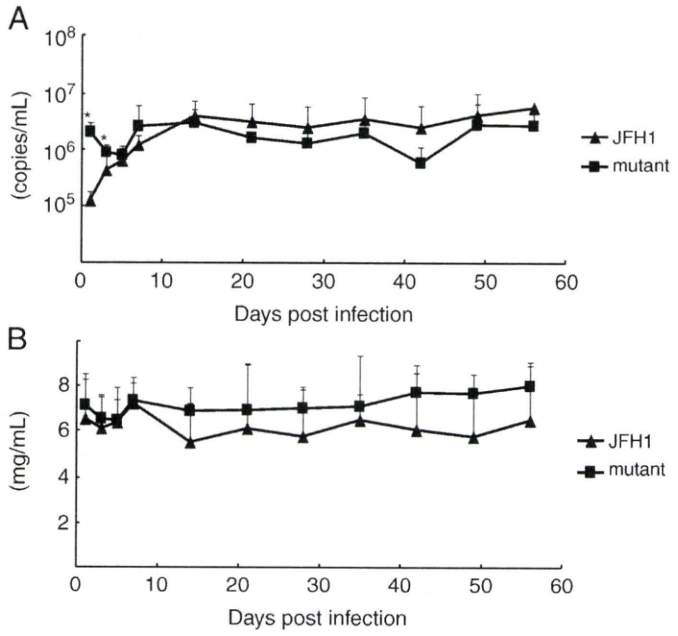

Fig. 6. In vivo analysis of cytopathic JFH1 mutants using human hepatocyte chimeric mice. A. Serial changes in HCV RNA in the sera of mice inoculated with the culture media from JFH1 mutants. The data shows the average of 2 mice for JFH1, and 3 mice for the mutant. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Levels of human albumin in the sera of mice inoculated with the culture media from JFH1 mutants.

inoculated with the cytopathic mutant virus showed conservation of the mutations in codons 2441, 2938 and 2985. However, on days 21 and later, the mutation at codon 2985 had reverted to the wild type JFH1 sequence in all the mutant-injected mice and the mutation at codon 2938 had reverted to the wild type JFH1 sequence in two of the three mice. The C2441S mutation was more stable in the mutant-injected mice, but one mouse had lost it at day 56 (Fig. 8).

Discussion

In this study, we investigated the significance of genetic mutations in plaque-purified, cytopathic HCV-JFH1 subclones. Genetically engineered JFH1-mutants encoding C2441S, P2938S, and R2985P led to much more cell death than the wild type JFH1, and also produced significantly higher amounts of core antigen in the culture medium and inside the cells than the parental JFH1 clone. In the single-cycle production assay, which exploited a receptor-deficient Huh7 cell line, the three JFH1-mutants, JFH1-C2441S, P2938S, and R2985P produced significantly more core antigen in the culture medium and expressed equivalently higher amounts of viral genomic RNA in the cells. These data suggest that the three mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated directly with enhanced intracellular replication and resultant virion formation, which correlated with the extent of the cytopathic effects. Interestingly, inoculation of a cytopathogenic mutant, JFH1-C2441S/P2938S/R2985P, into human hepatocyte chimeric mice produced significantly higher plasma HCV RNA concentrations than JFH1 at ~7 days post inoculation. At a later phase of infection, however, the mutations in this mutant HCV reverted partially to the wild type sequences. Taking all things together, it is suggested that in vitro-isolated, genetically modified cytopathic HCV subclones replicate robustly in the acute phase of in vivo infection but are eliminated rapidly and substituted by in vivo adapted clones.

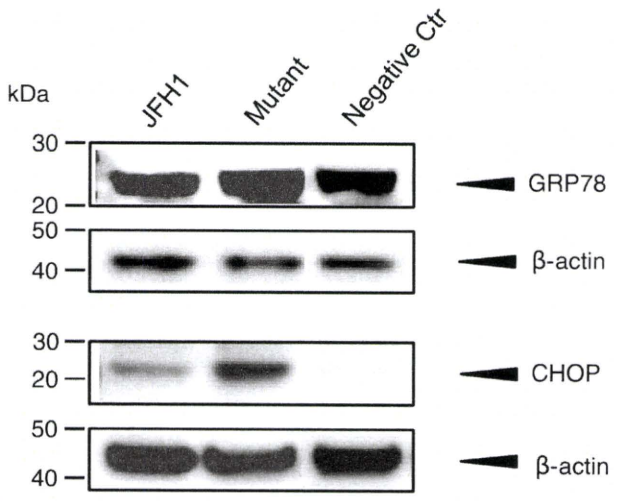

Fig. 7. Expression of ER stress-related proteins in human hepatocytes of chimeric mice infected with JFH1 or the mutant in the early phase. Western-blot analysis of the liver tissues of infected chimeric mice using anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody and anti-beta-actin. Liver samples were obtained at 5 days after inoculation. The negative control liver samples for this study was from uninfected human hepatocyte chimeric mouse.

Four of the five NS5B mutations appeared independently in several isolated subclones. This made us speculate that these amino acid substitutions may affect the enzymatic activity of RdRp. Mapping of the amino acid substitutions in the RdRp tertiary structure revealed that amino acid 2441 is located on the finger domain, and three amino acids, 2938, 2964, and 2985, are on the outer surface of the thumb domain, which corresponds to the opposite side of the nucleotide tunnel. The other substitutions, 3004 and 3005, are within the domain of the polypeptide linking the polymerase to the membrane anchor (Lesburg et al., 1999). Our preliminary study has shown that the NS5B mutations, P2938S and R2985P, did not affect cell-free enzymatic activities of the RNA polymerase. Thus, it is speculated that these mutations may affect the stability of the HCV replicase complex by altering surface affinity to other nonstructural proteins.

There are several reports on cell culture adaptive mutations in the HCV-JFH1 genome that gave more vigorous and consistent virus expression. Most studies involved prolonged cell culture of HCV-JFH1 or multiple rounds of successive passage onto naïve cells. Zhong et al. detected the E2-G451R mutation after culture for more than 60 days. The mutation led to more efficient production of infectious viral particles than wild type JFH1 (Zhong et al., 2006). Delgrange et al. conducted successive virus infections of naïve cells and identified the E2-N534K mutation that facilitated virus-CD81 attachment, and core-F172C and -P173S that increased secretion of virions (Delgrange et al., 2007). Using a similar method, Russell et al. identified E2-N417S that improved virus–cell attachment, and p7-N765D and NS2-Q1012R that increased virion production (Russell et al., 2008). Kaul et al. reported the NS5A-V2440L mutation, that was close to the C terminus and increased virion production (Kaul et al., 2007). Yi et al. used a chimeric virus of genotype 1a and JFH1 and identified the NS3-Q1251L mutation that resulted in enhanced virus production, possibly through improved interactions between NS2 and NS3 that were required for virion formation (Yi et al., 2002). Han et al. used EGFP-tagged virus and identified the mutually dependent mutations, NS3-M1290K and NS5A-T2438I, which improved virus production synergistically (Han et al., 2009).

Of note is that all of the mutations reported above promoted virion secretion or virus–cell surface interaction and none of them showed any effect on intracellular replication of viral RNA or translation of virus proteins. None of the adaptive mutations reported above overlapped with our cytopathogenic mutations. The mutations that we have identified conferred enhanced virus replication and protein expression in the early/acute stages of infection and subsequently led to massive cell death. Our data and the reports of other groups suggest that the HCV genome evolves to adapt to the host cell environment. Mutations that optimize virus secretion or virus–cell entry may be

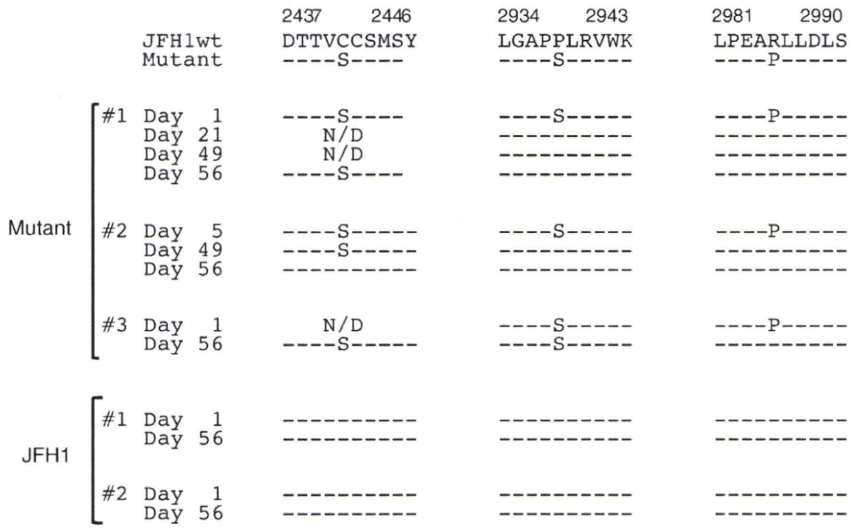

Fig. 8. Nucleotide sequence analysis of virus genomes circulating in the sera of infected mice. We extracted RNA from the sera of mice inoculated with culture media from JFH1 or JFH1-mutants and analyzed the viral sequence at the specified time points. N/D is not detectable. Wt: Wild type.

required for persistent infection in vitro, while those that affect cellular viral RNA replication may possibly promote viral genetic evolution and host cell damage.

The results of in vivo experiments using human hepatocyte chimeric mice were consistent with those of virus cell culture (Figs. 5, 6 and 7). The mutant JFH1 clones showed markedly higher levels of replication than the parental JFH1 in the acute phases. However, the serum HCV titers subsequently leveled out after two weeks of infection, concomitant with reversal of some cytopathic mutations to wild type sequences. Bukh et al. reported that inoculation of the HCV-1b genome into chimpanzee liver resulted in persistent infection, although the mutation reverted rapidly to wild type (Bukh et al., 2002). In this study, the NS5A-C2441S mutation was preserved in 2 of 3 mice, while NS5B-P2938S reverted to the wild type sequences in 2 of 3 mice and NS5B-R2985P reverted to wild type sequences in all 3 mice. These results suggest that the highly adapted JFH1 genome is infectious and viable in vivo, but is not as fit in vitro.

It is not clear why the subgenomic replicons with C2441S, P2938S or R2985P mutations did not show differences in replication levels compared to the wild type JFH1 subgenomic replicon. One may speculate that this discrepancy between the results using full-length HCV genomes and replicons might be the presence or absence of the HCV structural proteins. In addition, three individual substitutions G2964D, H3004Q and S3005N did not enhance viral replication as compared with the parental JFH1 nor did express detectable amounts of core protein. It is speculated that these mutants exist in host cells through co-infection with replication-competent viral clones resulting in enhanced replication.

There is clinical evidence that suggests the pathological outcomes of hepatitis C result from the immune response of the host rather than the direct cytopathic effects of the virus (Cerny and Chisari, 1999). However, several clinical studies have shown that fulminant hepatic failure (FHF, the HCV-JFH1 strain was isolated from such a case) featured massive hepatocyte apoptosis, as characterized by caspase activation and Fas–FasL expression (Leifeld et al., 2006; Mita et al., 2005; Ryo et al., 2000). The ER stress markers, GRP78 and ATF6 are upregulated in HCV-infected liver tissue as the histological grade advances (Shuda et al., 2003). This background and our results in vitro and in vivo suggest that HCV strains with highly infectious and cytopathic gene signatures may replicate aggressively in the acute phase of infection and that certain defects in innate or adaptive immune responses against the virus could lead to severe and persistent liver damage due to cytopathic effects induced directly by HCV. Such mechanisms might explain some rare clinical features of HCV infection, such as fulminant hepatic failure and post-transplantation severe fibrosing cholestatic hepatitis (Delladetsima et al., 1999; Dixon and Crawford, 2007).

In conclusion, we identified three substitutions in cytopathic HCV-JFH1 subclones derived from plaque assay. These substitutions directly enhanced virus replication in the early phases of virus infection in vitro and in vivo. This highly enhanced replication induced ER stress-mediated apoptosis and resulted in cytopathogenicity. Further analyses of cellular effects on HCV replication may elucidate the pathogenesis of HCV infection and may define novel host factors as targets of antiviral chemotherapeutics.

Materials and methods

Cells and cell culture

Huh-7.5.1 cells (Zhong et al., 2005) (kindly provided by Dr Francis V. Chisari) and CD81 deficient Huh7-S29 cells (Russell et al., 2008) (kindly provided by Dr Rodney S. Russell and Dr Robert H. Purcell) were maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum at 37 °C under 5.0% CO_2.

Sequence analysis

The cDNA from the isolated JFH1-plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequencing.

In vitro RNA synthesis and transfection

A plasmid, pJFH1full (Wakita et al., 2005), which encodes full-length HCV-JFH1 sequence, was used. In vitro RNA synthesis and transfection were conducted as previously described (Sekine-Osajima et al., 2008). Briefly, HCV RNA was synthesized from linearized pJFH1 plasmid as template and transfected into Huh-7.5.1 cells by electroporation. The transfected cells were split every 3 to 5 days. The culture media were subsequently transferred onto uninfected Huh-7.5.1 cells and Huh7-S29 cells. The levels of HCV replication and viral protein expression were detected by real-time PCR and western blotting.

Plaque assay

HCV plaque assays were performed as reported previously (Sekine-Osajima et al., 2008). Huh-7.5.1 cells were seeded in collagen-coated 60 mm-diameter plates. After overnight incubation, HCV-infected culture media were serially diluted in a final volume of 2 ml per plate and transferred onto the cell monolayer. After ~5 h of incubation, the inocula were removed and the cell monolayer was overlaid with 8 ml of culture medium containing 0.8% methylcellulose (Sigma). After 7 to 12 days culture, cytopathic plaques were visualized by staining with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (plaque-forming unit/ml).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5A and NS5B region of JFH1, plasmid pJFH1 was digested with HindIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescript II SK+ phagemid vector (Stratagene, La Jolla, CA). Mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-ChangeII Site-Directed Mutagenesis Kit, Stratagene) to generate the following codon changes: P2938S, G2964D, R2985P, H3004Q and S3005N. Finally, the HindIII–HindIII fragments were subcloned back into the parental plasmid, pJFH1. A PCR fragment (nt. 7421–7839) was subcloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI) and digested with RsrII and BsrGI. Finally, after introducing the codon change C2441S, the RsrII–BsrGI fragment was reinserted into the parental plasmid.

Quantification of HCV core antigen in the culture medium

The culture media from JFH1-RNA transfected Huh-7.5.1 cells and Huh7-S29 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 levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Itsui et al., 2009). Briefly, 10 μg of total cell lysate were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) Western Blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Bioscience, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody (Abcam, Cambridge, MA), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-beta-actin antibody (Sigma).

HCV subgenomic replicon constructs

The HCV subgenomic replicon plasmid, pRep-Feo, was derived from the HCV-N strain, pHCV1bneo-delS (Tanabe et al., 2004; Yokota et al., 2003). The replicon RNA was synthesized from pRep-Feo and transfected into Huh7 cells.

Luciferase reporter assay

Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX, Perkin Elmer, Waltham, MA) with a Bright-Glo Luciferase Assay System (Promega) (Tasaka et al., 2007). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assays

To evaluate cell viability, dimethylthiazol carboxymethoxy-phenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), as described previously (Sakamoto et al., 2007).

Real-time RT-PCR analysis

Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micro-grams of total cellular RNA were used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI 7500 Real-Time PCR System (Applied Biosystems). The primers used were as follows: HCV-JFH1 sense (positions 285 to 307; 5′-GGT-ACTGCCTGATAGGGTGCTT-3′), HCV-JFH1 antisense (positions 349 to 375; 5′-TGGTTTTTCTTTGAGGTTTAGGATTC-3′), GAPDH sense (5′-CCTCCCGCTTCGCTCTCT-3′), and GAPDH antisense (5′-GCTGGCGACG-CAAAAGA-3′).

HCV RNA inoculation into human hepatocyte chimeric mice

Housing, maintenance, and care of the mice used in this study conformed to the requirement for the humane use of animals in scientific research as defined by Animal Care and Use Committee of our institute. The culture media of Huh-7.5.1 cells transfected with parental JFH1 and JFH1 mutants were collected 10 days after transfection and passed through a 0.45 μm filter. The three mutations introduced in NS5A and NS5B were confirmed to conserve by the sequence analysis of virus genome of cell culture supernatants before inoculation. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff, Millipore). 100 μl of each culture medium was injected intravenously into human hepatocyte chimeric mice (PXB mice, Phenix Bio, Hiroshima, Japan) (Mercer et al., 2001). The rate of liver chimerism of these human hepatocyte chimeric mice was confirmed more than 70% by immunohistochemical analysis. After infection, blood samples were taken serially and levels for HCV RNA and human albumin were quantified using real-time RT-PCR and an enzyme immunoassay, respectively. RNA was extracted from serum samples and subjected to direct sequence determination.

Protein extraction from human hepatocyte chimeric mice and expression of ER stress-related proteins

5 days post inoculation, mice were sacrificed and proteins were extracted from liver samples with complete Lysis-M Reagent Kit (Roche Applied Science, Indianapolis, IN). One Mini Protease Inhibitor Cocktail Tablet was dissolved into 10 ml of Lysis-M Reagent and 500 μl of this fluid was added to 50 μg of each liver sample and homogenized. The lysate was transferred to a microcentrifuge tube and centrifuged at 14,000×*g* for 5 min. The supernatant containing soluble protein was transferred to a new reaction tube and 20 μg of each protein was used for western blotting to detect ER stress-related proteins.

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IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones

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ABSTRACT

Mechanisms of difference in interferon sensitivity between hepatitis C virus (HCV) strains have yet to be clarified. Here, we constructed an infectious genotype2b clone and analyzed differences in interferon-alpha sensitivity between HCV-2b and 2a-JFH1 clones using intergenotypic homologous recombination. The HCV-2b/JFH1 chimeric virus able to infect Huh7.5.1 cells and was significantly more sensitive to IFN than JFH1. IFN-induced expression of MxA and 25-OAS was significantly lower in JFH1 than in 2b/JFH1-infected cells. In JFH1-infected cells, expression of SOCS3 and its inducer, IL-6, was significantly higher than in 2b/JFH1-infected cells. The IFN-resistance of JFH1 cells was negated by siRNA-knock down of SOCS3 expression and by pretreatment with anti-IL6 antibody. In conclusion, intergenotypic differences of IFN sensitivity of HCV may be attributable to the sequences of HCV structural proteins and can be determined by SOCS3 and IL-6 expression levels.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). There is no therapeutic or prophylactic vaccine available for HCV and type I interferons have been the mainstay of HCV therapeutics (Hoofnagle and di Bisceglie, 1997). Antiviral therapeutic options against HCV are limited and yield unsatisfactory responses (Fried et al., 2002). Given these situations, gaining a detailed understanding of the molecular mechanisms of interferon resistance has been a high priority in academia and industry.

Molecular studies of HCV have been hampered by the lack of efficient *in vitro* and *in vivo* models of infection, which has been partly overcome by the development of HCV subgenomic replicons (Blight et al., 2000; Kato et al., 2003; Lohmann et al., 1999) and the HCV-JFH1 cell culture system (Wakita et al., 2005). HCV-JFH1 is an isolate of HCV genotype 2a that was obtained from a patient with fulminant hepatitis C. The full-length JFH1 genome has been shown to produce infectious particles in cell culture. Simultaneously, a robustly replicating intragenotypic chimera has been reported, which consists of the structural region of a genotype 2a, J6-clone and nonstructural region of JFH-1 (Lindenbach et al., 2005).

HCV isolates are classified into seven major genotypes and multiple subtypes (Gottwein et al., 2009). In infected individuals, HCV exists as quasispecies of closely related genomes (Bukh et al., 1995). A number of studies have suggested that the outcome of HCV infection, as well as the response to interferon treatment, depends on the genotype or quasispecies with which the patient is infected. However, it is not clear how these subtle genetic differences of HCV affect viral replication, infectivity and host responses. Thus, it is important to establish multiple cell culture-permissive strains of different genotypes and isolates of the same genotype for their potential value for characterizing the virus life cycle, drug sensitivity and virus-related cell signaling.

Our present work describes the generation of chimeric viruses with their structural regions from genotype 2b and non-structural genes from the HCV-JFH1 strain. The intergenotypic 2b/JFH1 viruses were compared in terms of intracellular replication, infectious virus production and sensitivity to interferon-alpha. Here we show that the differences in sensitivity to interferon are attributable to upregulated expression of the cellular interferon signal attenuator, SOCS3, and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

Abbreviations: HCV, hepatitis C virus; TLR, toll-like receptor; FBS, fetal bovine serum; ISG, interferon-stimulated gene; IFN, interferon; SOCS, suppressor of cytokine signaling; IL, interleukin; ALT, alanine aminotransferase; UTR, untranslated region; CLEIA, chemiluminescence enzyme immunoassay; PVDF, polyvinylidene fluoride; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha/beta receptor.

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Results

In vitro and in vivo infectivity analyses of HCV-2b and 2b/JFH1 intragenotypic chimeras

First, we investigated the infectivity of the full-length genotype 2b clone *in vitro* and *in vivo*. The full-length genotype 2b HCV clone was infectious after direct injection of RNA transcribed *in vitro* into the livers of human hepatocyte engrafted albumin-uPA/SCID mice (*see the* Supplementary Fig. 1). However, transfection of the HCV RNA into Huh7.5.1 cells did not lead to replication or secretion of virions. Knowing that the full-length genotype 2b HCV was not infectious *in vitro*, we constructed genotype2b/JFH1 intergenotypic recombinants. We constructed three recombinant clones of 2b/JFH1 (Fig. 1A), which were joined between E2 and p7 (JE31F), NS2 and NS3 (JE39F), and within NS2 at nt. 2867 (JEC3F). After transfection of these chimeric HCV RNAs and JFH-1 RNAs into Huh7.5.1 cells, all four clones expressed detectable amounts of HCV core protein in the cells (Fig. 1B) and culture fluid (Fig. 1C). Among the four clones, JEC3F produced the highest level of core protein in the cells and culture fluid. Similarly, in the reinfection assays, JEC3F infected naïve cells most efficiently (Figs. 1D and E). We then compared the infectivity of JEC3F with the other chimeric viruses, genotype2a J6/JFH1 and the JFH1 clone (Supplementary Fig. 2). Transfection of the individual clones into Huh7.5.1 cells showed that JEC3F and the 2b/JFH1 chimera secreted core protein into the medium most efficiently (Fig. 1C). We measured HCV core antigen and HCV-RNA levels in culture supernatant of JEC3F and JFH-1 infected cells. As shown in Fig. 1F, the ratio between supernatant HCV core antigen and HCV-RNA between JEC3F and JFH1 was well correlated each other.

Comparisons of sensitivity to IFN between intragenotypic chimeras and JFH1

Next, we investigated the interferon-alpha sensitivity of the three 2b/JFH1 chimeric viruses with different junctions, JE31F, JE39F and JEC3F, as well as JFH1. The four viral RNAs were transfected separately into Huh7.5.1 cells and were treated with 0, 1, 3 or 9 IU/mL of interferon-alpha-2b. Seventy-two hours after addition of interferon, core antigen was measured in the culture fluid. As shown in Fig. 2, all 2b/JFH1 chimeric clones showed significantly higher responses to interferon than JFH1 ($p<0.01$). These results indicate that the relative interferon sensitivity of 2b/JFH1 clones over JFH1 could be attributable to the sequences of HCV-2b-derived structural proteins, especially core, E1 or E2 protein.

Expression of IFN stimulated genes and STAT1 and 2 phosphorylation in HCV-infected cells

Knowing that the 2b/JFH1 chimeric clones are more sensitive to interferon than JFH1, we next analyzed the effects on cellular interferon signaling. We investigated the expression levels of the interferon-stimulated genes (ISGs), 25OAS and MxA mRNAs that mediate antiviral effects (Itsui et al., 2009; Itsui et al., 2006). Induction of 25OAS and MxA by IFN was significantly suppressed in cells infected with HCV-JFH1 and the JEC3F clones. Of note was that the induction of these ISGs was suppressed substantially in JFH1-infected cells compared to JEC3F-infected cells (Figs. 3A and B). We then detected IFN-induced phosphorylation of STAT1 and STAT2 to pSTAT1 and pSTAT2 in uninfected and JFH1- and JEC3F-infected cells. Phosphorylation of STAT1 and STAT2 occurs within minutes after addition of IFN and substantially decreased at time points later than 8 hours {Itsui, 2006 #1025}. Thus, we detected pSTAT1 and pSTAT2 before and at 15 minutes after IFN treatment. As shown in Figs. 3C and D, production of pSTAT1 and pSTAT2 was decreased substantially in JFH1-infected cells, compared with uninfected and JEC3F-infected cells. These finding indicated that the differences in sensitivity to interferon of JFH1 and JEC3F were closely associated with attenuation of the cellular IFN signaling pathway.

SOCS 3 is up-regulated in JFH-infected, IFN-resistant cells

We next investigated the effects of HCV replication on the expression of SOCS1 and SOCS3 that suppress IFN receptor-mediated signaling (Song and Shuai, 1998; Vlotides et al., 2004). While SOCS1 mRNA expression did not differ significantly between uninfected and JFH1- and JEC3F-infected cells, the SOCS3 mRNA expression level was significantly higher in JFH1-infected cells than in uninfected and JEC3F-infected cells (Figs. 4A and B).

Knock down of the SOCS3 gene

To verify that SOCS3 was the key molecule determining the sensitivity to IFN, we performed siRNA knock down of SOCS3 in the virus-infected cells. A SOCS3-directed siRNA was cotransfected with HCV-JFH1 or -JEC3F RNA into Huh7.5.1 cells. Three days after transfection we measured SOCS3 mRNA expression in JFH1 and JEC3F-transfected cells with or without SOCS3-siRNA. Interestingly, SOCS3-knock down in JFH1-transfected cells restored sensitivity of IFN to the same levels as JEC3F-transfected cells (Figs. 5A and B).

Interleukin-6 is involved in SOCS-mediated interferon resistance

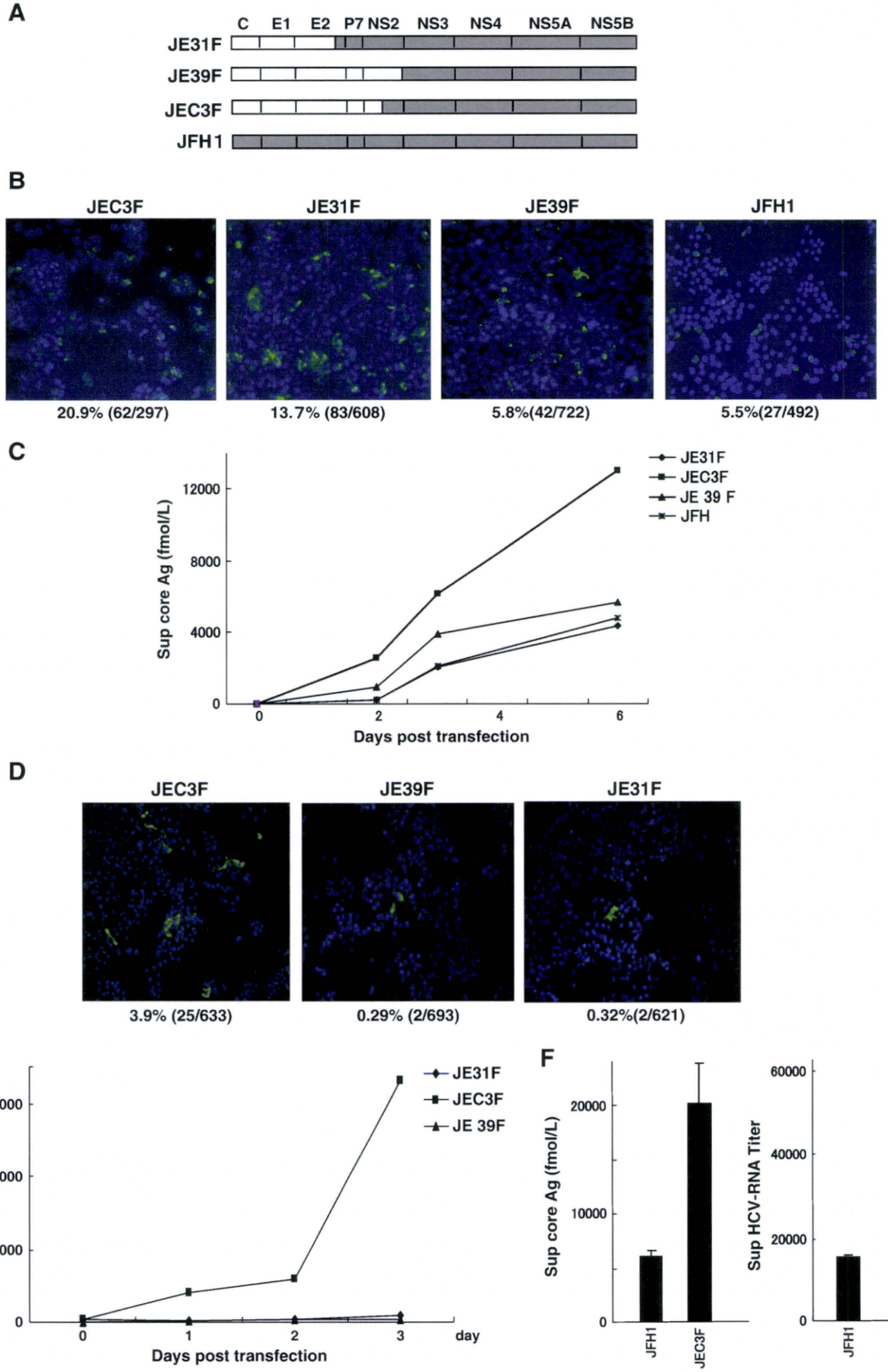
It has been reported that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (Hanada et al., 2003) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (Ramadori and Christ, 1999). This background led us to investigate whether overexpression of SOCS3 is associated with overproduction of IL-6. We investigated Phosphorylated STAT3 (pSTAT3) expression and IL-6 mRNA expression in JFH1- and JEC3F-transfected Huh7.5.1 cells. Phosphorylated STAT3 level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells and naïve Huh7.5.1cell (Fig. 6A). Moreover IL-6 gene expression level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells (Fig. 6B). Consistent with previous reports, treatment of the Huh7.5.1 cells with IL-6 induced expression of SOCS3 and SOCS1 mRNAs with SOCS3 being much stronger than SOCS1 (Fig. 6C).

Anti-IL-6 antibody restored IFN-resistance to HCV-infected cells

To investigate whether IL-6 is responsible for HCV infection-induced upregulation of SOCS and for resistance to interferon, JFH1 and JEC3F-infected Huh7.5.1 cells were pretreated with antibodies directed against IL-6 and subsequently treated with interferon. Interestingly, anti-IL-6-treated HCV-infected cells became significantly more susceptible to IFN treatment (Fig. 6D) without affecting viral expression levels in the absence of interferon (Fig. 6E). Cellular levels of SOCS3 mRNA were significantly lower in anti-IL-6-treated cells than untreated cells (Fig. 6F). These results strongly suggested that the interferon resistance of HCV-infected cells and the difference between the two viral strains are partly mediated by internal overproduction of IL-6 and subsequent upregulation of SOCS3.

Determination of the HCV structural region that induced SOCS3 and IL6

We studied further which part of HCV structural polyprotein is responsible for the difference in interferon-sensitivity. We constructed two additional chimeric clones between HCV-2b and JFH1. The 2bCoreJFH1 had the 2b-core region followed by the JFH1-structural and nonstructural regions. JCoreC3F was derived from JEC3F by exchanging the 2b-core with the JFH1-core (Fig. 7A). As


A
C E1 E2 P7 NS2 NS3 NS4 NS5A NS5B
JE31F
JE39F
JEC3F
JFH1
B
JEC3F
JE31F
JE39F
JFH1
20.9% (62/297)
13.7% (83/608)
5.8%(42/722)
5.5%(27/492)
C
JE31F
JEC3F
JE 39 F
JFH
Sup core Ag (fmol/L)
12000
8000
4000
0
0
2
4
6
Days post transfection
D
JEC3F
JE39F
JE31F
3.9% (25/633)
0.29% (2/693)
0.32%(2/621)
E
JE31F
JEC3F
JE 39F
Sup core Ag fmol/L
3000
2000
1000
0
0
1
2
3
day
Days post transfection
F
Sup core Ag (fmol/L)
20000
10000
0
JFH1
JEC3F
Sup HCV-RNA Titer
60000
40000
20000
0
JFH1
JEC3F

shown in Fig. 7B JFH1 and JCoreC3F, which had a JFH1-derived core region, were significantly more resistant to IFN than JEC3F and 2bCoreJFH1, with a 2b-derived core (Fig. 7B). Consistent with the interferon sensitivity results, JFH1 and JcoreC3F-infected cells expressed SOCS3 and IL6 mRNAs at significantly higher levels than JEC3F and 2bCoreJFH1-infected cells (Figs. 7C and D). These differences in gene expression were inversely associated with the cellular expression levels of each HCV chimeric clone (Fig. 7E). These results indicate that the amino acid sequence of the core protein is responsible for IL-6 and SOCS3-medited interferon resistance.

Discussion

In this study, we succeeded in establishing a new genotype 2b infectious HCV clone and genotype 2b/JFH1 cell culture-competent intragenotypic chimeric viruses (Fig. 1). Relative interferon sensitivities of 2b/JFH1 chimeras, compared with HCV-JFH1 virus (Fig. 2), led us to conduct a series of assays to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN-alpha receptor-mediated cellular responses were more attenuated in HCV-JFH1- and 2b/JFH1 chimera-infected than in uninfected Huh7.5.1 cells, but more potently for HCV-JFH1. Precise intragenotypic recombination analyses showed that the amino acid sequence of the HCV core protein is responsible for the differences in interferon sensitivity (Figs. 2, 7). The differences in the interferon-mediated antiviral effects were demonstrated further by the different rates of induction of interferon-inducible MxA and 25-OAS mRNAs (Figs. 3A and B) and IFN induced phosphorylation of STAT1 and STAT2 (Figs. 3D and E). We have demonstrated further that the expression of an interferon signal attenuator, SOCS3, was significantly higher in JFH1 than in 2b/JFH1-infected cells (Song and Shuai, 1998; Vlotides et al., 2004). Indeed, the siRNA-knock down of SOCS3 in JFH1 and 2b/JFH1-infected cells resulted in responsiveness to IFN (Fig. 5). Moreover, cellular expression of IL-6, which increases cytoplasmic phospho-STAT3 (Fig. 6A) and induces SOCS3 expression (Ramadori and Christ, 1999) was significantly higher in JFH1 transfected cells (Fig. 6B). Furthermore, by pre-treatment with anti-IL-6 antibody, JFH1- and 2b/JFH1-infected cells partially recovered elevation of SOCS3 expression and unresponsiveness to IFN (Fig. 6D). Taking all these things together, it is strongly suggested that the differences in IFN sensitivity between genotypes or isolates could be explained by SOCS3-mediated attenuation of interferon responses and, more importantly, IL-6 may constitute a molecular target to reverse such cellular interferon resistance.

Vast numbers of studies have failed to construct infectious HCV clones, other than HCV-JFH1. Murayama, et al. have conducted intragenotypic homologous recombination analyses between HCV-J6 and -JFH1 and have reported that that the NS3 protease and NS5B polymerase are essential for replication of the recombinant virus (Murayama et al., 2007). Up to now, several JFH1-based chimeric viruses have been reported, which include genotypes 4a (Scheel et al., 2008), genotype 1a, 1b, 2a (Pietschmann et al., 2006), genotype 3a (Gottwein et al., 2007), genotype 5 a (Jensen et al., 2008) and, genotype 2b, 6a, 7a (Gottwein et al., 2009). Gottwein, et al. constructed intergenotypic chimeric HCV from JFH1 and genotypes 1 through 7 and analyzed differences in sensitivity to antiviral drugs (Gottwein et al., 2009). However, intergenotypic differences in sensitivity to IFN-alpha and the molecular mechanisms involved have not been well characterized. In this study, we constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), which showed variable sensitivity to IFN and confirmed that the core region is responsible for such IFN sensitivity. This study may support the feasibility of such inter and intragenotypic homologous recombination approaches to characterize differences in viral kinetics and drug responses.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (Chang et al., 1991; Kalvakolanu, 2003; Ronni et al., 1998). On binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases-1 and -2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (Samuel, 2001; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak–STAT pathway and subsequent expression of ISGs (Vlotides et al., 2004). In HCV subgenomic replicon-expressing cells, expression levels of SOCS3 were inversely correlated with sensitivity to IFN to suppress viral RNA replication (Zhu et al., 2005).

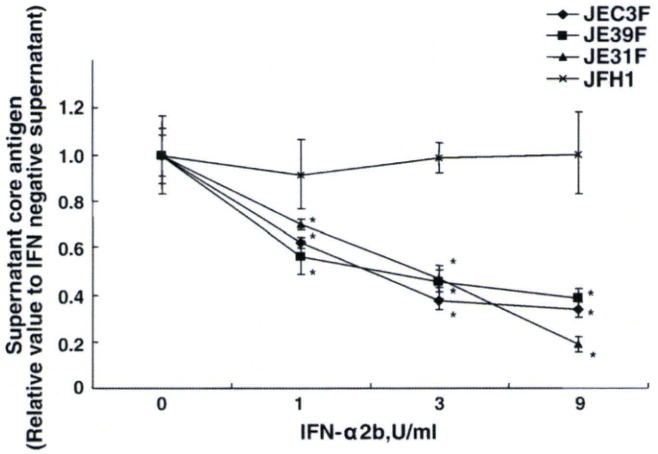


Fig. 2. Comparison IFN-alpha sensitivity among 2b/JFH1 chimeric viruses and JFH1. Ten μg of JE31F, JE39F, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells. The transfected cells were divided into 12 wells. Forty eight hours after transfection, cells were washed twice with PBS and treated with 0, 1, 3 and, 9 U/ml IFN-alpha-2b. Seventy-two hours after IFN-alpha 2b addition, quantification of HCV core antigen in culture fluids was conducted. The experiments were conducted twice by using Huh 751 cells of different passage, and a representative data was shown. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

Fig. 1. Replication and infection competency of HCV-2b/JFH1 chimeric viruses A. Genomic structures of HCV-JFH1, HCV-2b and 2b/JFH1 chimeric viruses. Intergenotypic homologous recombination was conducted between the HCV-2b and JFH1 (2a) clones and three chimeric clones were constructed that were joined between NS2-NS3 (JE39F), and within E2 at nt2541 (JE31F) and NS2 at nt. 2867 (JEC3F). B. Immunocytochemistry of HCV core. HCV RNA-transfected Huh7.5.1 cells were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after transfection using mouse-anti-core antibody(*green*) and DAPI(*blue*). Numbers at the bottom denote percentages of HCV core-positive cells. E. Time courses of 2b/JFH1 infected cells. JE31F, JE39F, JEC3F RNA-transfected cell culture fluids were used to infect naïve Huh7.5.1 cells in 60 mm-diameter plates at density of 3×10^5 cells per plate. Quantification of HCV core antigen in culture supernatants was carried out at 24 hours, 48 hours, 72 hours and 144 hours after inoculation. The experiment was done three times with similar results independently. Panel E shows representative date. F. Comparison between JFH1 and JEC3F supernatant HCV-RNA titer and core antigen. Four days after JFH1 and JEC3F RNA transfection, culture supernatant was harvested and subjected to both HCV core antigen assay and realtime RT-PCR of HCV-RNA. Assays were done in triplicate and the data are shown as mean ± sd.

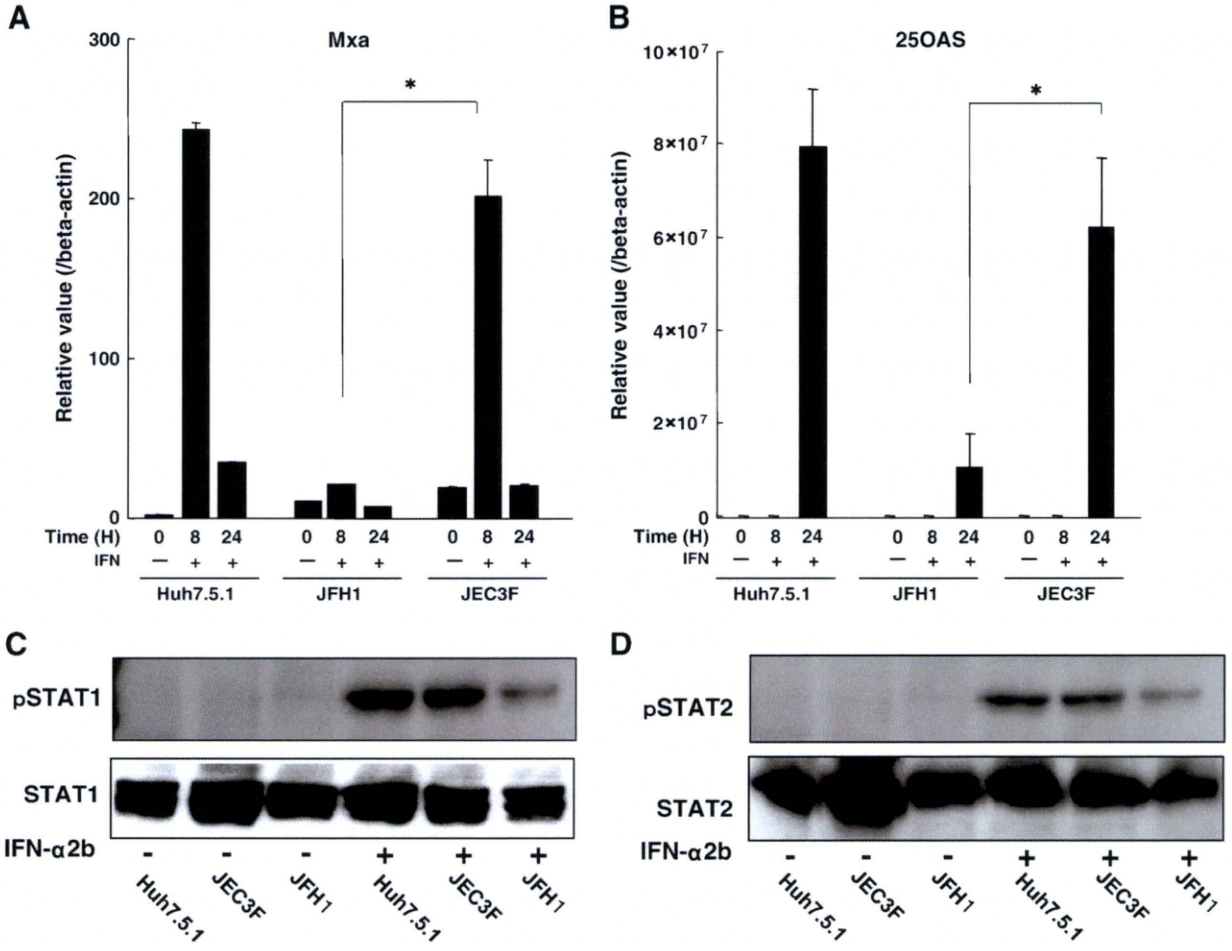


Fig. 3. Induction by interferon of the interferon-inducible genes, MxA (panel A), 25-OAS (panel B) and phosphorylated STAT1 (panel C) and STAT2 (panel D). JEC3F and JFH1 10 μg RNA was transfected into Huh7.5.1 cells. Forty-eight hours after transfection, the cells were treated with 25 U/ml IFN-alpha 2b. Total cellular RNA was isolated before and 8 and 24 hours after IFN treatment. Relative gene expression levels of MxA (panel A) and 25-OAS (panel B) were determined by real-time PCR at the time points indicated. JEC3F and JFH1 RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours after transfection, the cells were treated with 25 U/ml IFN-alpha 2b. Total cellular protein was isolated before and 15 minutes after IFN treatment. Ten μg of extracted protein were used for analysis of phosphorylated STAT1, STAT2 protein and STAT1, STAT2 protein as controls. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

HCV, on the other hand, counteracts such IFN-mediated antiviral pathways. The NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (He and Katze, 2002; Taylor et al., 1999). NS5A also induced expression of IL-8 and attenuated expression of ISGs (Polyak et al., 2001). HCV core protein has been reported to bind the STAT1-SH domain (Lin et al., 2006) or destabilize STAT1 (Lin et al., 2005) to block IFN signaling. It also has been reported that overexpression of core protein upregulated SOCS3 expression (Bode et al., 2003). In this study, we used full-length HCV cell culture and found, *for the first time*, that SOCS3 expression is upregulated differently depending on the genetic sequences of HCV strains and that these differences in SOCS3 expression are associated with sensitivity to IFN. Moreover, overexpression and knock down of SOCS3 expression were closely associated with the IFN sensitivity of the HCV-infected cells. These results indicate that interferon-resistance of HCV-infected cells is directed by overexpression of SOCS3, which may be upregulated by HCV proteins as reported (Bode et al., 2003) (Kawaguchi et al., 2004). A sequence comparison of our HCV2b and JFH1 clones has found 16 amino acid differences. These structural differences of the core protein might affect cellular responses to interferon (*see the* Supplementary Fig. 4).

It has been reported that IL-6 is the principal activator of STAT3 in hepatocytes through binding its receptor (Hanada et al., 2003; Ramadori and Christ, 1999). Furthermore, plasma IL-6 levels are elevated in

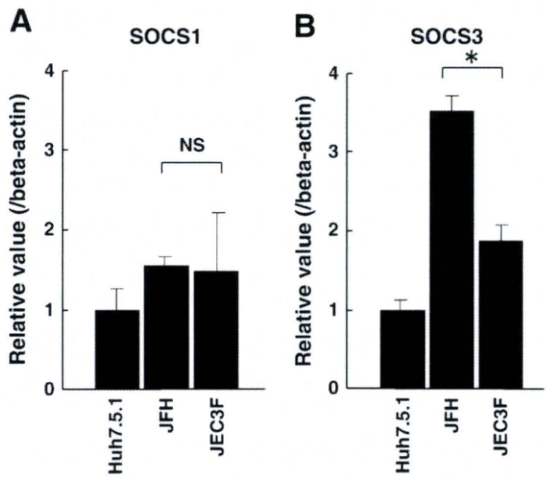


Fig. 4. Expression of SOCS1 mRNA (panel A), SOCS3 mRNA (panel B). Forty-eight hours after transfection of JEC3F, JFH1 10 μg RNA or mock transfection into Huh7.5.1 cells, total RNA and total protein were isolated. Relative gene expression levels of SOCS1 (panel A) and SOCS3 (panel B) and were determined 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.

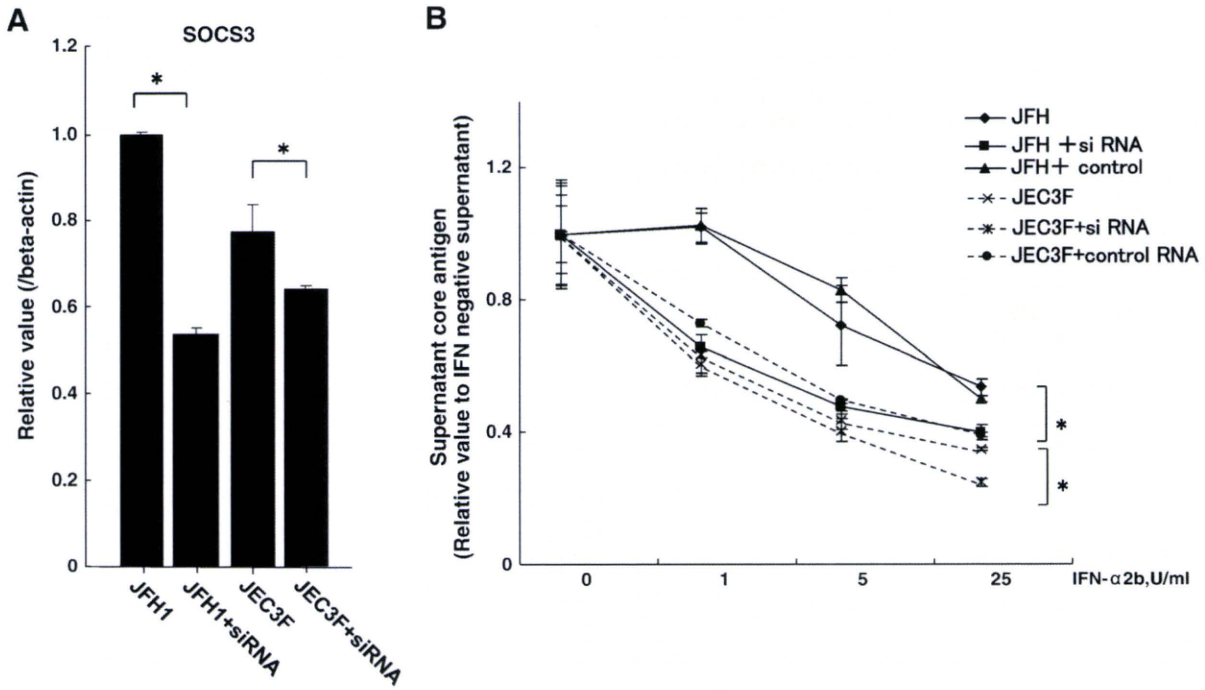


Fig. 5. Differences in sensitivities to IFN between SOCS3-knock down, HCV transfected cells. JFH1 or JEC3F 10 μg RNA, and 80 pmol siRNA SOCS3-HSS113312 or MOCK were electroporated into 5×10^6 uninfected Huh7.5.1 cells. A. Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight 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.

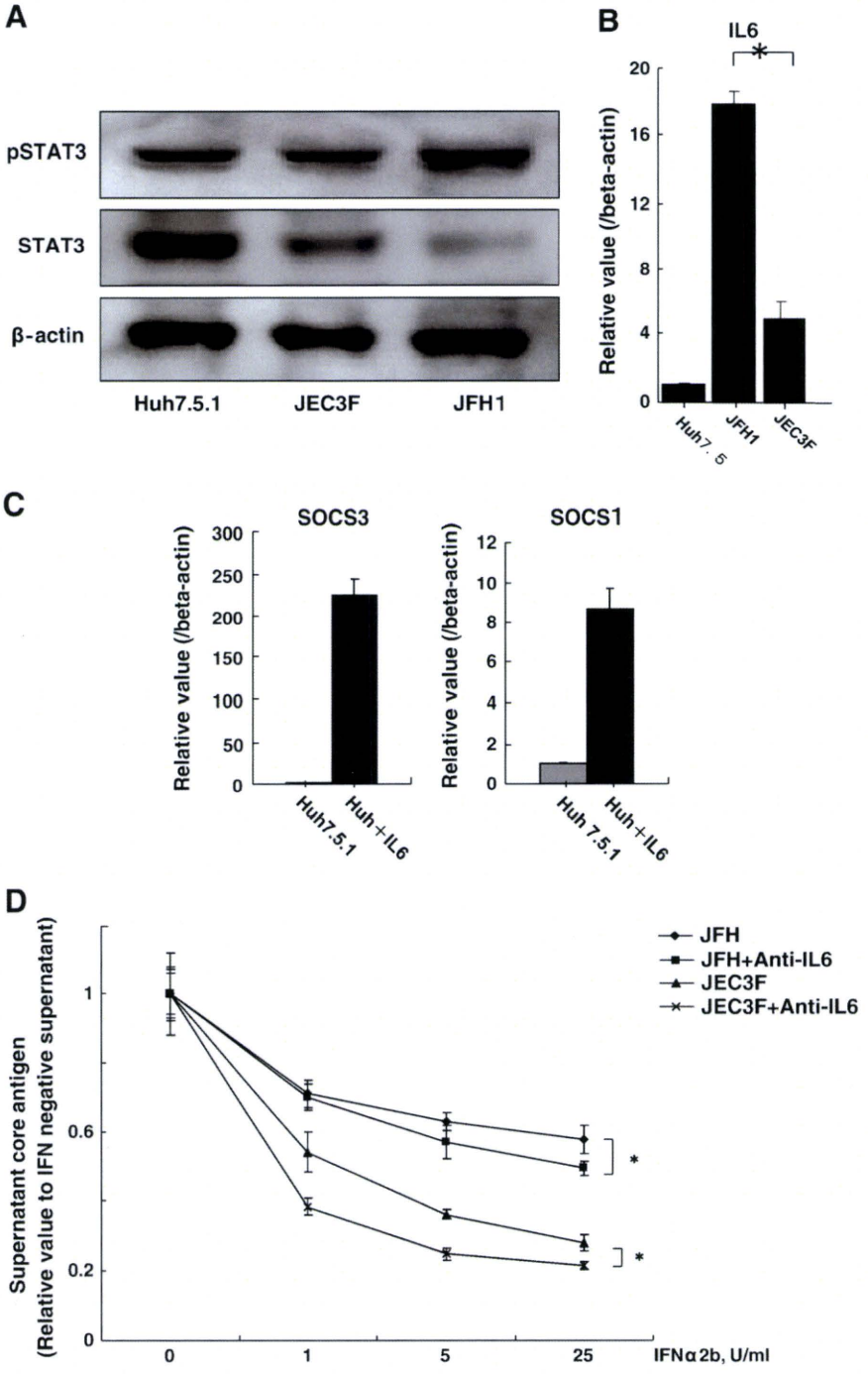


Fig. 6. IL-6 expression in HCV infected cells and change in IFN sensitivity by treatment with anti-IL6 antibody. A. Expression of cytoplasmic phospho-STAT3 in uninfected and HCV-infected Huh7 cells. JEC3F, JFH1 10 μg RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours total cellular protein 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.

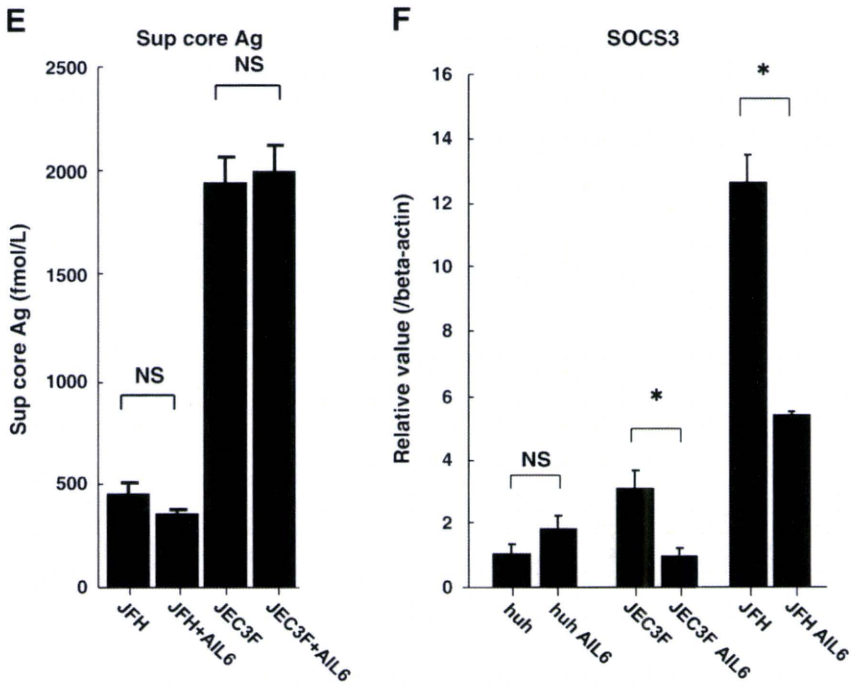


Fig. 6 (*continued*).

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,

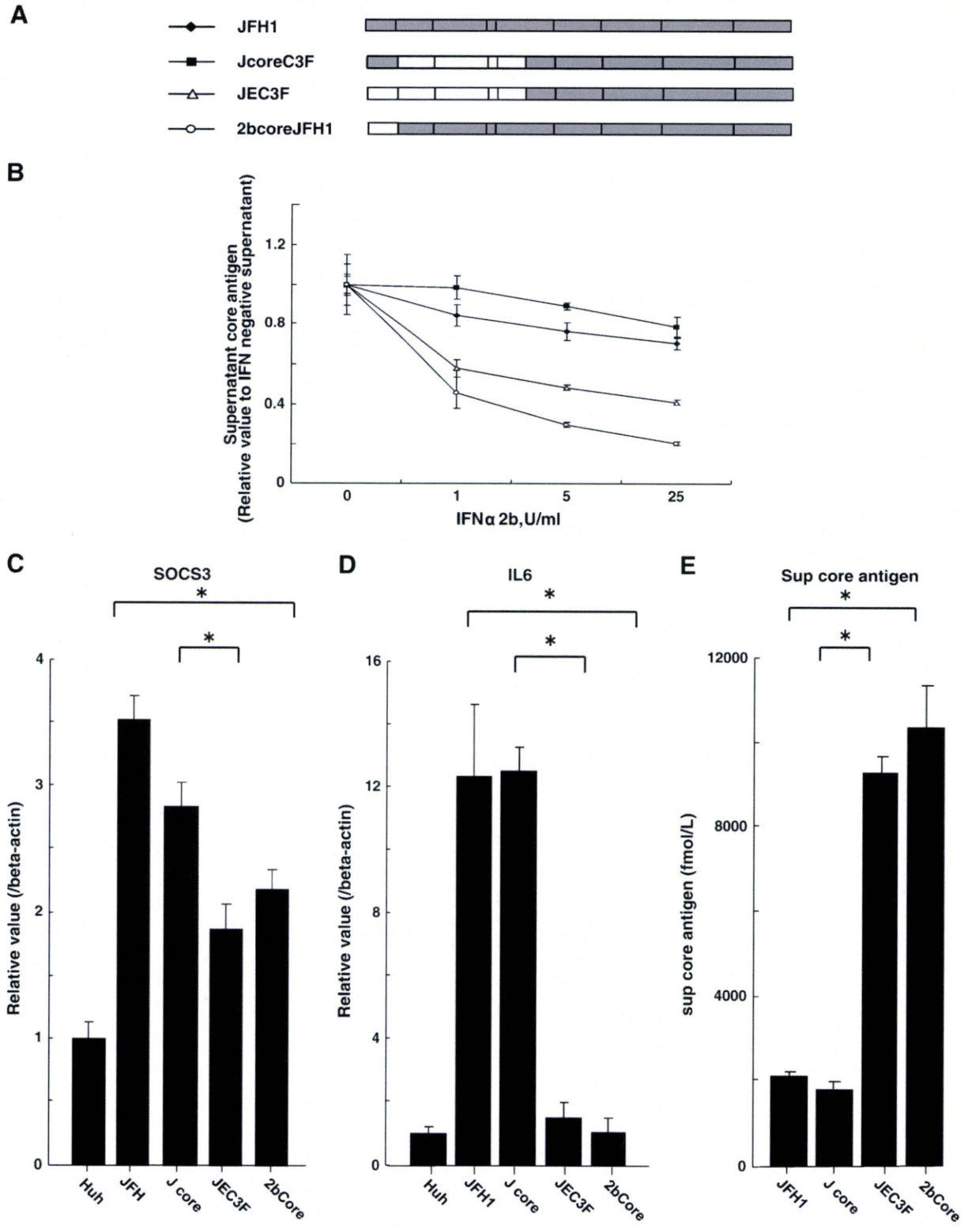


Fig. 7. Replacement of the HCV-2b-core region with JFH1-core causes upregulation of SOCS3 and IL-6 and restores resistance to IFN. A. Genome maps of JFH-1, JEC3F , J core C3F, 2b core JFH1 recombinant cDNA. J core C3 F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The 2b core 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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Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

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Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, p <0.0001) and sustained virological response (SVR) (odds ratio = 7.41, p <0.0001) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.
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Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

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Table 1. Baseline characteristics of all patients, and patients assigned to the model building or validation groups.

	All patients n = 496	Model group n = 331	Validation group n = 165
Gender: male	250 (50%)	170 (51%)	80 (48%)
Age (years)	57.1 ± 9.9	56.8 ± 9.7	57.5 ± 10.2
ALT (IU/L)	78.6 ± 60.8	78.1 ± 61.4	79.7 ± 59.6
GGT (IU/L)	59.3 ± 63.6	58.9 ± 62.0	60.2 ± 66.9
Platelets (10^9/L)	154 ± 53	153 ± 52	154 ± 56
Fibrosis: F3-4	121 (24%)	80 (24%)	41 (25%)
HCV-RNA: >600,000 IU/ml	409 (82%)	273 (82%)	136 (82%)
ISDR mutation: ≤1	220 (88%)	290 (88%)	145 (88%)
Core 70 (Arg/Gln or His)	293 (59%)/203 (41%)	197 (60%)/134 (40%)	96 (58%)/69 (42%)
Core 91 (Leu/Met)	299 (60%)/197 (40%)	200 (60%)/131 (40%)	99 (60%)/66 (40%)
IL28B: Minor allele	151 (30%)	101 (31%)	50 (30%)
SVR	194 (39%)	129 (39%)	65 (39%)
Relapse	152 (31%)	103 (31%)	49 (30%)
NVR	150 (30%)	99 (30%)	51 (31%)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Minor, heterozygote or homozygote of minor allele; SVR, sustained virological response; NVR, null virological response.

Japanese [6], European [7], and a multi-ethnic population [8,9]. The last three studies focused on the association of SNPs in the *IL28B* region with SVR [7–9] but we found a stronger association with NVR [6]. In addition to these host genetic factors, we have reported that mutations within a stretch of 40 amino acids in the NS5A region of HCV, designated as the IFN sensitivity determining region (ISDR), are closely associated with the virological response to IFN therapy: a lower number of mutations is associated with treatment failure [10–13]. Amino acid substitutions at positions 70 and 91 of the HCV core region (Core70, Core91) also have been reported to be associated with response to PEG-IFN/RBV therapy: glutamine (Gln) or histidine (His) at Core70 and methionine (Met) at Core91 are associated with treatment resistance [4,14]. The importance of substitutions in the HCV core and ISDR was confirmed recently by a Japanese multicenter study [15]. How these viral factors contribute to response to therapy is yet to be determined. For general application in clinical practice, host genetic factors and viral factors should be considered together.

Data mining analysis is a family of non-parametric regression methods for predictive modeling. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [16]. The major advantage of decision tree analysis over logistic regression analysis is that the results of the analysis are presented in the form of flow chart, which can be interpreted intuitively and readily made available for use in clinical practice [17]. The decision tree analysis has been utilized to define prognostic factors in various diseases [18–25]. We have reported recently its usefulness for the prediction of an early virological response (undetectable HCV-RNA within 12 weeks of therapy) to PEG-IFN/RBV therapy in chronic hepatitis C [26].

This study aimed to define the pre-treatment prediction of response to PEG-IFN/RBV therapy through the integrated analysis of host factors, such as the *IL28B* genetic polymorphism and various clinical covariates, as well as viral factors, such as mutations in the HCV core and ISDR and serum HCV-RNA load. In addition, for the general application of these results in clinical practice, decision models for the pre-treatment prediction of response were determined by data mining analysis.

Materials and methods

Patients

This was a multicentre retrospective study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 496 chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. Of these, 98 patients also were included in the original GWAS analysis [6]. The inclusion criteria in this study were as follows (1) infection by genotype 1b, (2) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (3) lack of other causes of liver disease, such as autoimmune hepatitis, and primary biliary cirrhosis, (4) completion of at least 24 weeks of therapy, (5) adherence of more than 80% to the planned dose of PEG-IFN and RBV for the NVR patients, (6) availability of DNA for the analysis of the genetic polymorphism of *IL28B*, and (7) availability of serum for the determination of mutations in the ISDR and substitutions of Core70 and Core91 of HCV. Patients received PEG-IFN alpha-2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily) which is the recommended dosage in Japan. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee. The baseline characteristics are listed in Table 1. For the data mining analysis, 67% of the patients (331 patients) were assigned randomly to the model building group and 33% (165 patients) to the validation group. There were no significant differences in the clinical backgrounds between these two groups.

Laboratory and histological tests

Blood samples were obtained before therapy and were analyzed for hematologic tests and for blood chemistry and HCV-RNA. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction as reported previously [4,11]. Genetic polymorphism in one tagging SNP located near the *IL28B* gene (rs8099917) was determined by the GWAS or DigiTag2 assay [27]. Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (TT) was

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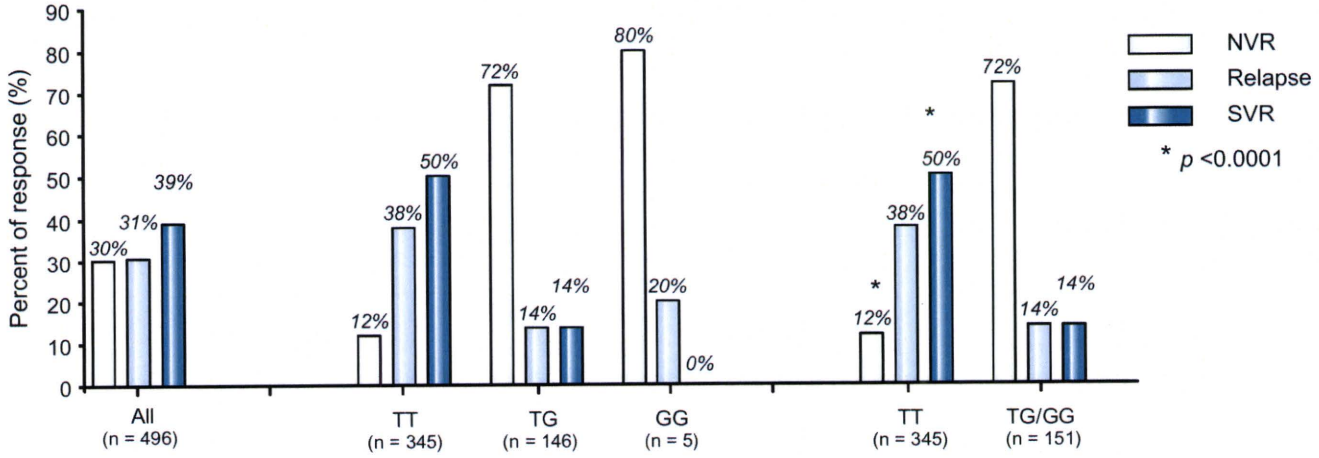

Fig. 1. Association between the IL28B genotype (rs8099917) and treatment response. The rates of response to treatment are shown for each rs8099917 genotype. The rate of null virological response (NVR), relapse, and sustained virological response (SVR) is shown. The *p* values are from Fisher's exact test. The rate of NVR was significantly higher ($p < 0.0001$) and the rate of SVR was significantly lower ($p < 0.0001$) in patients with the *IL28B* minor allele compared to those with the major allele. [This figure appears in colour on the web.]

defined as having the *IL28B* major allele. In this study, NVR was defined as a less than 2 log reduction of HCV-RNA at week 12 and detectable HCV-RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic systems, CA) at week 24 during therapy. RVR (rapid virological response) and complete early virological response (cEVR) were defined as undetectable HCV-RNA at 4 weeks and 12 weeks during therapy and SVR was defined as undetectable HCV-RNA 24 weeks after the completion of therapy. Relapse was defined as reappearance of HCV-RNA after the completion of therapy. The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Percentage of steatosis was quantified in 111 patients by determining the average proportion of hepatocytes affected by steatosis.

Statistical analysis

Associations between pre-treatment variables and treatment response were analyzed by univariate and multivariate logistic regression analysis. Associations between the *IL28B* polymorphism and sequences of HCV were analyzed by Fisher's exact test. SPSS software v.15.0 (SPSS Inc., Chicago, IL) was used for these analyses. For the data mining analysis, IBM-SPSS Modeler version 13.0 (IBM-SPSS Inc., Chicago, IL) software was utilized as reported previously [26]. The patients used for model building were divided into two groups at each step of the analysis based on split variables. Each value of each variable was considered as a potential split. The optimum variables and cut-off values were determined by a statistical search algorithm to generate the most significant division into two prognostic subgroups that were as homogeneous as possible for the probability of SVR. Thereafter, each subgroup was evaluated again and divided further into subgroups. This procedure was repeated until no additional significant variable was detected or the sample size was below 15. To avoid over-fitting, 10-fold cross validation was used in the tree building process. The reproducibility of the resulting model was tested with the data from the validation patients.

Results

Association between the IL28B (rs8099917) genotype and the PEG-IFN/RBV response

The rs8099917 allele frequency was 70% for TT ($n = 345$), 29% for TG ($n = 146$), and 1% for GG ($n = 5$). We defined the *IL28B* major allele as homozygous for the major sequence (TT) and the *IL28B* minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence. The rate of NVR was significantly higher (72% vs. 12%, $p < 0.0001$) and the rate of SVR was significantly lower (14% vs. 50%, $p < 0.0001$) in patients with the *IL28B* minor allele compared to those with the major allele (Fig. 1).

Effect of the IL28B polymorphism, substitutions in the ISDR, Core70, and Core91 of HCV on time-dependent clearance of HCV

Patients were stratified according to their IL28B allele type, the number of mutations in the ISDR, the amino acid substitutions in Core70 and Core91, and the rate of undetectable HCV-RNA at 4, 8, 12, 24, and 48 weeks after the start of therapy was analyzed (Fig. 2A–D). The rate of undetectable HCV-RNA was significantly higher in patients with the IL28B major allele than the minor allele, in patients with two or more mutations in the ISDR compared to none or only one mutation, in patients with arginine (Arg) at Core70 rather than Gln/His, and in patients with leucine (Leu) at Core91 rather than Met. The difference was most significant when stratified by the *IL28B* allele type. The rate of RVR and cEVR was significantly more frequent in patients with the *IL28B* major allele compared with those with the *IL28B* minor allele: 9% vs. 3% for RVR ($p < 0.005$) and 57% vs. 11% for cEVR ($p < 0.0001$). These findings suggest that IL28B has the greatest impact on early virological response to therapy.

Association between substitutions in the ISDR and relapse after the completion of therapy

Patients were stratified according to the *IL28B* allele, number of mutations in the ISDR, and amino acid substitutions of Core70 and Core91, and the rate of relapse was analyzed (Fig. 3A and B). Among patients who achieved cEVR, the rate of relapse was significantly lower in patients with two or more mutations in the ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.005$) (Fig. 3 B). On the other hand, the relapse rate was not different between the *IL28B* major and minor alleles within patients who achieved RVR (3% vs. 0%) or cEVR (28% vs. 29%) (Fig. 3A). Amino acid substitutions of Core70 and Core91 were not associated with the rate of relapse (data not shown).

Factors associated with response by multivariate logistic regression analysis

By univariate analysis, the minor allele of *IL28B* ($p < 0.0001$), one or no mutations in the ISDR ($p = 0.03$), high serum level of

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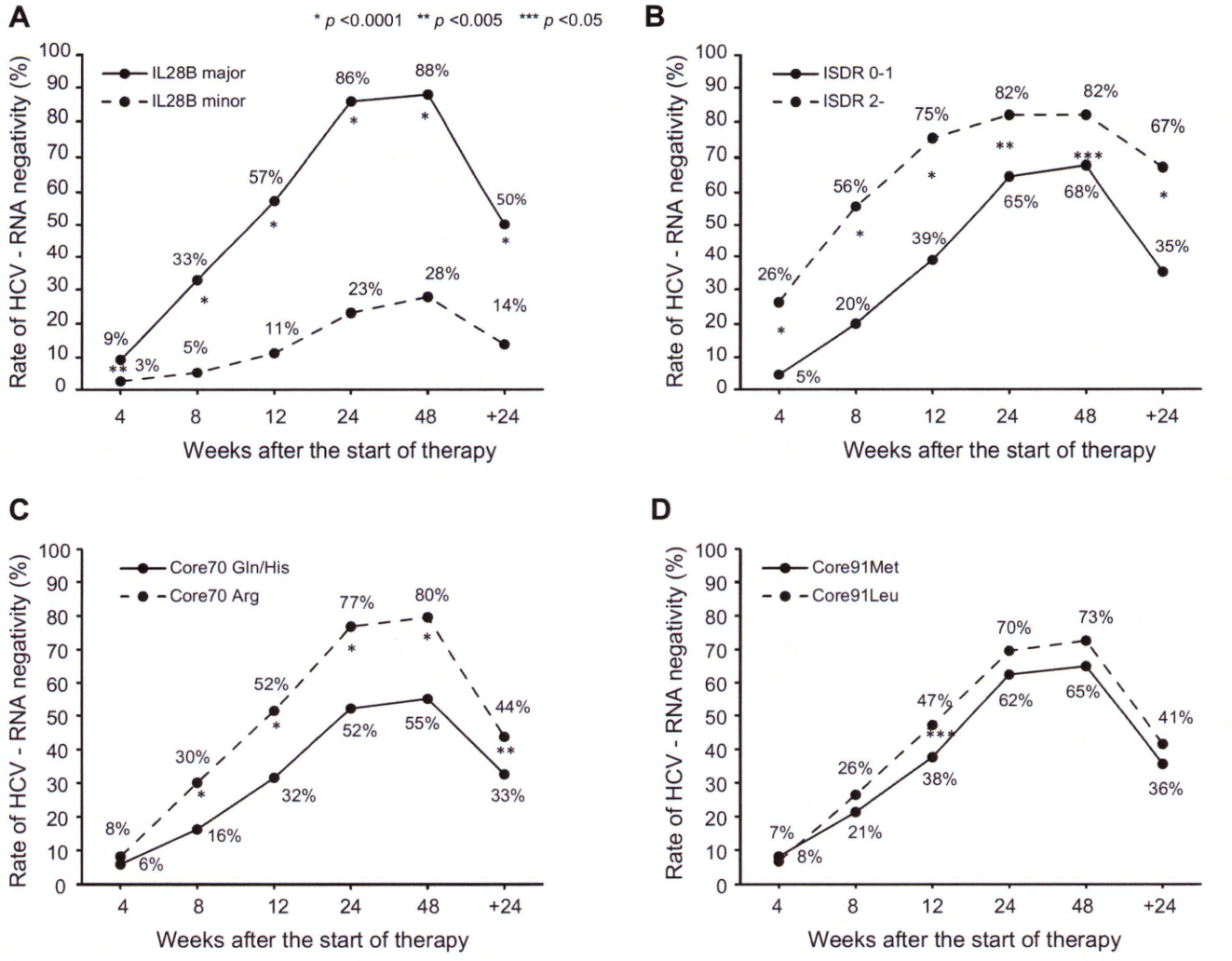

Fig. 2. Effect of *IL28B* mutations in the ISDR, Core70 and Core91 of HCV on time-dependent clearance of HCV. The rate of undetectable HCV-RNA was plotted for serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks) and for 24 weeks after the completion of therapy. Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele), (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations), amino acid substitutions of (C) Core70 (Gln/His vs. Arg), and (D) Core91 (Met vs. Leu). The *p* values are from Fisher's exact test.

HCV-RNA ($p = 0.035$), Gln or His at Core70 ($p < 0.0001$), low platelet counts ($p = 0.009$), and advanced fibrosis ($p = 0.0002$) were associated with NVR. By multivariate analysis, the minor allele of *IL28B* (OR = 20.83, 95%CI = 11.63–37.04, $p < 0.0001$) was associated with NVR independent of other covariates (Table 2). Notably, mutations in the ISDR ($p = 0.707$) and at amino acid Core70 ($p = 0.207$) were not significant in multivariate analysis due to the positive correlation with the *IL28B* polymorphism ($p = 0.004$ for ISDR and $p < 0.0001$ for Core70, Fig. 4).

Genetic polymorphism of *IL28B* also was associated with SVR (OR = 7.41, 95% CI = 4.05–13.57, $p < 0.0001$) independent of other covariates, such as platelet counts, fibrosis, and serum levels of HCV-RNA. Mutation in the ISDR was an independent predictor of SVR (OR = 2.11, 95% CI = 1.06–4.18, $p = 0.033$) but the amino acid at Core70 was not (Table 3).

Factors associated with the IL28B polymorphism

Patients with the *IL28B* minor allele had significantly higher serum level of gamma-glutamyltransferase (GGT) and a higher frequency of hepatic steatosis (Table 4). When the association between the *IL28B* polymorphism and HCV sequences was analyzed, Gln or His at Core70, that is linked to resistance to PEG-IFN and RBV therapy [4,14,15], was significantly more frequent in patients with the minor *IL28B* allele than in those with the major allele (67% vs. 30%, $p < 0.0001$) (Fig. 4). Other HCV sequences with an IFN resistant phenotype also were more prevalent in patients with the minor *IL28B* allele than those with the major allele: Met at Core91 (46% vs. 37%, $p = 0.047$) and one or no mutations in the ISDR (94% vs. 85%, $p = 0.004$) (Fig. 4).

Data mining analysis

Data mining analysis was performed to build a model for the prediction of SVR and the result is shown in Fig. 5. The analysis selected four predictive variables, resulting in six subgroups of patients. Genetic polymorphism of *IL28B* was selected as the best predictor of SVR. Patients with the minor *IL28B* allele had a lower probability of SVR and a higher probability of NVR than those with the major *IL28B* allele (SVR: 14% vs. 50%, NVR: 72% vs.

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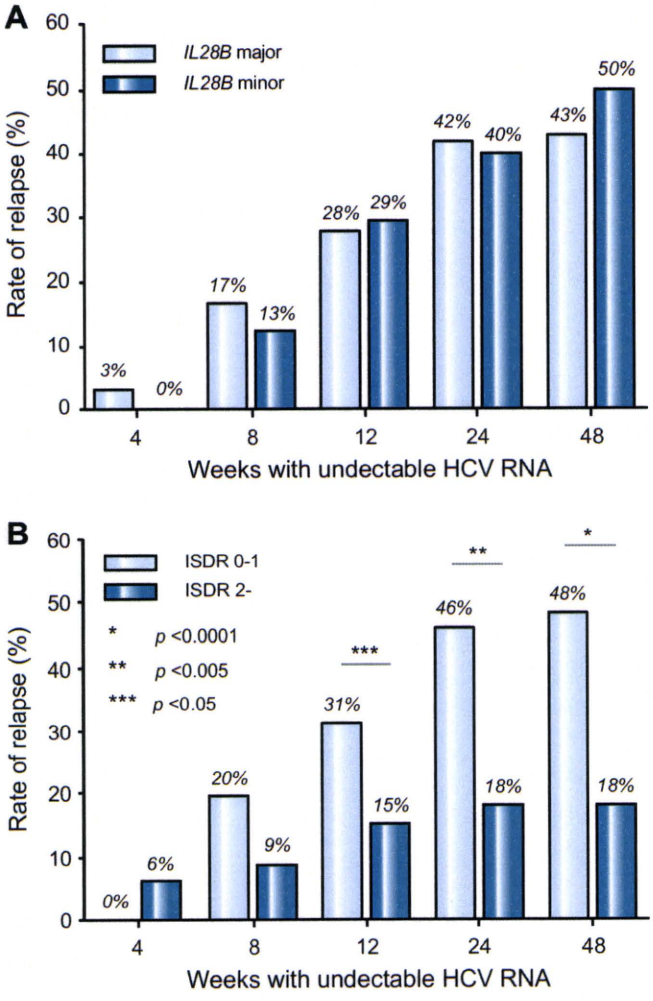

Fig. 3. Association between relapse and the *IL28B* allele or mutations in the ISDR. The rate of relapse was calculated for patients who had undetectable HCV-RNA at serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks). Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele) and (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations). The *p* values are from Fisher's exact test. [This figure appears in colour on the web.]

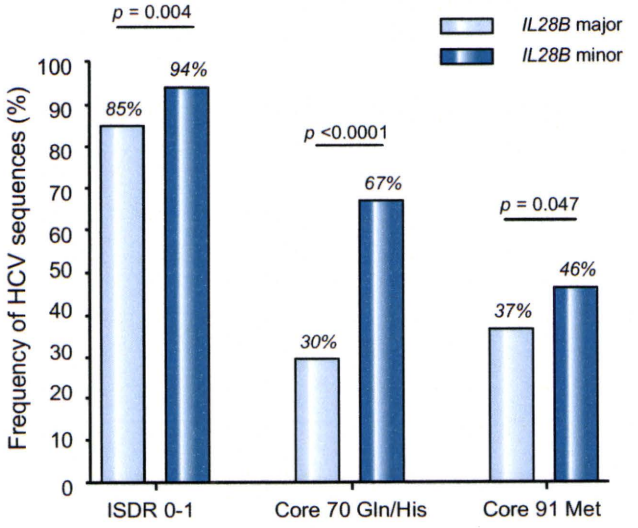

Fig. 4. Associations between the *IL28B* allele and HCV sequences. The prevalence of HCV sequences predicting a resistant phenotype to IFN was higher in patients with the minor *IL28B* allele than those with major allele. (A) 0 or 1 mutation in the ISDR of NS5A, (B) Gln or His at Core70, and (C) Met at Core91. *p* values are from Fisher's exact test. [This figure appears in colour on the web.]

12%). After stratification by the *IL28B* allele, patients with low platelet counts ($<140 \times 10^9$/L) had a lower probability of SVR and higher probability of NVR than those with high platelet counts ($\geqslant 140 \times 10^9$/L): for the minor *IL28B* allele, SVR was 7% vs. 19%, and NVR was 84% vs. 62%, and for the major *IL28B* allele, SVR was 32% vs. 66% and NVR was 16% vs. 8%. Among patients with the major *IL28B* allele and low platelet counts, those with two or more mutations in the ISDR had a higher probability of SVR and lower probability of relapse than those with one or no mutations in the ISDR (SVR: 75% vs. 27%, and relapse: 8% vs. 57%). Among patients with the major *IL28B* allele and high platelet counts, those with a low HCV-RNA titer (<600,000 IU/ml) had a higher probability of SVR and lower probability of NVR and relapse than those with a high HCV-RNA titer (SVR: 90% vs. 61%, NVR: 0% vs. 10%, and relapse: 10% vs. 29%). The sensitivity and specificity of the decision tree were 78% and 70%, respectively. The area under the receiver operating characteristic (ROC) curve of the model was 0.782 (data not shown). The proportion of patients with advanced fibrosis (F3-4) was 39% (84/217) in patients with low platelet counts ($<140 \times 10^9$/L) compared to 13% (37/279) in those with high platelet counts ($\geqslant 140 \times 10^9$/L).

Validation of the data mining analysis

The results of the data mining analysis were validated with 165 patients who differed from those used for model building. Each patient was allocated to one of the six subgroups for the validation using the flow-chart form of the decision tree. The rate of SVR and NVR in each subgroup was calculated. The rates of SVR and NVR for each subgroup of patients were closely correlated between the model building and the validation patients ($r^2 = 0.99$ and 0.98) (Fig. 6).

Discussion

The rate of NVR after 48 weeks of PEG-IFN/RBV therapy among patients infected with HCV of genotype 1 is around 20–30%. Previously, there have been no reliable baseline predictors of NVR or SVR. Because more potent therapies, such as protease and polymerase inhibitor of HCV [28,29] and nitazoxanide [30], are in clinical trials and may become available in the near future, a pre-treatment prediction of the likelihood of response may be helpful for patients and physicians, to support clinical decisions about whether to begin the current standard of care or whether to wait for emerging therapies. This study revealed that the *IL28B* polymorphism was the overwhelming predictor of NVR and is independent of host factors and viral sequences reported previously. The *IL28B* encodes a protein also known as IFN-lambda 3, which is thought to suppress the replication of various viruses including HCV [31,32]. The results of the current study and the findings of the GWAS studies [6–9] may provide the rationale for developing diagnostic testing or an IFN-lambda based therapy for chronic hepatitis C in the future.

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