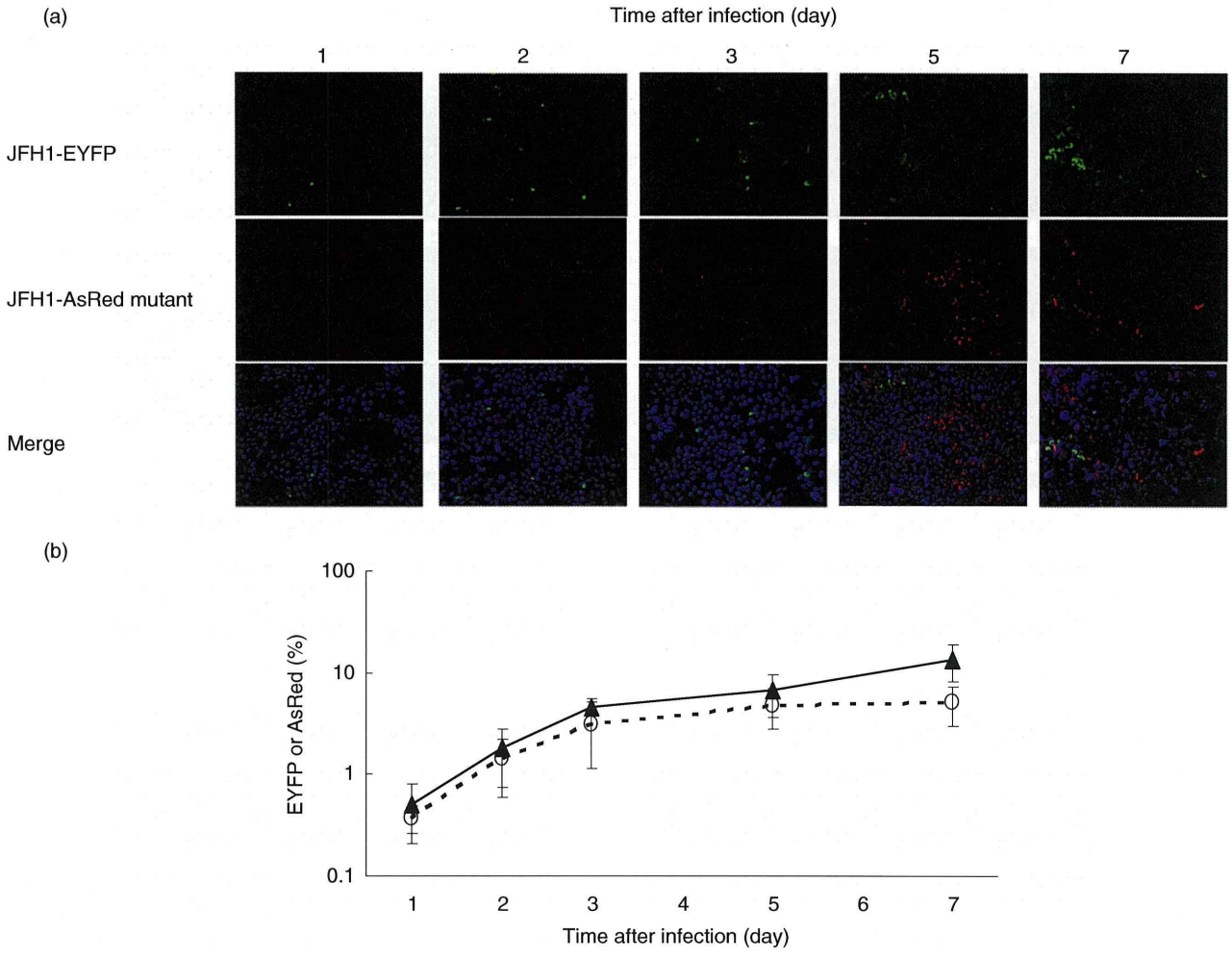


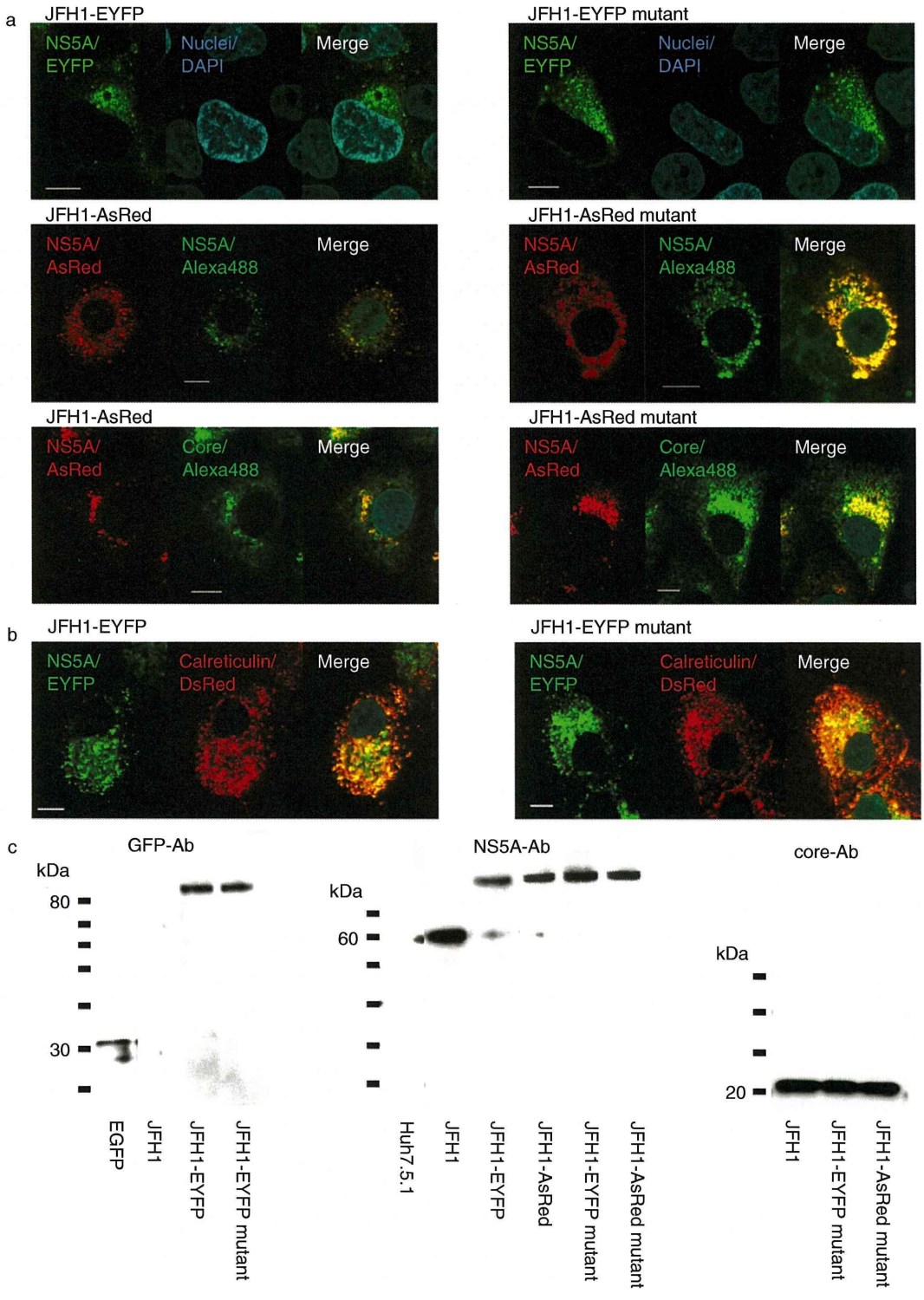
Figure 3 Intracellular *trans*-complementation of virus proteins. (a) Culture media from JFH1-EYFP and JFH1-AsRed mutant-transfected cells at 3 days post-transfection were added onto uninfected Huh7.5.1 cells. At the days indicated, EYFP or AsRed-directed-fluorescence was visualized directly. (b) The ratio of EYFP or AsRed-positive cells in Fig. 2a is calculated and plotted vs time. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —▲— JFH1-AsRed mutant; ···○··· JFH1-EYFP.

RESULTS

Infectious HCV reporter virus with robust virus production capability

FIRST, WE STUDIED whether the JFH1-EYFP mutant viruses are able to secrete sufficient amounts of infectious virus particles. Full-length HCV RNA was transcribed *in vitro* and transfected into Huh7.5.1 cells. Culture media were collected from cells transfected with JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, or JFH1-AsRed mutant, respectively, and inoculated into uninfected Huh7.5.1 cells. EYFP- or AsRed-positive cells were

Figure 4 Localization and expression of NS5A-EYFP and NS5A-AsRed fusion proteins. (a,b) Huh7.5.1 cells transfected with JFH1-EYFP, AsRed and their mutant RNA genomes were fixed at 3 days post-transfection. NS5A-EYFP and NS5A-AsRed fusion proteins were visualized using EYFP and AsRed, respectively. DsRed auto-fluorescence was observed directly. NS5A and core proteins were immunostained with Alexa Fluor 488-labeled goat antimouse immunoglobulin G (green). 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) staining revealed the nuclear chromatin. Bars represent 10 μm. (c) Cells were transfected with EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant or JFH1-AsRed mutant. Cells were harvested at 3 days post-transfection, and western blotting was performed by using anti-GFP, NS5A or core antibodies.



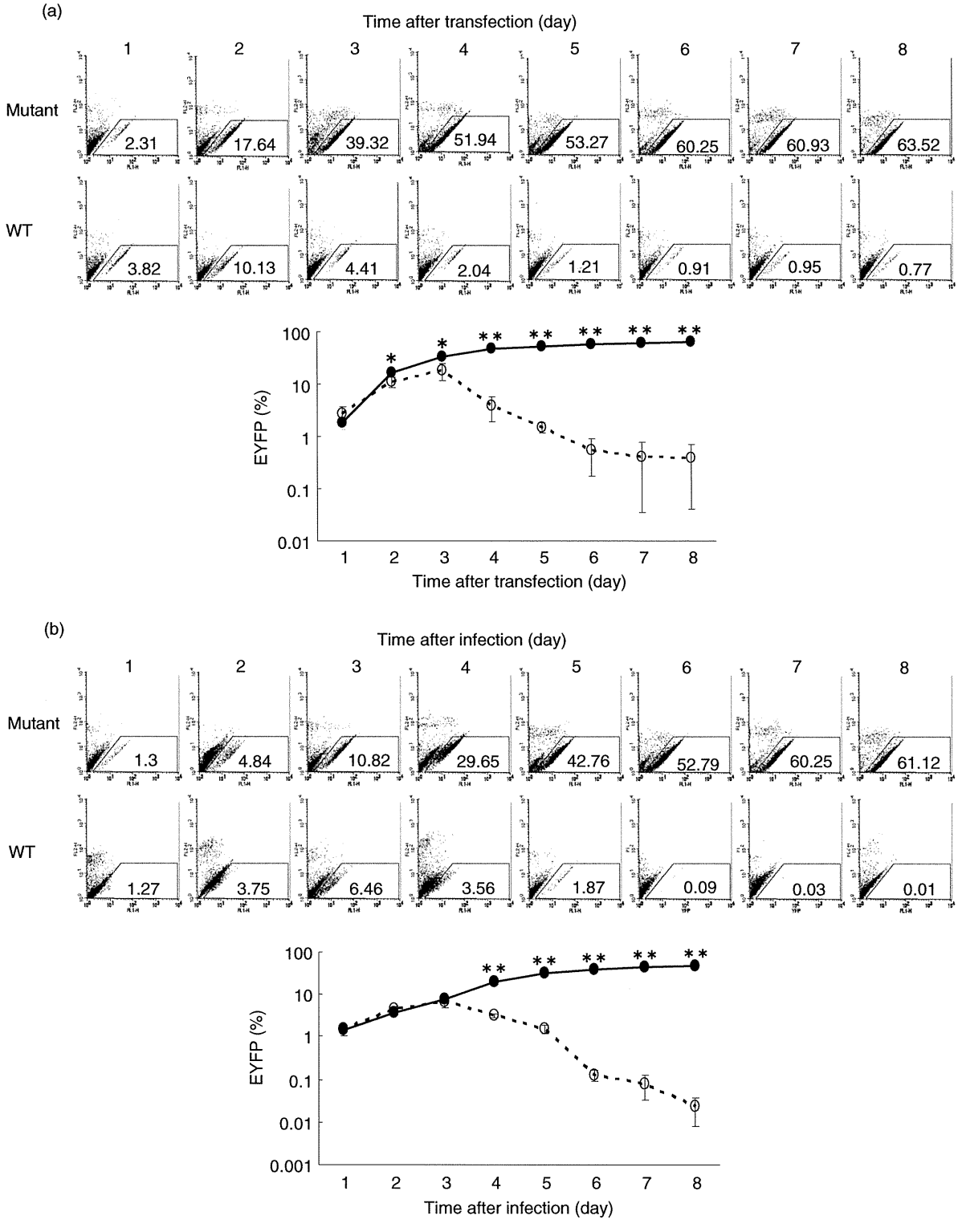
directly visualized by fluorescence microscopy on days 1–7. As shown in Figure 2(a), the number of cells positive for both JFH1-EYFP and JFH1-AsRed mutants, but not for JFH1-EYFP and JFH1-AsRed-infected cells, increased in a time-dependent manner. In JFH1-EFYP mutant-transfected cells, the proportion of EYFP-positive cells on days 3, 5 and 7 post-infection was 4.4%, 29% and 41%, respectively. In contrast, only 4.9% of JFH1-EYFP-transfected cells became EYFP-positive at 3 days post-infection, and the percentage of these fluorescence-positive cells decreased rapidly thereafter (Fig. 2b). Similarly, the percentage of cells infected with JFH1-AsRed mutant but not JFH1-AsRed increased exponentially. These results indicated that the two fluorescence virus clones with mutations are able to secrete infectious virus particles. We next compared levels of HCV core antigen in culture medium of cells infected with JFH1, JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, and JFH1-AsRed mutant viruses. The mutant viruses, but not the wild-type, produced amounts of core protein comparable to that of the parental JFH1 (Fig. 2C). In HCV-JFH1, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells, the core protein reached a peak of 1.25, 1.35 and 1.34 fmol/L, respectively, at 14 days post-transfection, while that of JFH1-EYFP JFH1-AsRed-transfected cells became undetectable at 10 days post-transfection. These results indicated that the mutant type is capable of producing an amount of viral particles comparable to that of the parental JFH1.

Using the two fluorescence-tagged viruses, we conducted co-infection of two virus strains, JFH1-AsRed mutant, which secreted infectious virus particles, and JFH1-EYFP, in which virus particle secretion was impaired. We collected culture media from cells transfected with JFH1-AsRed-mutant or JFH1-EYFP on day 2 post-transfection and infected both media onto uninfected Huh7.5.1 cells at a multiplicity of infection (moi, focus forming unit per cell) of 0.01. The number of JFH1-AsRed mutant-infected cells increased exponentially until day 3 but reached a plateau on days 5 and 7 post-infection. Interestingly, the number of cells positive for viral secretion-impaired JFH1-EYFP also increased in a manner similar to that of the JFH1-AsRed mutant (Fig. 3a). The percentage of AsRed mutant-positive cells was 4.6%, 6.7% and 14.8% at days 3, 5 and 7, respectively, while the percentage of EYFP-positive cells at the corresponding days was 3.1%, 4.8% and 5.1%, respectively (Fig. 3b). These results suggest that, in the co-culture of two HCV clones with and without virus particle secretion, a secretion-impaired virus clone is able to replicate and produce infectious particles possibly through the complementation of the intact virus.

Expression and subcellular localization of NS5A-fluorescence proteins

We next used fluorescence microscopy to study the subcellular localization of fluorescence and viral proteins. In cells transfected with JFH1-EYFP, JFH1-AsRed, and the respective mutants, EYFP and AsRed, were clearly visualized as dot-like structures in the perinuclear area (Fig. 4a). To determine if the NS5A-AsRed fusion protein indicates the subcellular localization of NS5A, we performed immunofluorescence staining of JFH-AsRed- and JFH1-AsRed mutant-infected cells using NS5A and HCV-core antibodies. Fluorescence of AsRed was co-localized precisely with NS5A and partially with core proteins. The fluorescence intensities of the JFH1-EYFP and -AsRed mutants within the cells were equal to that of the wild-type constructs. EYFP-NS5A of wild type and mutant JFH1 were localized in the ER (Fig. 4b). Western blotting was performed by using anti-GFP and anti-HCV-NS5A antibodies. As shown in Figure 4(c), three bands of the expected molecular weights of 27, 58 and 85 kDa, which corresponded to EGFP, NS5A and NS5A-EYFP fusion protein, were detected in EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells. The expression levels of core protein in JFH1-EYFP mutant- and JFH1-AsRed mutant-transfected cells were almost the same as those transfected with parental JFH1. These results indicate

Figure 5 Kinetics of hepatitis C virus (HCV)-infected cells. (a) Huh7.5.1 cells were infected with JFH1-EYFP or JFH1-EYFP mutant HCV RNA. At the days indicated, cells were harvested and subjected to flow cytometry. EYFP-positive cells were sorted based on EYFP activating (*x*-axis) and staining with a marker of dead cells (*y*-axis). The results are depicted as density plots. The ratios of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $*P < 0.05$. $**P < 0.01$. —•—, JFH1-EYFP mutant; -○-, JFH1-EYFP. (b) Culture media from JFH1-EYFP or mutant-transfected cells were added onto uninfected Huh7.5.1 cells at a moi of 0.01. At the days indicated, infected cells were analyzed using flow cytometry. The results are depicted as density plots. The ratio of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $*P < 0.05$. $**P < 0.01$. WT, wild type. —•—, JFH1-EYFP mutant; -○-, JFH1-EYFP.



that the fusion proteins of NS5A and the fluorescent proteins remain intact within cells and serve as accurate markers of infection and as indicators of the subcellular localization of HCV-NS5A proteins.

Kinetics of HCV infection

Using those fluorescence-tagged HCV constructs, we analyzed more precisely the ratio and kinesics of HCV RNA-transfected cells and virus-infected cells by flow cytometry. After HCV RNA transfection, the percentages of JFH1-EYFP and JFH1-EYFP mutant-transfected cells were almost equivalent up to 2 days. Thereafter, JFH1-EYFP-positive cells began to decrease in number, while the mutant-transfected cells increased exponentially until 5 days post-transfection and then reached a plateau, when 52.2% of the cells were EYFP-positive (Fig. 5a). We collected the media from JFH1-EYFP and mutant-transfected cells at 2 days post-transfection, and added it onto uninfected Huh7.5.1 cells at a moi of 0.01. Similar to the results of the transfection assay, the population of JFH1-EYFP mutant-infected cells increased exponentially and reached a stable state at 6 days post-infection, when 39.2% of the cells were EYFP-positive (Fig. 5b). Calculating from the above data, the rate of expansion of HCV-infected cells was $2^{1.5}$/day. The cell-to-cell expansion of the JFH1-EYFP mutant infection was blocked by prior treatment of cells with anti-CD81 antibody (data not shown). This finding indicated that the expansion of the EGFP-positive cells was due to cell–cell spread of EYFP-tagged HCV and not the division of the virus-positive cells.

To further refine the calculation of the rate of cell-to-cell spread of infection, we carried out JFH1-EYFP mutant infection of uninfected Huh7.5.1 cells seeded at various densities from 2×10^3 to 2×10^5 cells/mL (Fig. 6). Flow cytometry showed that the rates of expansion of HCV-infected cells were $2^{1.5}$, $2^{2.3}$ and $2^{2.5}$/day at 2×10^3, 2×10^4 and 2×10^5 cells/cm^2, respectively. The ability of JFH1-EYFP to spread is greater in cells seeded at higher density. The maximum rate of expansion of HCV-infected cells was calculated as $2^{2.5}$/day.

Effects of antiviral drugs on HCV-infected cells

We next investigated the effects of antiviral agents on the infection kinetics of tagged-HCV. Eighteen hours after transfection of EYFP-tagged HCV RNA, the cells were treated with 10, 30 or 50 U/mL of IFN-α-2b or with 10 μM of protease inhibitor, BILN2061. JFH1-EYFP mutant-transfected cells were analyzed using flow

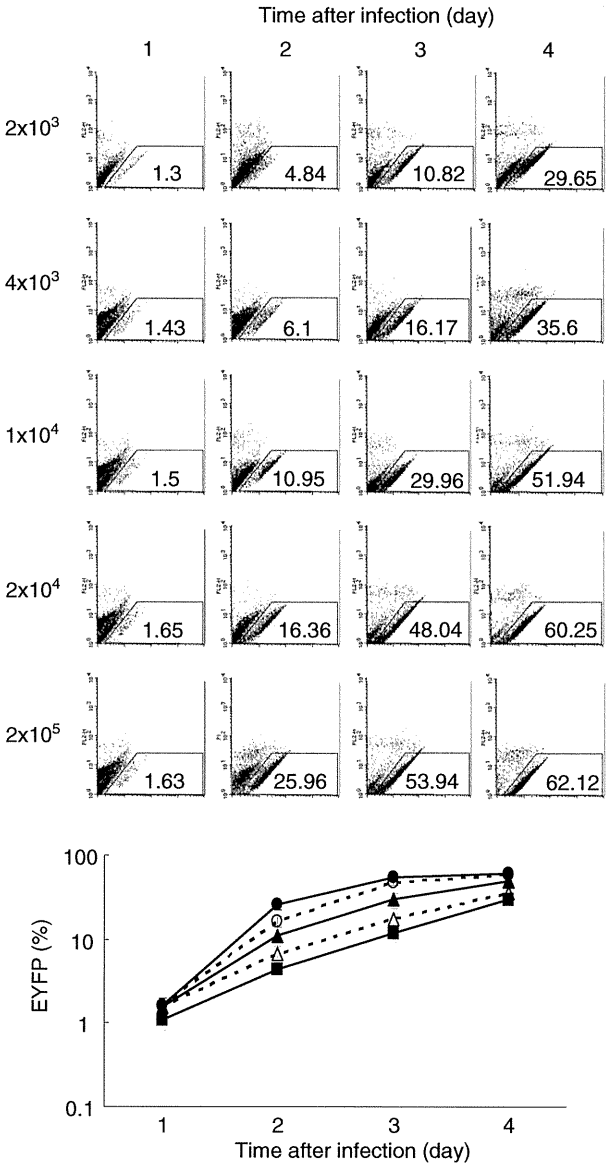


Figure 6 Rate of expansion of hepatitis C virus (HCV)-infected cells. The medium from JFH1-EYFP mutant was inoculated onto Huh7.5.1 cells seeded at different densities (2×10^3, 4×10^3, 1×10^4, 2×10^4 and 2×10^5 cells/cm^2) with core antigen adjusted doses. The results of flow cytometric analysis are depicted as density plots. The ratios of EYFP-positive cells vs time are shown beneath. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —■—, 2×10^3; - -△- -, 4×10^3; —▲—, 1×10^4; - -○- -, 2×10^4; —●—, 2×10^5.

cytometry. As shown in Figure 7, treatment of cells with the two compounds suppressed the time-dependent increase of HCV propagation. In addition, IFN-α-2b suppressed the dose-dependent increase of HCV

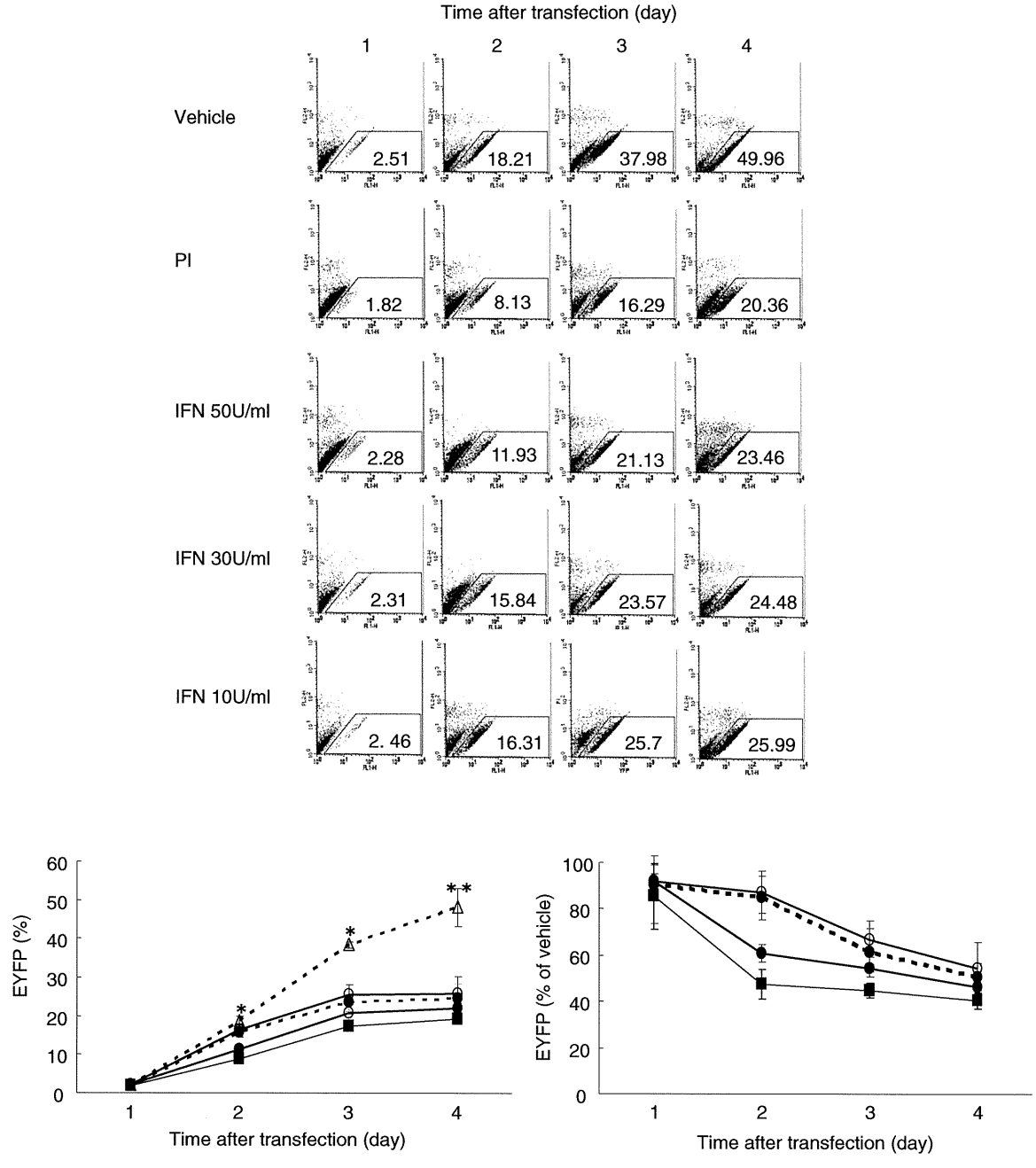


Figure 7 Effect of antiviral drugs on hepatitis C virus (HCV)-infected cells. Huh7.5.1 cells were transfected with JFH1-EYFP mutant RNA. Eighteen hours after transfection, cells were cultured with 10, 30 or 50 U/mL of interferon (IFN)-α-2b or 10 μM of the protease inhibitor BILN-2061. The cells were harvested at the days indicated and flow cytometry was performed. Ratios of EYFP-positive cells over time are shown at lower left. Plot values of 100% in each curve represent the EYFP expression levels in untreated cells (lower right). Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^{*}P < 0.05$. $^{**}P < 0.01$. PI, protease inhibitor. -☆-, vehicle; —●—, 50 U/mL; -●-, 30 U/mL; —○—, 10 U/mL; —■—, PI.

propagation. At all time points, the number of infected cells was significantly lower in the culture treated with the two compounds than in the untreated culture. The protease inhibitor suppressed infection faster than did IFN-α-2b. These data indicate that IFN and the protease inhibitor were not only able to suppress intracellular HCV replication levels but also to inhibit virus particle secretion and expansion of HCV-infected cell populations.

DISCUSSION

IN THIS STUDY, we used fluorescence-tagged HCV, in which virus assembly, particle secretion and re-infection functions are fully preserved (Fig. 1).[17] Utilizing the fluorescence-tagged HCV, we analyzed the rate of expansion of HCV infection using live-cell flow cytometric analyses (Figs 5–7). In the early periods of infection, the expansion of the virus-positive cell population increased exponentially and the maximum rate of expansion was calculated as $2^{2.5}$/day. It is not clear why HCV propagation reaches a plateau, but this observation, where HCV replication is limited in confluent cells, has been made previously.[26] Possible explanations include cell death due to over-confluence, depletion of the nucleoside triphosphate pools in resting cells, and/or cell cycle-dependent effects on virus RNA replication and translation.

Co-infection of the two virus clones, EYFP-JFH1 and AsRed mutant JFH1, showed that viruses with impaired particle secretion were able to replicate and expand virus-infected cells (Fig. 3). Although we have found no clear mechanism to explain those effects, we speculate that the secretion-defective virus (JFH1-EYFP) may assemble into infectious virus through *trans*-complementation of virus proteins via co-infection in a single cell or recombination of mutant and wild-type virus genomic RNA. The co-infection experiment showed that the increase of JFH1-AsRed mutant-positive cells was slower than in the single clone infection experiment (Fig. 2a). These findings suggest that viruses with impaired particle secretion (JFH1-EYFP) partially suppressed expansion of viruses with intact particle secretion (JFH1-AsRed mutant) through *trans*-suppression of cellular virus replication or competitive binding to cellular virus entry receptors.

After the development of HCV-JFH1 cell culture,[11] many variations of HCV cell culture systems have been developed. Lindenbach *et al.* developed a genotype 2a intragenotypic chimera, J6/JFH, in which the JFH1 structural region was replaced with that of J6, isolated from a patient with chronic hepatitis.[27] The J6/JFH chimera is able to produce virus particles more efficiently than JFH1 but does not produce virus-induced cytopathic effects (CPE). Several marker protein-tagged viruses have been reported, in which viral infection could be visualized readily in living cells. A subgenomic replicon that expressed an NS5A-GFP fusion protein was reported first.[12] However, the clone lacked the structural regions that are required for virus propagation. Subsequently, full-length HCV reporter viruses were developed in which the EGFP gene was inserted into the NS5A-C-terminus of JFH1[13] or JC-1.[14] Jones *et al.* inserted the *renilla* luciferase gene into P7 of J6/JFH.[15] Unfortunately, the efficiency of virus production by the recombinant reporter viruses was greatly reduced compared to wild-type viruses. Very recently, it has been reported that a JFH1-based adaptive strain of a HCV reporter virus can produce infectious HCV particles as robustly as the JFH1 wild-type strain.[17] This virus system has overcome the serious limitations associated with the use and application of other reporter viruses.

Compared with the other HCV reporter viruses, the JFH1-EYFP/AsRed mutant is capable of producing amounts of HCV virus equivalent to that of the parental JFH1, which enables continuous passage of infection in cell culture and analyses using various research modalities, including flow cytometry and live-cell microcopy. Considering the current situation regarding the lack of singly effective, proven antiviral agents against HCV, other than IFN formulations, the search for potential antiviral agents will continue to be a dominant goal of research to improve clinical anti-HCV chemotherapeutics. This tagged HCV culture system may provide a very convenient tool for studies of the complete virus life cycle in live cells and of virus–host interactions, and it may be useful for high-throughput screening of drugs.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

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Abstract
Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.
Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.
Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates ($P < 0.01$). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).
Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥ 2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

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ALT	Alanine transaminase
dM	Double mutant
ITT analysis	Intention-to-treat analysis
PP analysis	Per protocol analysis
SVR	Sustained virological response
ETR	End of treatment response
PKR	Double stranded RNA-dependent protein kinase
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88

Introduction

Hepatitis C virus (HCV) is one of the major pathogens causing chronic hepatitis [1, 2] and eradication of the virus by the host occurs infrequently during the natural course of infection once it becomes chronic. Interferon (IFN) has been used widely as the most effective antiviral agent for chronic hepatitis C. Although ribavirin (RBV), a synthetic guanosine analog, alone does not decrease the serum HCV RNA level [3–5], it has been shown that combination therapy with IFN-alpha (given 3 times weekly) and daily RBV gives a higher sustained response rate than IFN monotherapy [6–8]. Pegylation is the process by which an inert molecule of polyethylene glycol (PEG) is covalently attached to a protein, and the addition of PEG to IFN produces a biologically active molecule with a longer half-life and more favorable pharmacokinetics than the natural molecule. These characteristics allow more convenient, once-weekly dosing [9]. Pegylated (PEG)-IFN plus RBV is significantly more effective than IFN plus RBV or PEG-IFN alone for the treatment of chronic hepatitis C, with sustained virological response rates of $\sim$50% in patients infected with HCV genotype 1b [10].

We reported previously a close correlation between the number of mutations in the nonstructural 5A (NS5A) region of the HCV genome encoding amino acids (aa) at positions 2209–2248 [the IFN sensitivity determining region (ISDR)] and IFN efficacy in patients with HCV genotype 1b infection [11–13]. The aims of this study were to analyze clinical and virological factors associated with a higher rate of response by patients with HCV genotype 1b infection who were treated with combination therapy with pegylated-IFN-alpha 2b (PEG-IFN) plus RBV, and to clarify the relationship between ISDR mutations and virological response to the combination therapy.

Methods

Patients and methods

We analyzed, retrospectively, 239 patients with chronic HCV-1b infection who received combination therapy with PEG-IFN plus RBV between December 2004 and April 2008 at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated hospitals participating in the Ochanomizu-Liver Conference Study Group. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by reverse transcription polymerase chain reaction (RT-PCR). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age; gender; body mass index (BMI); previous IFN therapy; grade of inflammation and stage of fibrosis on liver biopsy; pretreatment biochemical parameters, such as hemoglobin, alanine transaminase (ALT) level, platelet count, low density lipoprotein (LDL) cholesterol, serum HCV RNA level (Log IU/ml); and the amino acid sequence of the IFN sensitivity determining region (aa 2209–2248, ISDR). Liver biopsy specimens were evaluated according to the grade of inflammation and the stage of fibrosis; this was done blindly by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of our hospital, and informed written consent was obtained from each patient.

Nucleotide sequencing of the *NS5A* gene

The serum samples were frozen at −80°C until use. Extraction of RNA from serum and RT-PCR were performed as described previously [14]. The PCR and sequencing primers were synthesized with a DNA synthesizer (model 391; Applied Biosystems Japan, Chiba, Japan).

To determine the nucleotide sequence of the *NS5A 2209–2248* region, we amplified nucleotides (nt) 7296–7320 of HCV complementary DNA by using the outer pair of primers [5′ outer primer, 5′-TGG ATG GAG TGC GGT TGC ACA GGT A-3′ (nt 6703–6727 of HC-J4); 3′ outer primer, 5′-TCT TTC TCC GTG GAG GTG GTA TTG C-3′ (nt 7296–7320)]. We transferred 1 μl of the first PCR product to the second PCR reaction along with the nested 5′ and 3′ primers [5′ inner primer, 5′-TGT AAA ACG ACG GCC AGT CAG GTA CGC TCC GGC GTG CA-3′ (nt 6722–6741), with the M13 forward primer sequence underlined; and 3′ inner primer, 5′-CAG GAA ACA GCT ATG ACC GGG GCC TTG GTA GGT GGC AA-3′ (nt 7275–7294), with the M13 reverse primer sequence underlined]. An M13 forward primer and an M13 reverse primer were attached to the 5′ terminal of the 5′ and 3′ inner primers, respectively, to facilitate direct sequencing with an automated DNA sequencer (model 373S; Applied Biosystems Japan).

Both strands of the PCR products were sequenced with the PRISM dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions. The sequencing primer was the M13 forward primer for the sense strand and the M13 reverse primer for the antisense strand. Deduced aa sequences of *NS5A* 2209–2248 were compared with the *NS5A* 2209–2248 sequences of HCV-J [15], which are prototypic sequences of HCV-1b. The results of the sequencing analysis were confirmed as consistent for each sample by repeating the experiment twice with different PCR products, to rule out the possibility of selection and amplification of minor NS5A quasi species variants in the low-titer specimens.

Nucleotide sequencing of the core gene

Substitutions of amino acids 70 and 91 in HCV-core region were determined according to core sequences obtained as described previously [16, 17]. The pattern of glutamine/histidine (mutant) at aa 70 and methionine (mutant) at aa 91 was evaluated as the double-mutant (dM) type, while the other patterns were non-double-mutant (non dM) type. Two patterns of mutants and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type (dW), while the other patterns were considered non-double-wild-type (non dW).

Study design and treatment regimens

Patients were treated with combination therapy with PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg subcutaneously and RBV (Rebetol; Schering-Plough Nordic Biotech) (body weight [b.w.] < 60 kg, 600 mg po daily; b.w. 60–80 kg, 800 mg po daily; b.w. > 80 kg, 1000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 48 weeks. Treatment reduction was permitted, to escape side effects, but extended treatment of 72 weeks is not included in this analysis. Achieved rates of PEG-IFN and RBV administration were calculated as the percentage of the actual total dose administered of a standard total dose of 48 weeks according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was done by a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4-weekly intervals, and after therapy at 4-weekly intervals for 24 weeks, by a quantitative PCR assay with a sensitivity of 100 copies/ml (National Genetics Institute, Los Angeles, CA, USA).

Outcomes

The primary end point was a sustained biochemical and virological response. Sustained virological response (SVR) was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were end-of-treatment virological responses (HCV RNA undetectable in serum). In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response were explored.

Statistical analysis

SPSS software package (SPSS 12J for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis, which was carried out using the χ^2 or Fisher's exact probability test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P* values were two-tailed and those lessthan 0.05 were considered statistically significant.

Results

Clinical characteristics and response to therapy

The clinical characteristics of the 239 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, serum HCV RNA levels were undetectable by the end of treatment in 172 of the 239 patients (72%) who were treated with PEG-IFN plus RBV, and among them, 98 of the 239 patients (41%) had an SVR (Table 2). The SVR rate decreased with drug discontinuation and dose

Table 1 Baseline characteristics of participating patients infected with HCV genotype 1b

Total number	239
Age (years)[a]	57 (21–78)
Gender (male/female)	142/97
Body mass index (kg/m^2)[a]	23.3 (15.3–31.0)
Previous interferon therapy (no/yes)	167/72
Histology at biopsy	
Grade of inflammation	
A0/1/2/3	3/65/102/10
Stage of fibrosis	
F0/1/2/3/4	4/73/57/37/9
Hemoglobin (g/dl)[b]	14.3 ± 1.3
ALT (IU/L)[b]	86 ± 67
Platelet count ($\times 10^3/\mu l$)[b]	160 ± 58
LDL cholesterol (mg/dl)[b]	74 ± 19
Serum HCV-RNA level (Log(IU/ml))[b, c]	6.1 ± 0.6
Type of mutations in the core (dM/non dM)	30/166
Type of mutations in the core (dW/non dW)	65/131
Type of ISDR sequence (0/1/2/3/4 or more)	126/45/11/5/18

HCV hepatitis C virus, *LDL* low density lipoprotein, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209–2248}$, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 2 Sustained response rates to treatment according to drug adherence

Characteristic	Number/total number (%)
Overall	
End of treatment	172/239 (72)
End of follow up	98/239 (41)
PEG-interferon-α2b adherence	
End of treatment	
>80%	131/154 (85)
60–80%	19/27 (70)
<60%	22/58 (38)
End of follow up	
>80%	86/154 (56)
60–80%	7/27 (26)
<60%	5/58 (9)
Ribavirin adherence	
End of treatment	
>80%	113/134 (84)
60–80%	37/46 (80)
<60%	22/59 (37)
End of follow up	
>80%	74/133 (56)
60–80%	18/47 (38)
<60%	6/59 (10)

PEG pegylated

reduction. The SVR rates of patients who received a total cumulative treatment dose of PEG-IFN of more than 80% were almost twice as high as the rates of patients who received less than 80% (56%, 26%, and 9% with >80%, 60%–80% and <60% of the PEG-IFN dose, $P < 0.001$). The SVR rates did not decrease with RBV reduction, as long as the cumulative treatment dose of RBV was more than 60%, but when the RBV reduction fell below 60%, the SVR rates were significantly lower (56%, 38%, and 10% with >80%, 60%–80%, and <60% of the RBV dose, $P < 0.001$).

Factors associated with sustained virological response

Seven parameters that influenced the SVR rate were identified by univariate analysis, including stage of fibrosis at liver biopsy, hemoglobin, platelet count, serum HCV RNA level, the type of ISDR sequence, and adherence to PEG-IFN plus RBV (Table 3). On the other hand, the SVR rate was not related to gender ($P = 0.07$), age or BMI. The amino acid substitution pattern was not significant in the overall analysis, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve SVR. As shown in Table 4, gender differences existed in the mutations in ISDR and core regions based on therapeutic responses. Because there were rather fewer female than male patients, the type of ISDR sequence did not significantly influence the SVR in females. We also analyzed types of mutations in the core, and the amino acid substitution pattern was not significant in the male patients, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve an SVR, as mentioned above. We also compared results between treatment-naïve patients and those who had failed previous IFN therapy (Table 5). As there were some differences in stage of fibrosis, platelet count, grade of inflammation, and gender in univariate analysis, treatment was comparably effective in both groups.

Finally we performed multivariate analysis in subjects with good drug adherence (Table 6), which identified only one parameter that influenced the SVR rate independently by variable selection: the number of mutations in the ISDR sequence (two or more: odds ratio [OR] = 5.181, $P < 0.05$). This regression model was always obtained regardless of the variable selection method used, including conditional parameter estimation, Wald statistic, and

Table 3 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
Age (years)[a]	56 (27–69)	58 (23–72)	NS
Gender (male/female)	65/33	77/64	0.070
Previous interferon therapy (no/yes)	68/30	99/42	NS
Grade of inflammation (A0–1/2–3)	31/50	37/62	NS
Stage of fibrosis (F0–2/3–4)	68/13	67/33	*0.009*
Body mass index (kg/m^2)[a]	23.3 (15.5-28.1)	23.3 (15.3-31.0)	NS
Pretreatment Hemoglobin (g/dl)[b]	14.6 ± 1.1	14.0 ± 1.4	*<0.001*
Pretreatment ALT (IU/ml)[b]	87 ± 68	86 ± 67	NS
Pretreatment platelet count ($\times 10^3/\mu l$)[b]	178 ± 63	148 ± 51	*<0.001*
Pretreatment LDL cholesterol (mg/dl)[b]	78 ± 21	72 ± 18	NS
Pretreatment serum HCV-RNA level (Log(IU/ml))[b, c]	5.9 ± 0.7	6.2 ± 0.4	*<0.001*
No. of mutations in the ISDR (0–1/2 or more)	66/23	105/11	*0.002*
Type of mutations in the core (dM/non dM)	9/76	21/90	NS
Type of mutations in the core (dW/non dW)	31/54	34/77	NS
PEG-interferon adherence (>80/60–80/<60%)	85/7/6	68/20/53	*<0.001*
Ribavirin adherence (>80/60–80/<60%)	72/19/7	60/28/53	*<0.001*

IFN interferon, *RBV* ribavirin, *SVR* sustained virological response, *NS* not significant, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209–2248}$, *core* substitution of amino acids 70 and 91, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 4 Mutations in the ISDR and core regions analyzed separately for gender based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
No. of mutations in the ISDR (0–1/2 or more)			
Male	36/21	56/8	*0.002*
Female	30/2	49/3	NS
Type of mutations in the core (dM/non dM)			
Male	8/46	11/48	NS
Female	1/30	10/42	*0.026*
Type of mutations in the core (dW/non dW)			
Male	18/36	16/43	NS
Female	13/18	18/34	NS

likelihood ratio statistic in combination with forward or backward variable selection methods.

Comparison of SVR rates according to the number of mutations in the ISDR sequence

We analyzed first the percentage of patients with more than two mutations in the ISDR among 762 patients who received IFN therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. The percentage of patients with more than two mutations in the ISDR was between about 20% and 30% for all ages (Fig. 1a).

Secondly, we analyzed responses to PEG-IFN plus RBV treatment and serum levels of HCV RNA in relation to the number of mutations in the ISDR. In Fig. 1b, patients with SVR are indicated by open circles and those with non-SVR, by closed circles. Although the rate of SVR tended to be higher in patients with increasing numbers of mutations in the ISDR, 5 patients with more than two mutations in the ISDR who experienced drug discontinuation and dose reduction resulted in non-SVR.

We confirmed changes over time in VR rates in patients treated with PEG-IFN plus RBV (Fig. 1c). Patients with more than two mutations in the ISDR are indicated in the figure by open circles and those with none or one mutation in the ISDR, by closed circles. The VR rates tended to be high early in the treatment in patients with more than two mutations in the ISDR.

Finally we compared the PEG-IFN plus RBV treatment efficacy in two groups, divided based on ISDR mutations. Patients with more than two mutations in the ISDR had a significantly higher tendency to achieve SVR in both ITT and per-protocol (PP) analyses ($P < 0.01$) (Fig. 1d), and

Table 5 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on previous interferon therapy

Previous interferon therapy	No (n = 167)	Yes (n = 72)	P value
Sustained response rates	68/167 (41)	30/72 (42)	NS
Age (<65/≥65)	127/40	57/15	NS
Gender (male/female)	93/74	49/23	0.074
Grade of inflammation (A0–1/2–3)	55/72	13/40	*0.018*
Stage of fibrosis (F0–2/3–4)	103/24	32/21	*0.003*
Pretreatment hemoglobin (<14.5/≥14.5)	93/74	41/31	NS
Pretreatment platelet count (<160/≥160 $\times 10^3$)	84/83	50/22	*0.006*
Pretreatment Serum HCV RNA level[a] (<6/≥6)	54/112	25/46	NS
No. of mutations in the ISDR (0–1/2 or more)	116/22	55/12	NS
PEG-interferon adherence (>80/60–80/<60%)	110/18/39	43/9/20	NS
Ribavirin adherence (>80/60–80/<60%)	97/30/40	35/17/20	NS

[a] Data are shown as Log(IU/ml)

Table 6 Multivariate analysis for the clinical and virological factors related to sustained response to PEG-IFN plus RBV therapy in 104 patients who were not intolerant to PEG-IFN plus RBV therapy

Factor	Category	Odds ratio (95% CI)	P value
(a) Five-factor model			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	4.486 (0.922–21.74)	0.063
Pretreatment Hemoglobin (g/dl)		1.250 (0.853–1.833)	NS
Pretreatment Serum HCV RNA level[a]		0.510 (0.224–1.159)	NS
Stage of fibrosis	F 0/1/2	1	
	F 3/4	0.460 (0.153–1.382)	NS
Pretreatment Platelet count ($\times 10^3/\mu l$)		1.022 (0.949–1.101)	
(b) Step-wise variable selection			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	5.181 (1.129–23.81)	*0.034*

CI confidence interval, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209–2248}$

[a] Data are shown as Log(IU/ml)

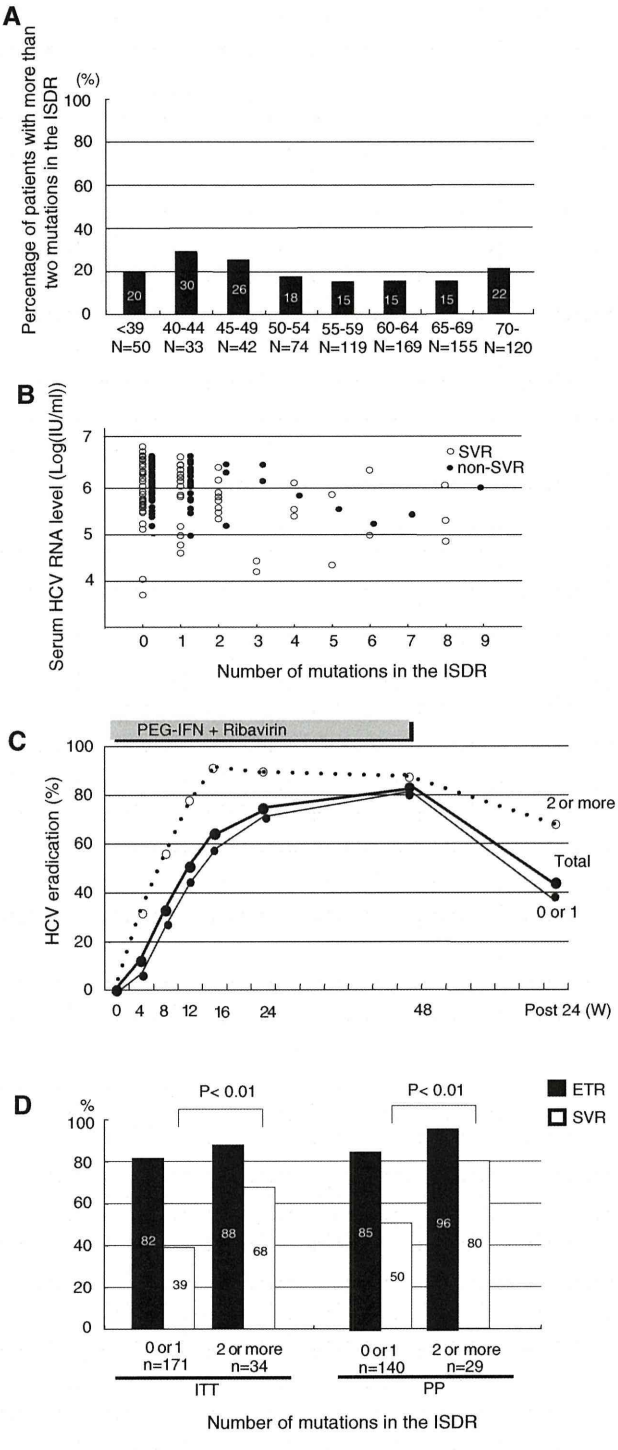
the SVR rates of the patients with good drug adherence was 80%.

Side effects

Side effects leading to treatment discontinuation occurred in 53 patients (22%). Overall, 109 patients (46%) required reduction of the dose of one or both drugs during the treatment regimens (23% required PEG-IFN reduction and 35% required RBV reduction). The most common events leading to drug withdrawal were general fatigue and appetite loss (n = 15), hematologic abnormalities (n = 6), dermatological symptoms (n = 5), retinopathy (n = 5), neuro-psychiatric events (n = 4), and interstitial pneumonia, including severe cough (n = 4).

Discussion

Although the relationship between ISDR mutations and the clinical efficacy of IFN has been conflicting in Western countries [18–24], our results support previous studies reporting a close correlation between the number of mutations in the ISDR and IFN efficacy in patients with chronic HCV-1b infection [11–13]. Because most patients with 4 or more mutations in the ISDR (hereafter classified as the mutant type) experienced SVR with conventional IFN monotherapy, we reported previously that the number of amino acid substitutions in the ISDR was an independent predictor of the response to IFN therapy [12]. In the present study, we demonstrate that ISDR mutations are the most effective predictors of treatment outcome of 48-week



◀ **Fig. 1** **a** The percentages of patients with more than two mutations in the interferon sensitivity determining region in NS5A $_{2209–2248}$ (*ISDR*), according to age (*horizontal axis*) among 762 patients who received interferon (IFN) therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. **b** Responses to pegylated (PEG)-IFN plus ribavirin (RBV) treatment and serum levels of hepatitis C virus (HCV) RNA in relation to the number of mutations in the ISDR. Patients with sustained virological response (*SVR*) are indicated by *open circles* and those with non-SVR by *closed circles*. **c** Changes over time in VR rates in patients treated with PEG-IFN plus RBV. Patients with more than two mutations in the ISDR are indicated by *open circles* and those with no or one mutation in the ISDR by *closed circles*, *W* weeks. **d** PEG-IFN plus RBV treatment efficacy divided into two groups based on ISDR mutations. End-of-treatment response (*ETR*) and SVR are shown in both intention-to-treat (ITT) analysis (*left*) and per-protocol (PP) analysis (*right*)

PEG-IFN plus RBV therapy in patients with HCV genotype 1binfection.

In the present study, the SVR rate was not related to gender, age, or previous IFN therapy by univariate analysis. First of all, in regard to gender ($P = 0.07$), as male patients had a higher tendency to achieve SVR than female patients, further validation in larger-scale studies is required to clarify the significance of gender. Secondly, in regard to age, there was no relation to SVR in overall analysis with continuous variables, but younger patients, aged less than 65 years, had a higher rate of response than those aged more than 65 years ($P < 0.05$, data not shown). Actually there are some reports suggesting the relationship of age and SVR [25, 26]. Finally, in regard to previous IFN therapy, as shown in Table 5, treatment was comparably effective in both groups; previous IFN therapy did not affect the SVR rate. The reasons for equivalent response rates in subjects with prior IFN history, which was not expected, are unclear. In our study, the group with prior IFN history had more advanced liver fibrosis and a low platelet count, and stage of fibrosis was one of the factors extracted by univariate analysis as a useful pretreatment marker predicting SVR. We also analyzed the other three parameters extracted by univariate analysis. Although there was no difference in pretreatment hemoglobin, or number of ISDR mutations, the group with prior IFN history tended to have a low serum HCV-RNA level. Further, the group with prior IFN history had a high proportion of male patients. Although the SVR rate was not related to gender, male subjects had a higher tendency to achieve SVR than female subjects.

In our present study, the SVR rate was not related to core mutations. As described in previous reports [17, 27, 28], amino acid substitutions in the core region are regarded as predictors of response to PEG-IFN plus RBV therapy in Japanese patients infected with HCV genotype 1b. In the present study, the SVR rate was not related to the pattern of amino acid substitution in the overall analysis. The reasons for these discrepant results are unclear, but females with dual substitutions at amino acids 70 and 91 had a lower tendency to achieve SVR. Further studies are necessary to clarify the mechanism of action for amino acid substitutions in the core region of HCV.

Recent studies suggest that the mutations in the ISDR are associated with response to combination therapy with IFN and RBV [29–32]. Most recently, it has been reported

that amino acid substitutions in the core and mutations in the ISDR are predictive of virological response to the combination therapy in patients with HCV genotype 1b and a high viral load [28]. There are some reports suggesting that the mutations in the ISDR may not serve as a predictor for treatment outcome [33, 34], but as the numbers of subjects in these studies were around 30, a number which is not sufficient to evaluate the results, this factor may explain these discrepant results.

The mechanisms of IFN sensitivity in relation to the sequence of the HCV $NS5A_{2209–2248}$ region are not clear. However the "mutant-type" ISDR correlates with a low viral load, as reported previously [12, 35, 36]; most patients in the present study with two or more mutations in the ISDR had high levels of virus. Furthermore, stepwise multiple logistic regression analysis of the factors, including substitution of the ISDR and the viral load, revealed that both of them were independent predictive variables of SVR, and the odds ratio of the number of mutations in the ISDR was the highest in the pretreatment factors associated with SVR by multivariate analysis. The precise mechanism involved must be elucidated in further in vitro studies.

There have been several reports that suggest biological roles of the ISDR in the response to IFN and in HCV infection. Double-stranded RNA-dependent protein kinase (PKR) is a critical component of the cellular antiviral responses induced by IFN. Gale et al. [37, 38] have reported that mutations within the PKR-binding region of NS5A, including ISDR, can disrupt the NS5A–PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN. Toll-like receptor (TLR) has also been reported to play various roles in many viral infections, and it has been reported that NS5A bound MyD88, a major adaptor molecule of TLR-mediated signaling, and inhibited the TLR–MyD88 signaling pathway by a direct interaction with the death domain of MyD88 through the ISDR [39]. Furthermore, it has been reported that the lipid droplet is an important organelle for HCV production, and NS5A is a key protein that recruits replication complexes to lipid droplets for the production of infectious viral particles [40]. While the mechanism of action of the ISDR in the response to IFN or viral replication remains to be proven, these findings suggest new aspects of HCV infections.

In our previous report [12], patients with 4 or more mutations in the ISDR experienced SVR with conventional IFN monotherapy, but in more effective therapy with PEG-IFN plus RBV combination therapy, the number of mutations as a predictor of SVR decreased from 4 to 2. Watanabe et al. [41] have also reported that the number and position of mutations in the ISDR correlated with IFN efficacy in HCV-1b infection. Moreover, it has been reported that patients with viruses mutated at positions 2209, 2216, or 2227 more frequently experienced SVR than did those without these mutations. Another group has also reported regarding statistical analysis, using a database of 675 individual ISDR sequences in HCV-NS5A and the IFN response [42]. They have shown that IFN-sensitive viruses contain a larger and more diverse collection of substitutions than IFN-resistant viruses. While it remains unknown how the numbers of mutations are involved in the biological role of ISDR, or which sites of mutation and changes of amino acid are also important for the response to IFN-based treatment, it is thought that the functional importance of numbers or sites of mutations can be explained in terms of interaction between NS5A and some target molecules such as PKR, MyD88, and lipid droplets.

In vitro studies have shown that the introduction of NS5A mutations enables an HCV replicon to replicate efficiently [10, 43, 44]. In our previous report, site-specific mutation of the ISDR also modulated HCV replication [45]. The ISDR was identified originally as the site that determines the sensitivity of HCV to IFN [12]. This indicates that the ISDR mutations are not lethal in vivo. Furthermore, mutations in the ISDR are closely associated clinically with decreased serum HCV RNA levels [42], whereas ISDR mutations in the HCV replicon enhance replication. While the explanation for this paradox has not become clear, a big difference between the environment of cultured cells and that in the human liver is thought contribute to this phenomenon.

We found that the percentage of patients with more than two mutations in the ISDR was between 20% and 30% for all ages; thus, around one-fifth of patients are thought likely to experience SVR. Indeed, the SVR rate among patients with two or more mutations in the ISDR sequence was 68% (ITT) and 80% (PP) compared to 39% (ITT) and 50% (PP) among those patients with no or one mutation in the present study. Furthermore, predictive factors such as serum HCV RNA level, stage of fibrosis, and hemoglobin also aid in the assessments of treatment, and we can use these parameters to develop a treatment strategy.

Several prospective randomized trials have shown that 72-week extended therapy improves SVR by 7.5%–12% in late viral responders [46, 47]. One cohort study showed that 72-week treatment for late viral responders achieved an even higher SVR, of 67.1%, which was 21% higher than the SVR achieved with 48-week treatment [48]. These reports demonstrate that tailoring of treatment duration by on-treatment viral response can further improve the outcomes of antiviral therapy. In our 48-week based treatment, 90% of patients with more than 2 ISDR mutations cleared the virus within 12 weeks of treatment (early viral response; EVR) and consequently achieved 30% higher SVR than those with 1 or no ISDR mutation. These results

suggest that ISDR mutations will remain a significant predictor of good response to IFN therapies, including 72-week extension.

In conclusion, ISDR mutations are the most effective predictors of treatment outcomes in multivariate analysis. The number of mutations in the ISDR sequence of HCV-1b ($\geq$2) is the most effective parameter which will facilitate further the selection of patients with a high likelihood of response to PEG-IFN plus RBV treatment.

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Sequences in the Interferon Sensitivity-Determining Region and Core Region of Hepatitis C Virus Impact Pretreatment Prediction of Response to PEG-Interferon Plus Ribavirin: Data Mining Analysis

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The aim of the present study was to clarify the significance of viral factors for pretreatment prediction of sustained virological response to pegylated-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C using data mining analysis. Substitutions in the IFN sensitivity-determining region (ISDR) and at position 70 of the HCV core region (Core70) were determined in 505 patients with genotype 1b chronic hepatitis C treated with PEG-IFN plus RBV. Data mining analysis was used to build a predictive model of sustained virological response in patients selected randomly (n = 304). The reproducibility of the model was validated in the remaining 201 patients. Substitutions in ISDR (odds ratio = 9.92, $P < 0.0001$) and Core70 (odds ratio = 1.92, $P = 0.01$) predicted sustained virological response independent of other covariates. The decision-tree model revealed that the rate of sustained virological response was highest (83%) in patients with two or more substitutions in ISDR. The overall rate of sustained virological response was 44% in patients with a low number of substitutions in ISDR (0–1) but was 83% in selected subgroups of younger patients (<60 years), wild-type sequence at Core70, and higher level of low-density lipoprotein cholesterol (LDL-C) (≥120 mg/dl). Reproducibility of the model was validated ($r^2 = 0.94$, $P < 0.001$). In conclusion, substitutions in ISDR and Core70 of HCV are significant predictors of response to PEG-IFN plus RBV therapy. A decision-tree model that includes these viral factors as predictors could identify patients with a high probability of sustained virological response. ***J. Med. Virol. 83:445–452, 2011.***

KEY WORDS: data mining; decision-tree model; ISDR; core region; PEG-interferon

INTRODUCTION

The combination of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) is currently the most effective therapy for chronic hepatitis C, but the rate of sustained virological response after 48 weeks of therapy is about 50% in patients with HCV genotype 1b and a high HCV

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RNA titer [Manns et al., 2001; Fried et al., 2002]. The most reliable means to predict sustained virological response is to monitor the viral response during the early weeks of treatment. The early virological response, defined as undetectable HCV RNA at week 12, is associated with a high rate of sustained virological response [Davis et al., 2003; Lee and Ferenci, 2008]. The rapid virological response, defined as undetectable HCV RNA at week 4 of therapy, is even more predictive of sustained virological response than the early virological response [Jensen et al., 2006; Yu et al., 2008; Izumi et al., 2010]. However, there is no established means that predicts the virological response before commencing treatment. Recent reports have revealed that single nucleotide polymorphisms located near the *IL28B* gene show a strong association with the response to PEG-IFN plus RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Kurosaki et al., 2010c]. These findings indicate that the host factor is an important determinant of the treatment response. On the other hand, the present study's authors have reported that a stretch of 40 amino acids in the NS5A region of HCV, designated as the interferon sensitivity-determining region (ISDR), has a close association with the virological response to interferon mono-therapy [Enomoto et al., 1995, 1996; Kurosaki et al., 1997]. More recently, amino acid substitutions at positions 70 and 91 of the core region have been reported to be associated with response to PEG-IFN plus RBV combination therapy [Akuta et al., 2005, 2007a]. The impact of these HCV substitutions on treatment response is yet to be validated.

Decision-tree analysis is a core component of data mining analysis that can be used to build predictive models [Breiman et al., 1980]. This method has been used to define prognostic factors in various diseases such as prostate cancer [Garzotto et al., 2005], diabetes [Miyaki et al., 2002], melanoma [Averbook et al., 2002; Leiter et al., 2004], colorectal carcinoma [Zlobec et al., 2005; Valera et al., 2007], and liver failure [Baquerizo et al., 2003]. The major advantage of decision-tree analysis over logistic regression analysis is that the results of analysis are easy to understand. The simple allocation of patients into subgroups by following the flowchart form could define the predicted possibility of outcome [LeBlanc and Crowley, 1995].

Decision-tree analysis was used for the prediction of early virological response (undetectable HCV RNA within 12 weeks of therapy) to PEG-IFN and RBV combination therapy in chronic hepatitis C [Kurosaki et al., 2010a], and more recently for the pretreatment prediction of sustained virological response [Kurosaki et al., 2010b]. In the latter model, simple and noninvasive standard tests were used as parameters; specialized tests such as viral mutations and host genetics, or invasive tests such as liver histology, were not included because the aim of that model was for use in general medical practice, especially in some countries or areas where resources are limited. Thus, the impact of viral mutations or liver histology was not considered in that model.

The present study examined whether including viral substitutions in ISDR and the core region of HCV in the decision-tree model could improve its predictive accuracy over the previous model to identify chronic hepatitis C patients who are likely to respond to PEG-IFN plus RBV therapy.

MATERIALS AND METHODS

Patients

This multicenter retrospective cohort study included 505 chronic hepatitis C patients who were treated with PEG-IFN alpha-2b and RBV at Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University Graduate School of Medical Sciences, Yamanashi University, Osaka City University, and their related hospitals. The inclusion criteria were: (1) genotype 1b, (2) HCV RNA titer higher than 100 kIU/ml by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic Systems, Pleasanton, CA), (3) no co-infection with hepatitis B virus or human immunodeficiency virus, (4) no other causes of liver disease, (5) patients having undergone liver biopsy prior to IFN treatment, (6) number of substitutions in ISDR having been determined, (7) substitutions in the amino acid positions 70 and 91 of the core region having been determined, and (8) completion of at least 12 weeks of therapy. Patients were treated with PEG-IFN alpha-2b (1.5 μg/kg) weekly plus RBV. The daily dose of RBV was adjusted by weight: 600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg. For the analysis, patients were assigned randomly to either the model building (304 patients) or validation (201 patients) groups. There were no significant differences in the clinical backgrounds between these two groups (Table I). Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committees of all concerned hospitals.

Laboratory Tests

Hematological tests, blood chemistry, and HCV RNA titer were analyzed before therapy and at least once every month during therapy. 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. At position 70 of the core region (Core70), arginine was defined as the wild type, and glutamine or histidine was defined as the mutant type. At position 91 of the core region, leucine was defined as the wild type and methionine was defined as the mutant type, as described previously [Akuta et al., 2005]. Fibrosis and activity were scored according to the METAVIR scoring system [Bedossa and Poynard, 1996]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of