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)

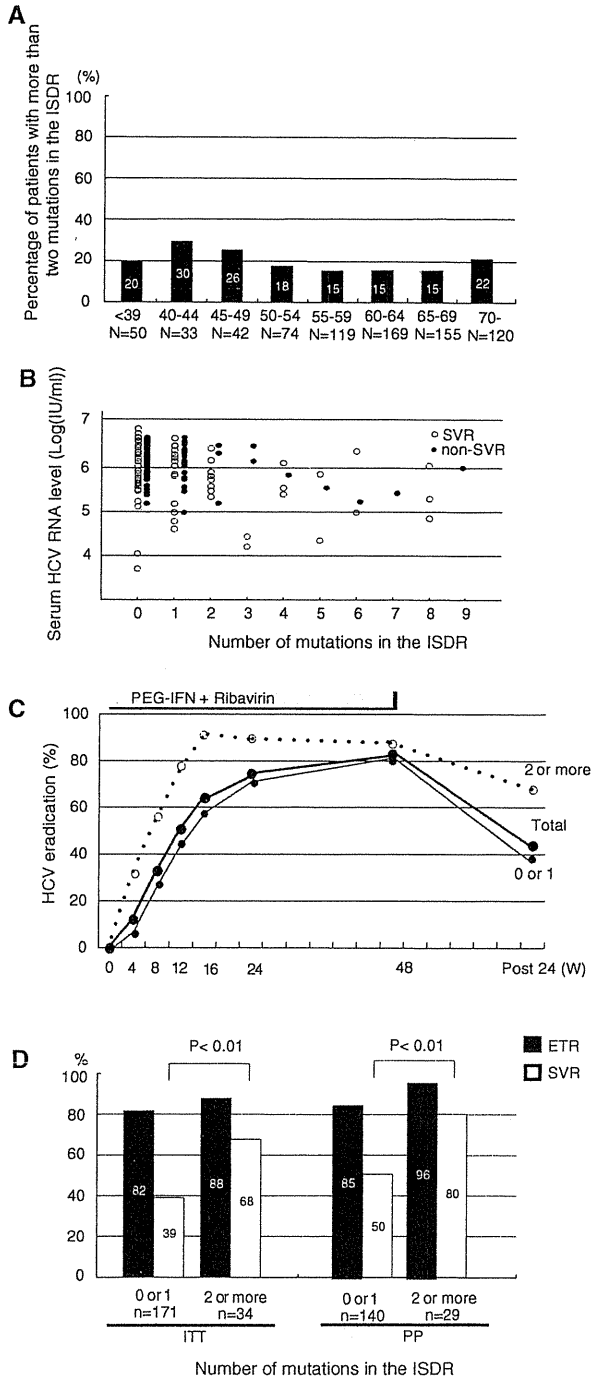
the SVR rates of the patients with good drug adherence was 80%.

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.

Acknowledgments This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology-Japan; the Japan Society for the Promotion of Science; the Ministry of Health, Labour and Welfare-Japan; the Japan Health Sciences Foundation; the Miyakawa Memorial Research Foundation; and the National Institute of Biomedical Innovation. The following hospitals participated in the Ochanomizu-Liver Conference Study Group: Oume City General Hospital, Kashiwa City Hospital, Kudanzaka Hospital, Showa General Hospital, Shuwa General Hospital, Soka Municipal Hospital, Tama-Nambu Chiiki Hospital, Tuchiura Kyodo General Hospital, Tokyo Kyosai Hospital, Tokyo Metropolitan Ohtsuka Hospital, Tokyo Metropolitan Fuchu Hospital, Tokyo Metropolitan Bokutoh Hospital, Toride Kyodo General Hospital, Nakano General Hospital, Hokushin General Hospital, Mishima Social Insurance Hospital, Musashino Red Cross Hospital, Yokosuka Kyosai Hospital, Yokohama City Minato Red Cross Hospital.

References

1. Major ME, Feinstone SM. The molecular virology of hepatitis C. Hepatology. 1997;25:1527–38.
2. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 1989;244:359–62.
3. Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. Lancet. 1991;337:1058–61.
4. Dibisceglie AM, Shindo M, Fong TL, Fried MW, Swain MG, Bergasa NV, et al. A pilot-study of ribavirin therapy for chronic hepatitis-C. Hepatology. 1992;16:649–54.
5. Dusheiko G, Main J, Thomas H, Reichard O, Lee C, Dhillon A, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol. 1996;25:591–8.
6. Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O, et al. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. Lancet. 1998;351:83–7.
7. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomised trial of interferon alpha 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha 2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet. 1998;352:1426–32.
8. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med. 1998;339:1485–92.
9. Glue P, Fang JWS, Rouzier-Panis R, Raffanel C, Sabo R, Gupta SK, et al. Pegylated interferon-alpha 2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Clin Pharmacol Ther. 2000;68:556–67.
10. Fried MW, Shiffman ML, Reddy KR, Smith C, Marionos G, Goncales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
11. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest. 1995;96:224–30.
12. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
13. Kurosaki M, Enomoto N, Murakami T, Sakuma I, Asahina Y, Yamamoto C, et al. Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology. 1997;25:750–3.
14. Enomoto N, Kurosaki M, Tanaka Y, Marumo F, Sato C. Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. J Gen Virol. 1994;75:1361–9.
15. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular-cloning of the human hepatitis-C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA. 1990;87:9524–8.
16. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology. 2005;48:372–80.
17. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology. 2007;46:1357–64.
18. Zeuzem S, Lee JH, Roth WK. Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon Alfa. Hepatology. 1997;25:740–4.
19. Khorsi H, Castelain S, Wyseur A, Izopet J, Canva V, Rombout A, et al. Mutations of hepatitis C virus 1b NS5A 2209–2248 amino acid sequence do not predict the response to recombinant interferon-alfa therapy in French patients. J Hepatol. 1997;27:72–7.
20. Squadrito G, Leone F, Sartori M, Nalpas B, Berthelot P, Raimondo G, et al. Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alfa. Gastroenterology. 1997;113:567–72.
21. Hofgartner WT, Polyak SJ, Sullivan DG, Carithers RL, Gretch DR. Mutations in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. J Med Virol. 1997;53:118–26.
22. Squadrito G, Orlando ME, Cacciola I, Rumi MG, Artini M, Picciotto A, et al. Long-term response to interferon alpha is unrelated to "interferon sensitivity determining region" variability in patients with chronic hepatitis C virus-1b infection. J Hepatol. 1999;30:1023–7.
23. Chung RT, Monto A, Dienstag JL, Kaplan LM. Mutations in the NS5A region do not predict interferon-responsiveness in American patients infected with genotype 1b hepatitis C virus. J Med Virol. 1999;58:353–8.
24. Sarrazin C, Berg T, Lee JH, Teuber G, Dietrich CF, Roth WK, et al. Improved correlation between multiple mutations within the NS5A region and virological response in European patients chronically infected with hepatitis C virus type 1b undergoing combination therapy. J Hepatol. 1999;30:1004–13.
25. Honda T, Katano Y, Urano F, Murayama M, Hayashi K, Ishigami M, et al. Efficacy of ribavirin plus interferon-alpha in patients aged $\geq$60 years with chronic hepatitis C. J Gastroenterol Hepatol. 2007;22:989–95.

26. Hung CH, Chen CH, Lee CM, Wu CM, Hu TH, Wang JH, et al. Association of amino acid variations in the NS5A and E2-PePHD region of hepatitis C virus 1b with hepatocellular carcinoma. J Viral Hepatitis. 2008;15:58–65.
27. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype1b and high viral load. J Med Virol. 2006;78:83–90.
28. Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol. 2009;81:640–9.
29. Yen YH, Hun CH, Hu TH, Chen CH, Wu CM, Vvang JH, et al. Mutations in the interferon sensitivity-determining region (nonstructural 5A amino acid 2209–2248) in patients with hepatitis C-1b infection and correlating response to combined therapy of pegylated interferon and ribavirin. Aliment Pharmacol Ther. 2008;27:72–9.
30. Hung CH, Lee CM, Lu SN, Lee JF, Wang JH, Tung HD, et al. Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. J Viral Hepatitis. 2003;10:87–94.
31. Murayama M, Katano Y, Nakano I, Ishigami M, Hayashi K, Honda T, et al. A mutation in the interferon sensitivity-determining region is associated with responsiveness to interferon-ribavirin combination therapy in chronic hepatitis patients infected with a Japan-specific subtype of hepatitis C virus genotype 1b. J Med Virol. 2007;79:35–40.
32. Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008;48:1753–60.
33. Murphy MD, Rosen HR, Marousek GI, Chou SW. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. Dig Dis Sci. 2002;47:1195–205.
34. Yang SS, Lai MY, Chen DS, Chen GH, Kao JH. Mutations in the NS5A and E2-PePHD regions of hepatitis C virus genotype 1b and response to combination therapy of interferon plus ribavirin. Liver Int. 2003;23:426–33.
35. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, et al. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology. 1997;25:745–9.
36. Watanabe H, Nagayama K, Enomoto N, Itakura J, Tanabe Y, Hamano K, et al. Sequence elements correlating with circulating viral load in genotype 1b hepatitis C virus infection. Virology. 2003;311:376–83.
37. Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology. 1997;230:217–27.
38. Gale M, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, et al. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol. 1998;18:5208–18.
39. Abe T, Kaname Y, Hamamoto I, Tsuda Y, Wen XY, Taguwa S, et al. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor–MyD88-dependent signaling pathway in macrophage cell lines. J Virol. 2007;81:8953–66.
40. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol. 2007;9:1089–97.
41. Watanabe H, Enomoto N, Nagayama K, Izumi N, Marumo F, Sato C, et al. Number and position of mutations in the interferon (IFN) sensitivity-determining region of the gene for nonstructural protein 5A correlate with IFN efficacy in hepatitis C virus genotype 1b infection. J Infect Dis. 2001;183:1195–203.
42. Witherell GW, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. J Med Virol. 2001;63:8–16.
43. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science. 2000;290:1972–4.
44. Maekawa S, Enomoto N, Sakamoto N, Kurosaki M, Ueda E, Kohashi T, et al. Introduction of NS5A mutations enables subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. J Viral Hepatitis. 2004;11:394–403.
45. Kohashi T, Maekawa S, Sakamoto N, Kurosaki M, Watanabe H, Tanabe Y, et al. Site-specific mutation of the interferon sensitivity-determining region (ISDR) modulates hepatitis C virus replication. J Viral Hepatitis. 2006;13:582–90.
46. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
47. Ferenci P, Laferl H, Scherzer TM, Maieron A, Hofer H, Stauber R, et al. Peginterferon alfa-2a/ribavirin for 48 or 72 weeks in hepatitis C types 1 and 4 patients with slow virologic response. Gastroenterology 2009 [Epub ahead of print].
48. Watanabe S, Enomoto N, Koike K, Izumi N, Takikawa H, Hashimoto E, et al. Prolonged treatment with pegylated interferon a 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan. Hepatol Res 2009 [Epub ahead of print].

Virology 405 (2010) 361–369

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants☆,☆☆

Kako Mishima [a,1], Naoya Sakamoto [a,b,*,1], Yuko Sekine-Osajima [a], Mina Nakagawa [a,b], Yasuhiro Itsui [a,c], Seishin Azuma [a], Sei Kakinuma [a,b], Kei Kiyohashi [a], Akiko Kitazume [a], Kiichiro Tsuchiya [a], Michio Imamura [d], Nobuhiko Hiraga [d], Kazuaki Chayama [d], Takaji Wakita [e], Mamoru Watanabe [a]

[a] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[b] Department for Hepatitis Control, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[c] Department of Internal Medicine, Soka Municipal Hospital, 2-21-1 Soka, Soka City, Saitama 340-8560, Japan
[d] Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima 734-8551, Japan
[e] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 11 March 2010
Returned to author for revision 7 April 2010
Accepted 7 June 2010
Available online 6 July 2010

Keywords:
HCV-JFH1 cell culture
Plaque assay
Cytopathic effect
Adaptive mutations
Human hepatocyte chimeric mice

ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family Flaviviridae. One of the characteristics of the Flaviviridae is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

☆ The authors, K.M., N.S., Y.S., M.N., Y.I., S.A., S.K., K.K., A.K., K.T., M.I., N.H., K.C., T.W. and M.W. declare that there is no conflict of interest.

☆☆This study was supported by grants from Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan, Japan Health Sciences Foundation, and National Institute of Biomedical Innovation.

* Corresponding author. Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81 3 5803 0268.
E-mail address: nsakamoto.gast@tmd.ac.jp (N. Sakamoto).

[1] K. M. and N. S. contributed equally to this work.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.06.020

reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see Materials and methods). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

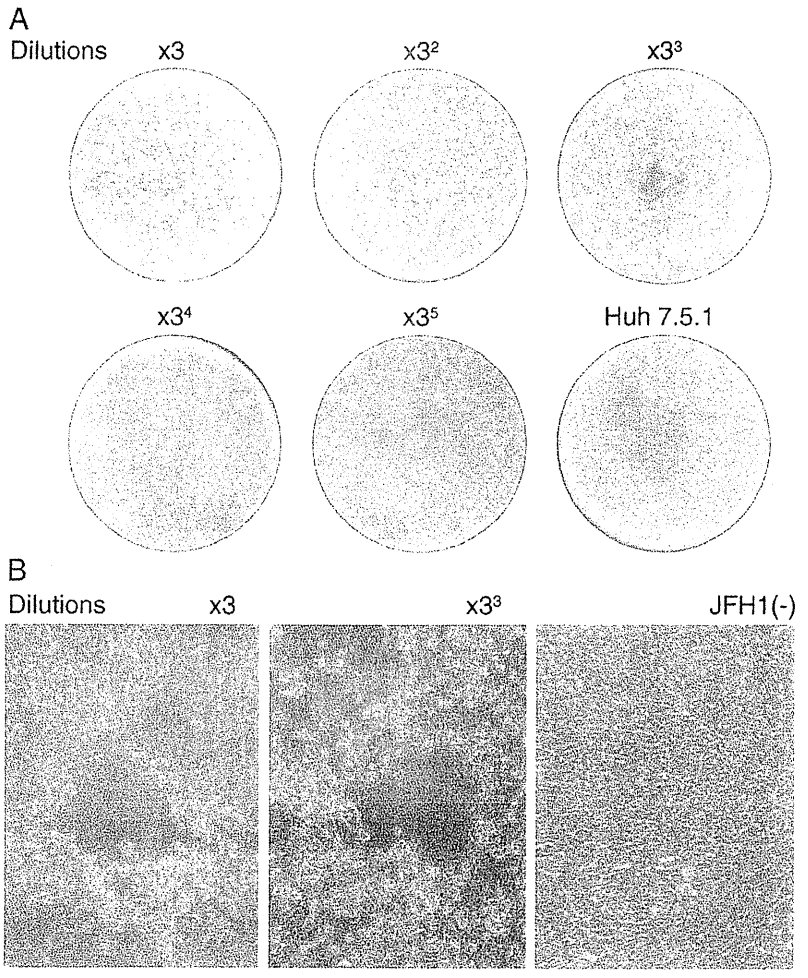


Fig. 1. The cytopathic effects of HCV-JFH1 in vitro. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

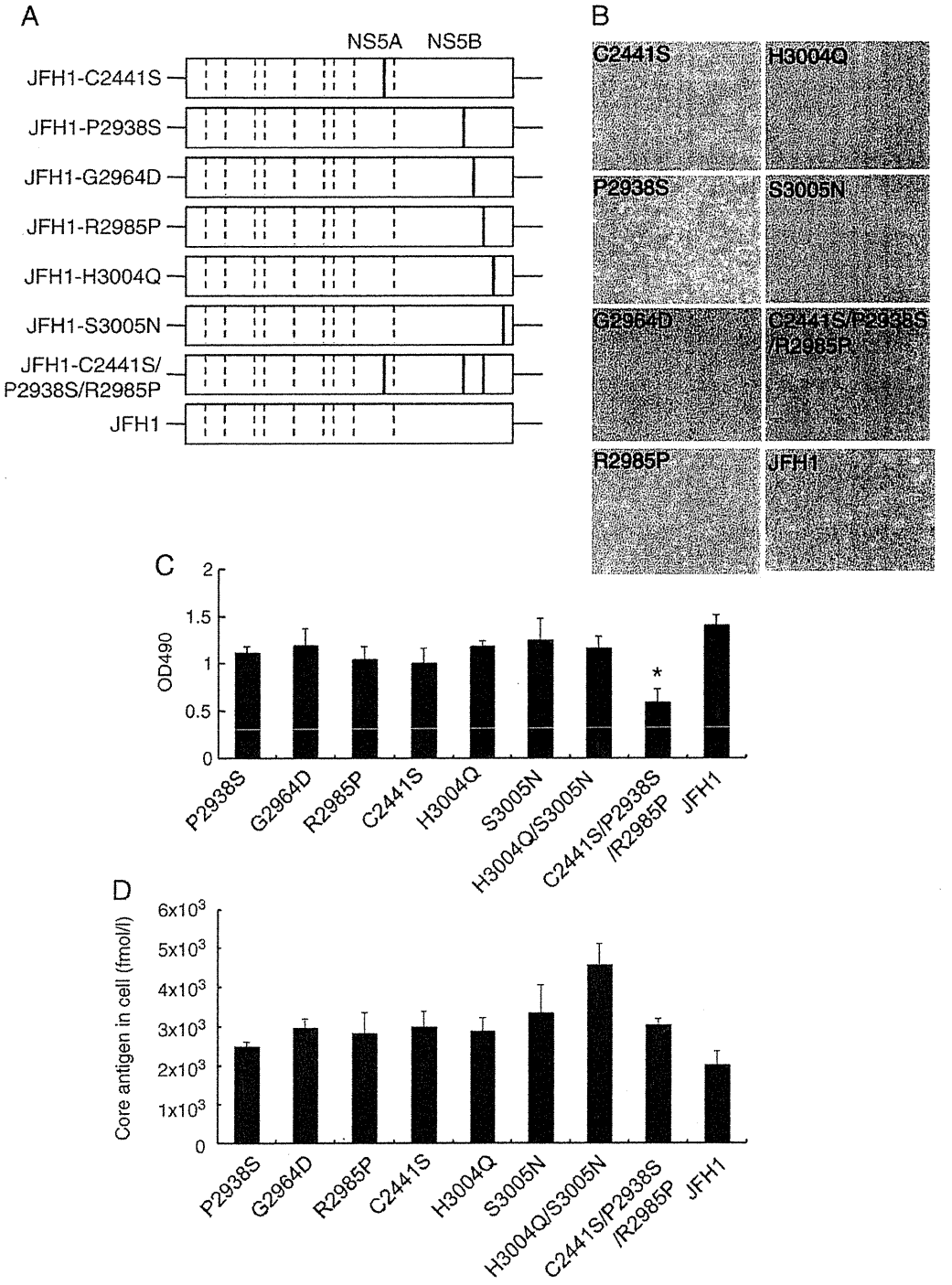


Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. C. MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. D. Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of re-infection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S,

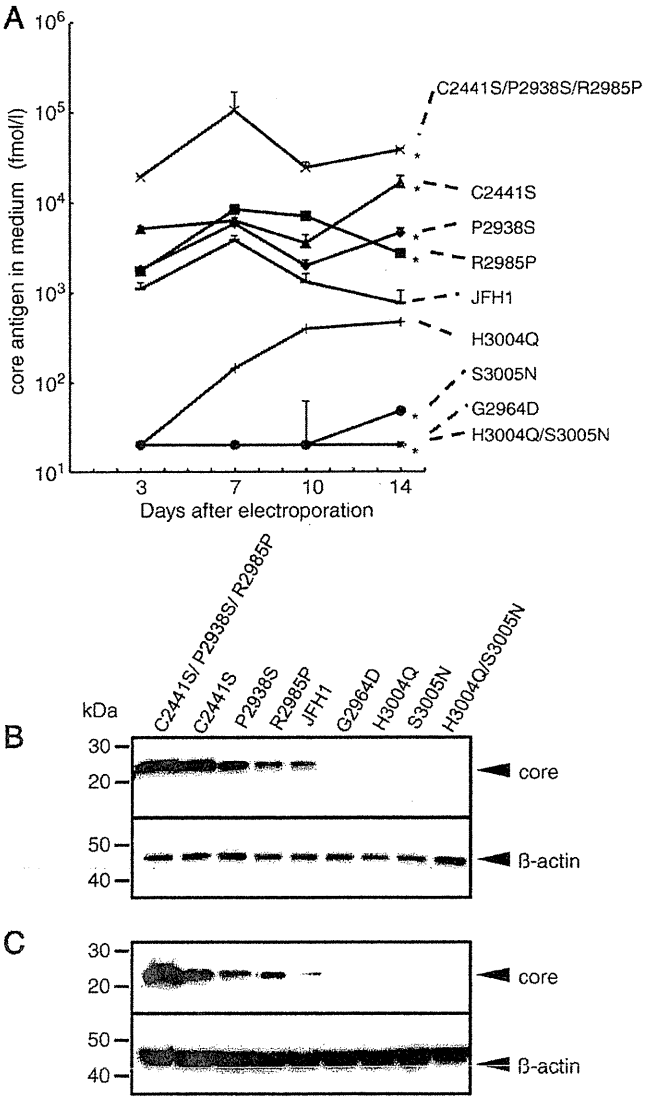


Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

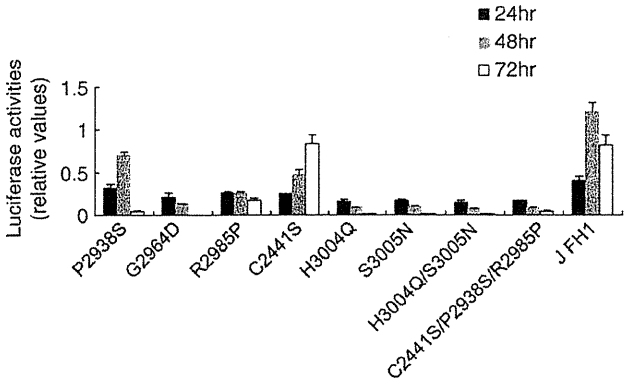


Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected

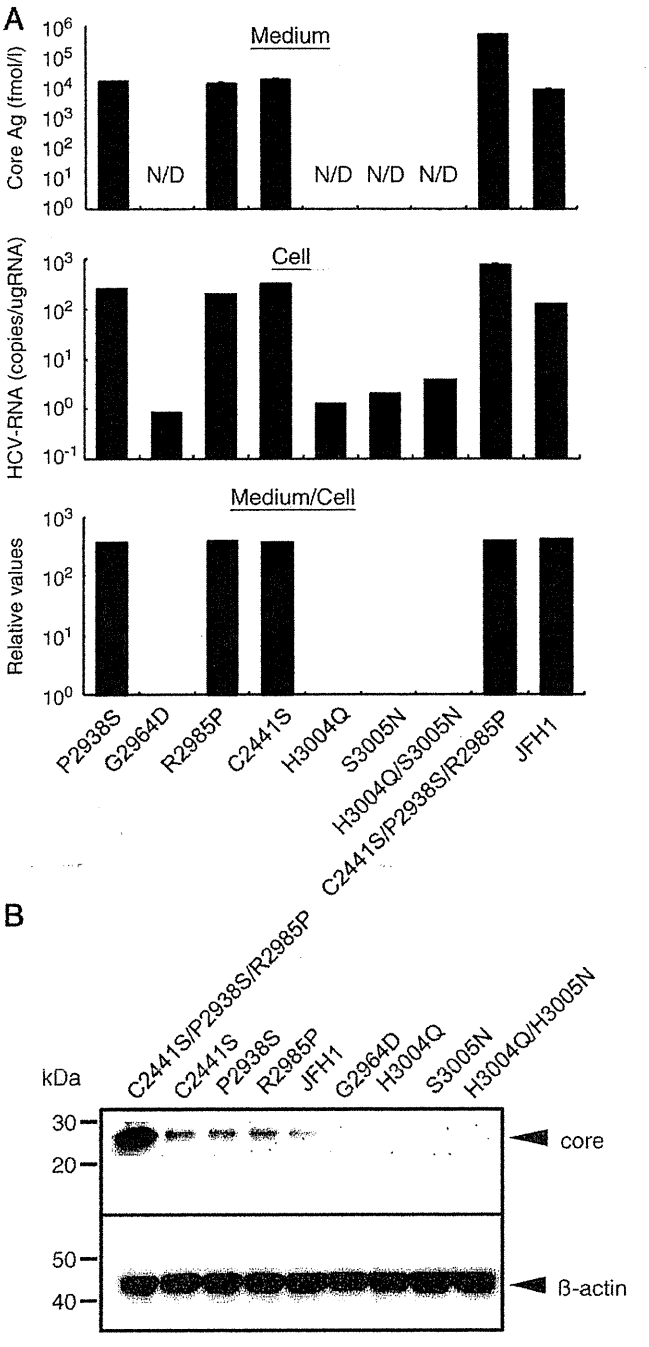


Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice

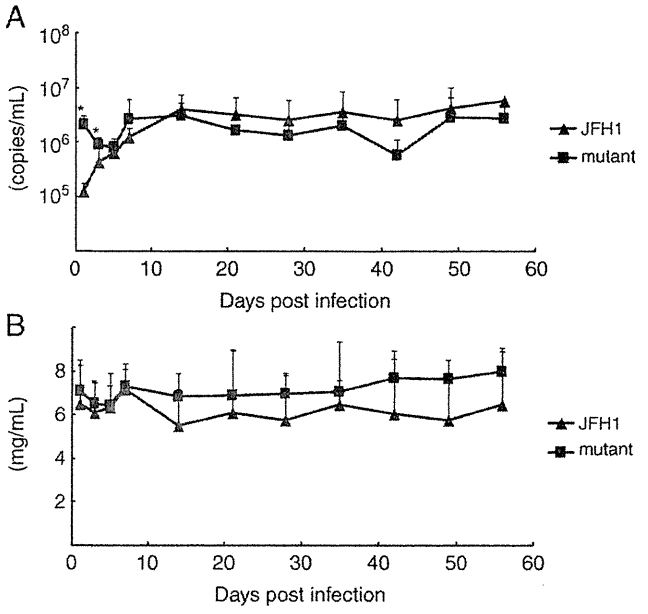


Fig. 6. In vivo analysis of cytopathic JFH1 mutants using human hepatocyte chimeric mice. A. Serial changes in HCV RNA in the sera of mice inoculated with the culture media from JFH1 mutants. The data shows the average of 2 mice for JFH1, and 3 mice for the mutant. Asterisks indicate p-values of less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

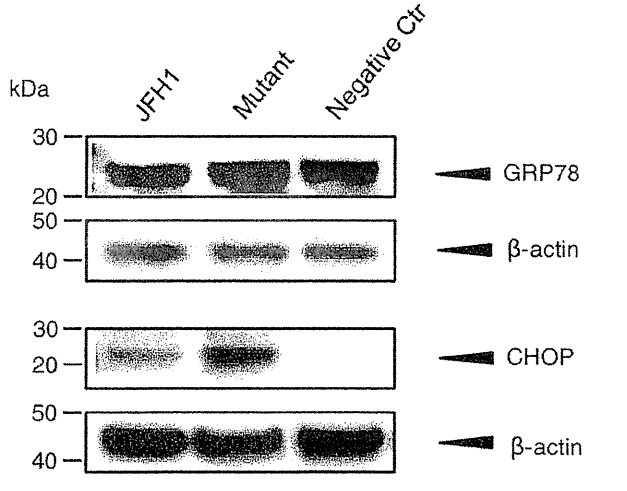


Fig. 7. Expression of ER stress-related proteins in human hepatocytes of chimeric mice infected with JFH1 or the mutant in the early phase. Western-blot analysis of the liver tissues of infected chimeric mice using anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody and 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

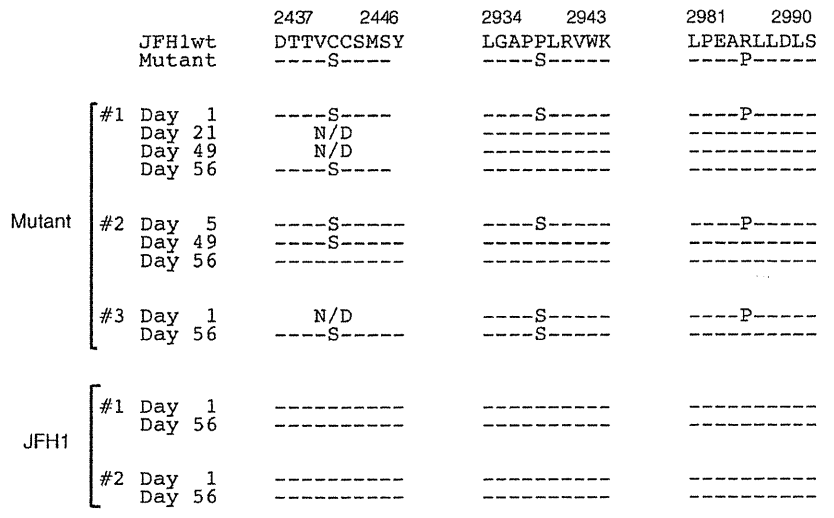


Fig. 8. Nucleotide sequence analysis of virus genomes circulating in the sera of infected mice. We extracted RNA from the sera of mice inoculated with culture media from JFH1 or JFH1-mutants and analyzed the viral sequence at the specified time points. N/D is not detectable. Wt: Wild type.

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.

Acknowledgments

We are indebted to Dr. Francis V. Chisari for providing the Huh-7.5.1 cell line and Dr. Rodney S. Russell for receptor-deficient Huh7-S29 cells. This study was supported by grants from Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and

Welfare-Japan, Japan Health Sciences Foundation, and National Institute of Biomedical Innovation.

References

Alter, M.J., 1997. Epidemiology of hepatitis C. Hepatology 26, 62S–65S.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81, 1631–1648.

Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R.E., Govindarajan, S., Shapiro, M., Claire, M.S., Bartenschlager, R., 2002. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc. Natl. Acad. Sci. U. S. A. 99 (22), 14416–14421.

Cerny, A., Chisari, F.V., 1999. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology 30 (3), 595–601.

Delgrange, D., Pillez, A., Castelain, S., Cocquerel, L., Rouille, Y., Dubuisson, J., Wakita, T., Duverlie, G., Wychowski, C., 2007. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J. Gen. Virol. 88 (Pt 9), 2495–2503.

Delladetsima, J.K., Boletis, J.N., Makris, F., Psichogiou, M., Kostakis, A., Hatzakis, A., 1999. Fibrosing cholestatic hepatitis in renal transplant recipients with hepatitis C virus infection. Liver Transpl. Surg. 5 (4), 294–300.

Dixon, L.R., Crawford, J.M., 2007. Early histologic changes in fibrosing cholestatic hepatitis C. Liver Transpl. 13 (2), 219–226.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marionos, G., Goncales, F.L., Häussinger, D., Diago, M., Garosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.

Han, Q., Xu, C., Wu, C., Zhu, W., Yang, R., Chen, X., 2009. Compensatory mutations in NS3 and NS5A proteins enhance the virus production capability of hepatitis C reporter virus. Virus Res. 145 (1), 63–73.

Itsui, Y., Sakamoto, N., Kakinuma, S., Nakagawa, M., Sekine-Osajima, Y., Tasaka-Fujita, M., Nishimura-Sakurai, Y., Suda, G., Karakama, Y., Yamamoto, M., Watanabe, T., Ueyama, M., Funaoka, Y., Azuma, S., and Watanabe, M. (2009). Antiviral effects of the interferon-induced protein GBP-1 and its interaction with the hepatitis C virus NS5B protein. Hepatology 50 (6), 1727–1737.

Jordan, R., Wang, L., Graczyk, T.M., Block, T.M., Romano, P.R., 2002. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J. Virol. 76 (19), 9588–9599.

Kato, N., 2001. Molecular virology of hepatitis C virus. Acta Med. Okayama 55 (3), 133–159.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125 (6), 1808–1817.

Kaul, A., Woerz, I., Meuleman, P., Leroux-Roels, G., Bartenschlager, R., 2007. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J. Virol. 81 (23), 13168–13179.

Koutsoudakis, G., Herrmann, E., Kallis, S., Bartenschlager, R., Pietschmann, T., 2007. The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. J. Virol. 81 (2), 588–598.

Leifeld, L., Nattermann, J., Fielenbach, M., Schmitz, V., Sauerbruch, T., Spengler, U., 2006. Intrahepatic activation of caspases in human fulminant hepatic failure. Liver Int. 26 (7), 872–879.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6 (10), 937–943.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309 (5734), 623–626.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285 (5424), 110–113.

Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7 (8), 927–933.

Mita, A., Hashikura, Y., Tagawa, Y., Nakayama, J., Kawakubo, M., Miyagawa, S., 2005. Expression of Fas ligand by hepatic macrophages in patients with fulminant hepatic failure. Am. J. Gastroenterol. 100 (11), 2551–2559.

Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M.R., Pedrazzini, E., Bonatti, S., Migliaccio, G., 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 293 (1), 31–43.

Russell, R.S., Meunier, J.C., Takikawa, S., Faulk, K., Engle, R.E., Bukh, J., Purcell, R.H., Emerson, S.U., 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. 105 (11), 4370–4375.

Ryo, K., Kamogawa, Y., Ikeda, I., Yamauchi, K., Yonehara, S., Nagata, S., Hayashi, N., 2000. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. Am. J. Gastroenterol. 95 (8), 2047–2055.

Sakamoto, N., Watanabe, M., 2009. New therapeutic approaches to hepatitis C virus. J. Gastroenterol. 44 (7), 643–649.

Sakamoto, N., Yoshimura, M., Kimura, T., Toyama, K., Sekine-Osajima, Y., Watanabe, M., Muramatsu, M., 2007. Bone morphogenetic protein-7 and interferon-alpha synergistically suppress hepatitis C virus replicon. Biochem. Biophys. Res. Commun. 357 (2), 467–473.

Sekine-Osajima, Y., Sakamoto, N., Mishima, K., Nakagawa, M., Itsui, Y., Tasaka, M., Nishimura-Sakurai, Y., Chen, C.H., Kanai, T., Tsuchiya, K., Wakita, T., Enomoto, N., Watanabe, M., 2008. Development of plaque assays for hepatitis C virus-JFH1 strain and isolation of mutants with enhanced cytopathogenicity and replication capacity. Virology 371 (1), 71–85.

Shuda, M., Kondoh, N., Imazeki, N., Tanaka, K., Okada, T., Mori, K., Hada, A., Arai, M., Wakatsuki, T., Matsubara, O., Yamamoto, N., Yamamoto, M., 2003. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J. Hepatol. 38 (5), 605–614.

Tanabe, Y., Sakamoto, N., Enomoto, N., Kurosaki, M., Ueda, E., Maekawa, S., Yamashiro, T., Nakagawa, M., Chen, C.H., Kanazawa, N., Watanabe, M., 2004. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J. Infect. Dis. 189, 1129–1139.

Tasaka, M., Sakamoto, N., Itakura, Y., Nakagawa, M., Itsui, Y., Sekine-Osajima, Y., Nishimura-Sakurai, Y., Chen, C.H., Yoneyama, M., Fujita, T., Wakita, T., Maekawa, S., Enomoto, N., Watanabe, M., 2007. Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response. J. Gen. Virol. 88 (Pt 12), 3323–3333.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.

Yi, M., Bodola, F., Lemon, S.M., 2002. Subgenomic hepatitis C virus replicons inducing expression of a secreted enzymatic reporter protein. Virology 304 (2), 197–210.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M., Mizusawa, H., 2003. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 4 (6), 602–608.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102 (26), 9294–9299.

Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J.A., Chisari, F.V., 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. 80 (22), 11082–11093.

Antiviral Effects of the Interferon-Induced Protein Guanylate Binding Protein 1 and Its Interaction with the Hepatitis C Virus NS5B Protein

Yasuhiro Itsui,[1,2]* Naoya Sakamoto,[1,3]* Sei Kakinuma,[1] Mina Nakagawa,[1,3] Yuko Sekine-Osajima,[1] Megumi Tasaka-Fujita,[1] Yuki Nishimura-Sakurai,[1] Gouki Suda,[1] Yuko Karakama,[1] Kako Mishima,[1] Machi Yamamoto,[1] Takako Watanabe,[1] Mayumi Ueyama,[1] Yusuke Funaoka,[1] Seishin Azuma,[1] and Mamoru Watanabe[1]

Interferons (IFNs) and the interferon-stimulated genes (ISGs) play a central role in antiviral responses against hepatitis C virus (HCV) infection. We have reported previously that ISGs, including guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27, inhibit HCV subgenomic replication. In this study we investigated the effects of these ISGs against HCV in cell culture and their direct molecular interaction with viral proteins. HCV replication and virus production were suppressed significantly by overexpression of GBP-1, IFI-6-16, or IFI-27. Knockdown of the individual ISGs enhanced HCV RNA replication markedly. A two-hybrid panel of molecular interaction of the ISGs with HCV proteins showed that GBP-1 bound HCV-NS5B directly. A protein truncation assay showed that the guanine binding domain of GBP-1 and the finger domain of NS5B were involved in the interaction. Binding of NS5B with GBP-1 inhibited its guanosine triphosphatase GTPase activity, which is essential for its antiviral effect. Taken together, interferon-induced GBP-1 showed antiviral activity against HCV replication. *Conclusion:* Binding of the HCV-NS5B protein to GBP-1 countered the antiviral effect by inhibition of its GTPase activity. These mechanisms may contribute to resistance to innate, IFN-mediated antiviral defense and to the clinical persistence of HCV infection. (HEPATOLOGY 2009;50:1727-1737.)

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality.[1] Hepatitis C is characterized by persistent infection of the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses, not only by way of therapeutic applications[2] but also as a natural cellular antiviral mechanism.[3,4] Interferons are produced naturally in response to virus infection and to cellular exposure to IFN itself. Binding of the IFNs to their receptors activates the Jak-STAT pathway to form a complex with IFN-stimulated gene factor-3 (ISGF3), which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of the IFN-stimulated genes (ISGs), and activates expression of ISGs.

HCV subgenomic replicons constitute *in-vitro* models that simulate cellular autonomous replication of HCV

Abbreviations: CLEIA, chemiluminescence enzyme immunoassay; Fluc, firefly luciferase; GBP-1, guanylate binding protein 1; GTPase, guanosine triphosphatase; HCV, hepatitis C virus; IFN, interferon; IgG, immunoglobulin G; ISG, interferon-stimulated gene; ISGF3, IFN-stimulated gene factor-3; IRF-1, interferon regulatory factor 1; ISRE, IFN-stimulated response element; NF-κB, nuclear factor-kappaB; NS, nonstructural.

From the [1]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; [2]Soka Municipal Hospital, Saitama, Japan; and [3]Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan.

Received March 12, 2009; accepted July 24, 2009.

Supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare, the Japan Health Sciences Foundation, and the National Institute of Biomedical Innovation.

**Yasuhiro Itsui and Naoya Sakamoto contributed to this work.*

Address reprint requests to: Naoya Sakamoto, M.D., Ph.D., Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: nsakamoto.gast@tmd.ac.jp; fax +81 3-5803-0268.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23195

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

genomic RNA. Replication of the HCV replicon can be abolished by treatment with small amounts of type-I and type-II IFNs.[5,6] These findings suggest that various molecules encoded by the ISGs have antiviral activities against HCV replication. We have reported previously that the baseline activities of ISG expression are substantially decreased in cells expressing HCV replicon and that this decrease is partly attributable to the transcriptional suppression of interferon regulatory factor 1 (IRF-1).[7] We performed expressional screening of ISGs to investigate their antiviral effects against HCV replication and showed that guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27 had novel activities against cells harboring an HCV replicon.[8] In this study we investigated the antiviral effects and molecular mechanism of GBP-1, IFI-6-16, and IFI-27 on HCV-JFH1-infected cells.

Materials and Methods

Cells and Cell Culture. Huh7, Huh7.5.1, and 293T 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. To maintain cell lines carrying the HCV replicon (Huh7/Rep-Feo cells), G418 (Nacalai Tesque, Kyoto, Japan) was added to the culture medium to a final concentration of 500 μg/mL.

HCV Replicon Constructs and Transfection. The HCV replicon plasmids, which contain Rep-Feo, were derived from the HCV-N strain, pHC1bneo/delS (1b-Feo) and HCV-JFH1 strain, pSGR-JFH1 (2a-Feo).[6,9] These constructs express a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase. The replicon RNA synthesis and transfection have been described (Huh7/Rep-1bFeo, Huh7/Rep-2aFeo).[10,11]

HCV Cell Culture System. A plasmid, pJFH1-full,[12] which encodes the full-length HCV-JFH1 sequence, was linearized and used as a template for synthesis of HCV RNA using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNaseI (RQ-1, RNase-free DNase, Promega) treatment, the transcribed HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh-7.5.1 cells were washed twice, 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 subjected to an electric pulse (1,050 μF and 270 V) using the Easy Ject system (EquiBio, Middlesex, UK). After electroporation, the cell suspension was left for 5 minutes at room temperature and then incubated under normal culture conditions in a 10-mm diameter cell culture dish. The transfected cells were split every 3 to 5 days. The culture supernatants were subsequently transferred onto uninfected Huh-7.5.1 cells.

Construction of Plasmids Expressing ISGs and Analysis of Their Effect on HCV Subgenomic and Genomic Replication. We constructed plasmids expressing GBP-1, IFI-6-16, IFI-27, and IRF-1. The full-length human ISGs were amplified by polymerase chain reaction (PCR) from Huh7 cells and cloned into pcDNA3.1D/V5-His-TOPO (pcDNA4/TO/myc-his for IRF-1) (Invitrogen) to yield the mammalian expression construct, pcDNA-ISG. The ISG-expression plasmid, pcDNA-ISG, was transfected into Huh7/Rep-1bFeo or Huh7/2aFeo cells, and the replication level of the HCV replicon was analyzed by luciferase assay. A plasmid, pcDNA3.1D/V5-His/lacZ (Invitrogen), was used as a control plasmid vector for mock transfection.

Another plasmid, pcDNA-ISG, was transfected into HCV-JFH1 cell culture systems. Forty-eight hours after transfection the culture supernatants, total cellular RNA, and protein, which were used for quantification of HCV core antigen, were harvested.

Luciferase Assays. Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual-Luciferase Reporter Assay System (Promega).

Real-Time Reverse Transcription (RT)-PCR Analysis. Total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate complementary DNA (cDNA) from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of messenger RNA (mRNA) was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers have been described.[13]

Quantification of HCV Core Antigen in Culture Supernatants. Culture supernatants of JFH1-RNA transfected Huh-7.5.1 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).

Synthetic Short Hairpin RNA (shRNA) and shRNA-Expression Plasmid. ISG-directed shRNA expression vectors (pUC19-shRNA-ISG) were designed and constructed as described.[11] Briefly, oligodeoxyribonucleotides encoding shRNA sequences were synthesized and cloned just downstream of the human U6 promoter in the

plasmid pUC19. To avoid problems of structural instability of DNA strands arising from the tight palindrome structure during transcription of shRNA, several point mutations were introduced into the sense strand of the shRNA sequences, which retained fully the silencing activity of the shRNA.[11] Sequences of the shRNAs are shown in Supporting Table 1.

Construction of Plasmids Expressing Full-Length and Truncated HCV-NS Proteins. Expression plasmids of HCV-NS3, NS4B, NS5A, and NS5B were constructed by inserting PCR-amplified fragments encoding each HCV-NS protein into pcDNA4/TO/myc-his (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively). The plasmids, which expressed truncated HCV-NS5B proteins, were generated by insertion of various fragments amplified by PCR using pcDNA-NS5B into pcDNA4/TO/myc-his.

Immunoprecipitation Assay. Plasmids expressing HCV-NS protein (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, or pcDNA-NS5B) and plasmids expressing ISG (pcDNA-GBP-1, pcDNA-IFI-6-16, or pcDNA-IFI-27) were cotransfected into HEK-293 T cells. Forty-eight hours after transfection, cellular proteins were harvested and immunoprecipitation assay was performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). The immunoprecipitated proteins were analyzed by western blotting.

Mammalian Two-Hybrid Assay. Mammalian two-hybrid assay (Stratagene, La Jolla, CA) is a method for detecting protein-protein interactions in vivo in mammalian cells. In this assay a gene encoding the HCV-NS protein (NS3, NS4B, NS5A, and NS5B) was fused to the DNA-binding domain of the yeast protein GAL4 (a bait plasmid, pcCMV-BD), whereas another gene (ISG; GBP-1, IFI-6-16, and IFI-27) was fused to the transcriptional activation domain of the mouse protein nuclear factor-kappaB (NF-κB) (an acceptor plasmid, pCMV-AD). These two-hybrid constructs are cotransfected into Huh-7 cells with a reporter plasmid encoding the firefly-luciferase gene. If the ISG protein and HCV-NS protein interact, they create a functional transcriptional activator by bringing the activation domain into close proximity with the DNA-binding domain; this can be detected by expression of the luciferase reporter gene. The ISG-encoding site was inserted into a plasmid, pCMV-AD, by cloning from a plasmid, pcDNA-ISG (pCMV-AD-ISG). The HCV-NS protein gene, which was subcloned from a plasmid, pcDNA-NS protein, was also inserted into a plasmid, pCMV-BD (pCMV-BD-NS protein). The plasmids pCMV-AD-ISG and pCMV-BD-NS protein were cotransfected with a reporter plasmid, pFR-luc encoding Fluc into Huh-7 cells. Cellular proteins were harvested after 48 hours and luciferase assays were performed.

Immunohistochemistry. Huh7.5.1 cells infected with HCV-JFH-1 were seeded onto 18-mm round microcover glasses (Matsunami, Tokyo, Japan). After transfection of plasmids expressing ISG, pcDNA-ISG, Huh7.5.1 cells were fixed with cold acetone. The cells were incubated with the primary antibodies for 1 hour at 37°C and with Alexa Flour 488 goat antimouse immunoglobulin G (IgG) antibody and Alexa 568 donkey antigoat IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VectaShield Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized with fluorescence microscopy (BZ-8000, Keyence, Osaka, Japan).

The Establishment of a Mutant Form of GBP-1. In order to introduce mutations into GBP-1, the full-length human ISGs were amplified by PCR from a human liver cDNA library (Invitrogen) and cloned into pCMV-Tag Epitope Tagging Mammalian Expression Vectors; pCMV-GBP-1 (Stratagene). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II Site-Directed Mutagenesis Kit; Stratagene): R48P; pCMV-GBP1.R48P.

Guanosine Triphosphatase (GTPase) Assay. Cellular proteins were harvested from Huh7 cells 48 hours after transfection of a plasmid expressing HCV-NS5B, pcDNA-NS5B, or a plasmid for mock transfection, pcDNA3. A GTPase assay was performed to examine GTPase activity of cellular proteins using GTPase ELIPA kits (Cytoskelton, Denver, CO). The assay is based on an absorbance shift (340 to 360 nm) that occurs when 2-amino-6-mercapto-7-methylpurine ribonucleoside is catalytically converted to 2-amino-6-mercapto-7-methyl purine in the presence of inorganic phosphate (Pi). The reaction is catalyzed by purine nucleoside phosphorylase. One molecule of inorganic phosphate will yield one molecule of 2-amino-6-mercapto-7-methyl in an essentially irreversible reaction. Thus, the absorbance at 360 nm is directly proportional to the amount of Pi generated in the reaction.

Transient Transfection. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Luciferase Assays. Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega).

Western Blot Analysis. Western blotting was performed as described.[10] Briefly, 10 μg of total cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a poly-