を促進する酵素，つまり，タンパク質の高次構造の形成において重要な役割を担う作用のことである．プロリンはプロリン環をもっていて，通常の条件下でもシス型とトランス型が立体障害をきたすことなく共存できるが，PPIase活性をもつシクロフィリンは，プロリン残基との間のペプチド結合を180度回転させて構造変換を促進し，タンパク質を正しい構造に折れたたむ広義の分子シャペロンと考えられる(図1)．CsA同様に免疫抑制剤としてしられるタクロリムス(FK506)も細胞内受容体であるFK binding protein（FKBP)と複合体を形成することが知られるが，FKBPもシクロフィリン同様にPPIase活性を有しており，タンパク質の高次構造の形成に重要な役割を担っている．

CsAとFK506はカルシニューリン(CN)を阻害することで免疫応答を抑える薬剤であることが広く知られている．図2に示すようにT細胞の受容体が抗原刺激を受けると，細胞内のCNが活性化し，転写因子NFATを脱リン酸化し，種々のサイトカイン遺伝子発現を誘導するが，構造上まったく異なるCsA-シクロフィリン，FK506-FKBPの複合体が同一のタンパク質CNの活性発現制御系に作用し，T細胞活性化の細胞内シグナル伝達系を抑制して免疫抑制作用を発現することはじつに興味深い．さらに，これら複合体の形成によりシクロフィリンやFKBPが持つPPIase活性が阻害されることも明らかになってきており，この活性阻害はCN系の阻害を介した免疫抑制効果とは関係がない．シクロフィリンやFKBPの基質や作用機序などこれから解明すべき点は多いが，タンパク質の高次構造の形成に重要な役割を担うシャペロン機能をもつ宿主因子として，多岐にわたる疾患への関与が報告されており，さまざまな分野の研究者にとって非常に興味深いタンパク質といえる．

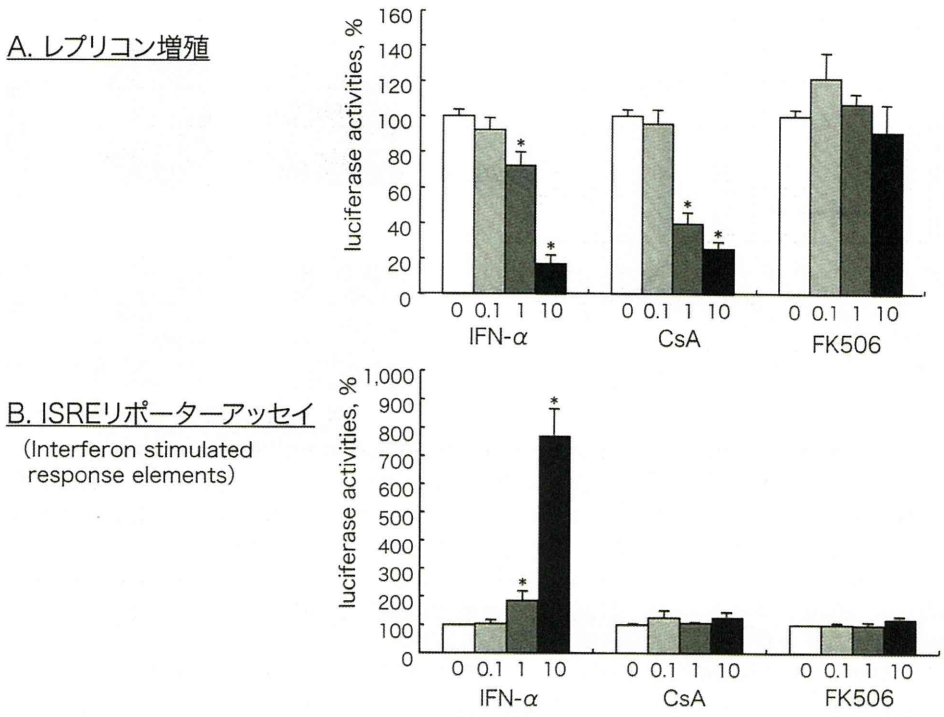

図4　抗HCV効果とISRE活性への影響

レプリコン増殖はCsAの濃度依存性に抑制されたが，FK506では臨床血中濃度をはるかに超えた濃度においても増殖抑制効果は認められない．HCV増殖を同様に強力に抑制するIFN αは，遺伝子転写調節領域にあるISREにより制御させるIFN誘導遺伝子の発現を介して，抗ウイルス作用を呈し，IFN α添加によってISRE活性は上昇する．しかし，CsAではこのようなISRE活性の上昇は認められず，そのHCV抑制効果はIFNとは独立した作用機構を介することが明らかとなった．

3　シクロスポリンAの持つHCV増殖抑制効果

2003年inoueらによりC型慢性肝炎患者においてインターフェロン(IFN) αとCsAの併用投与がIFN単独よりも治療効果が有意に上昇することが報告されたが[6]，その作用機序は十分明らかとされていなかった．われわれはHCVの培養細胞での複製増殖モデルであるHCVレプリコンシステムを改変したキメラリポーター発現レプリコンによるHCV発現定量系を用いて薬剤・サイトカインなどの抗ウイルス効果を解析したところ，CsAが濃度依存性にHCVレプリコン増殖を抑制し，臨床血中濃度域にてHCVの抗ウイルス効果を認めることを報告した(図3)[8]．ほぼ同時期に国内から同様の報告がなされ[7]，CsAの持つ抗HCV効果が注目された．

現在HCV治療の基軸となっているIFN αは，ISRE（=interferon stimulation response element)により制御されるIFN誘導遺伝子の発現を介して，抗ウイルス作用を呈する．われわれの検討ではIFN αによってISRE活性は濃度依存性に上昇するが，CsAではこのようなISRE活性の上昇は認められず，そのHCV抑制効果はIFNとは独立した作用機構を介すると考えられた(図4)[10]．さらに，FK506にはHCVの抑制効果がみられないこ

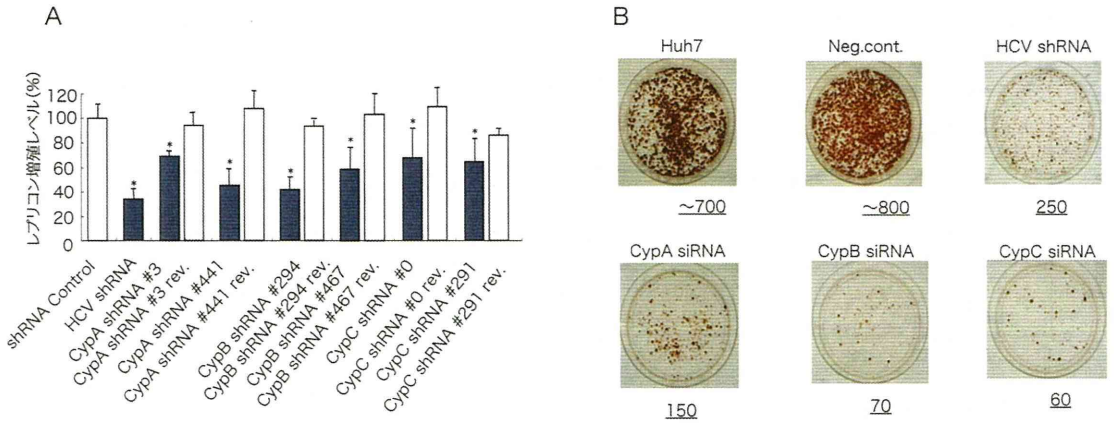

図5　shRNAによるサイクロフィリン発現抑制時のHCV増殖レベルの解析

シクロフィリンのサブタイプのうち，HCVゲノム増殖コンプレックスが存在するとされる細胞質，および小胞体に局在する，シクロフィリンA, B, Cについて，HCVレプリコン増殖に与える影響を検討した．それぞれ2種のshRNA発現ベクターを作成し，これを用いてノックダウン細胞を作成し，HCVレプリコンの増殖効果を解析したところ，シクロフィリンの発現抑制により，HCVレプリコン増殖は40～60％と有意に抑制された(A)．(Cyp:シクロフィリン，HCVshRNAはすでにHCVを抑制することが確認されているshRNA. 各shRNAのrev.はターゲット部位の配列を逆にしたコントロール)

また，シクロフィリンを恒常的にノックダウンした細胞の解析では，レプリコンの増殖によるコロニー形成能は明らかに低下し，シクロフィリンがHCV増殖に関与していると考えられた(B)．B各図の下段数字はコロニー数．

とから，両者の違いである細胞内受容体であるシクロフィリンにわれわれは注目した．

ヒトの場合，シクロフィリンとFKBPのサブタイプはそれぞれ15種類前後も存在することが報告されている．これらは組織特異性を示し，細胞質以外にも細胞膜や細胞内小器官に特異的に存在するが，ほぼすべての細胞に普遍的に存在するものから，組織あるいは細胞特異的に存在するものまで多種多様である．シクロフィリンとFKBPのPPIaseとしての違いに関しては，酵素の基質特異性にあるという報告があり[16]，FKBPはプロリンの前のアミノ酸が疎水性のものである場合にのみ活性を示すのに対して，シクロフィリンはプロリンの前のアミノ酸がほぼどの種類であっても高い酵素活性を示すためFKBPのような選択性をもたないことが両者の違いにつながるという．この基質特異性の違いが，生理的に何らかの意味を持っていると信じられているが，生体内での機能との関連性についてはいまだ十分に明らかにされていない．シクロフィリンは約15種類知られており，細胞内のあらゆる画分に存在している[17]．われわれの検討ではHCVレプリコンの複製増殖は，HCV増殖複合体が存在すると考えられる細胞質，小胞体などに多いといわれるシクロフィリンA, B, Cの関与が考えられる結果となった(図5)[10]．その後，HCVの複製増殖とシクロフィリンに関する報告が相次ぎ，HCV蛋白のひとつであるNS5Bとシクロフィリンの特異的な結合がHCV複製増殖に関与しているとする報告や[9,18]，最近ではシクロフィリンAの重要性も相次いで報告されており[19～23]，シクロフィリンがHCVライフサイクルに深く関与していることが示唆され今後の解析が待たれる．