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# HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy

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## ABSTRACT

**Background and aims** A number of recent studies have shown that human polymorphisms near the *IL28B* type III interferon (IFNλ) gene influence the response to peg-interferon plus ribavirin combination therapy for infection with chronic hepatitis C virus (HCV). Viral polymorphisms, including substitutions within the HCV core and NS5A proteins, have also been shown to influence treatment outcome, but it is not known whether these factors act independently of the IL28B polymorphism or if they reflect the same or a different underlying mechanism. Multiple logistic regression was used to determine whether host and viral polymorphisms independently predict sustained virological response (SVR).

**Methods** Two single nucleotide polymorphisms were genotyped in the IL28B locus (rs12979860 and rs8099917) from 817 patients with chronic HCV infection, and substitutions at amino acids 70 and 91 of the HCV core protein and within the NS5A interferon sensitivity-determining region (ISDR) were analysed.

**Results** It was found that independent predictors of an SVR included IL28B rs12979860 CC genotype (OR=4.98; p=4.00E-08), core amino acid 70 substitutions (OR=0.53; p=0.016), age and baseline viral load. For non-virological response, the IL28B rs12979860 CT/TT genotype (OR=0.23; p=1.96E-8) and age were independent predictors. IL28B rs12979860 genotype (p=1.4E-8), core amino acid 70 substitutions (p=0.0013), ISDR substitutions (p=0.0019), baseline viral load, γ-glutamyltranspeptidase, alanine aminotransferase and platelet count were independent predictors for change in viral load by week 4 of treatment.

**Conclusions** IL28B polymorphisms and HCV core amino acid 70 substitutions contribute independently to an SVR to peg-interferon plus ribavirin combination therapy.

### Significance of this study

**What is already known about this subject?**

- Clinical and viral factors influence the outcome of peg-interferon plus ribavirin combination therapy for chronic hepatitis C virus infection.
- Polymorphisms within the human IL28B locus strongly influence treatment outcome.
- Substitutions at amino acids 70 and 91 of the HCV core protein as well as within the interferon sensitivity-determining region (ISDR) also affect response to treatment.

**What are the new findings?**

- IL28B polymorphisms as well as substitutions at amino acid 70 both independently predict sustained virological response, suggesting that they influence treatment outcome through different mechanisms.
- IL28B polymorphisms, substitutions at core protein amino acid 70 and ISDR substitutions are each independent predictors for change in viral load after 4 weeks of treatment.

**How might it impact on clinical practice in the forseeable future?**

- The combination of IL28B genotyping and detection of core protein substitutions may yield more accurate pretreatment predictions of treatment efficacy.

## INTRODUCTION

Hepatitis C virus (HCV) is a primary cause of chronic hepatitis and often progresses to liver cirrhosis and hepatocellular carcinoma.[1 2] Peg-interferon plus ribavirin combination therapy (PEG-RBV) is the current standard of care, but it is only effective in 50% of patients and has severe side effects often requiring discontinuation or dose modification.[3] Consequently, reliable predictors are needed to identify unsuitable candidates as early as possible.

Genome-wide association studies have reported common single nucleotide polymorphisms (SNPs) predictive of response to interferon treatment. While polymorphisms in MxA,[4 5] interferon α-receptor 1,[6] osteopontin[7] and MAPKAPK3[8] have been reported to be associated with interferon response, several linked SNPs within the IL28B locus on chromosome 19 have recently been shown to be the strongest predictors of early viral kinetics, response to treatment and spontaneous viral clearance.[9–15]

Viral polymorphisms have also been shown to be associated with treatment response. HCV genotypes 1 and 4 in particular are considered more difficult to treat than genotypes 2 and 3,[16 17] and genotype 3 is associated with steatosis.[18] Within genotype 1b, amino acid substitutions at positions 70 and 91 of the HCV core protein and accumulation of substitutions in the interferon sensitivity-determining region (ISDR) of the NS5A protein[19 20] have also been shown to be associated with treatment outcome, especially among Japanese patients.

Consequently, a number of human and viral factors are now known to affect response to treatment, but in order to identify the most important independent predictors and to identify which, if any, may be useful in guiding clinical practice, it is necessary to analyse them simultaneously in a multivariate model. In this study we therefore attempted to identify host and viral factors that independently predict treatment outcome.

## MATERIALS AND METHODS

### Patients

Data from 817 patients who were treated with PEG-RBV combination therapy for chronic hepatitis C genotype 1b infection between 2002 and 2008 were collected from Toranomon Hospital (Tokyo) and hospitals that belong to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) in Hiroshima, Japan. Study subjects tested positive for HCV RNA over a span of >6 months, were negative for hepatitis B and HIV, and showed no evidence of other liver diseases. Patients received weekly injections of peg-interferon-α2b at 1.5 g/kg body weight for 48 weeks and ribavirin was administered orally. The amount of ribavirin was adjusted based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients with low baseline viral load (<5 log IU/ml) were excluded, as were patients who received <0.89 g/kg of peg-interferon or <8.3 mg/kg of ribavirin. Treatment success was evaluated based on a sustained virological response (SVR), defined as undetectable HCV RNA levels 24 weeks after cessation of treatment. Some patients showed a transient response (TR or relapser), in which HCV RNA dropped to undetectable levels during treatment but then later rebounded. In those with a non-viral response (NVR), HCV RNA levels failed to decline by 2 log10 IU/ml by week 12 of treatment and never dropped below detectable levels. Histopathological diagnosis was made according to the criteria of Desmet *et al*.[21] All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

### HCV RNA levels

HCV RNA levels were monitored throughout the course of treatment at 1 or 2 month intervals for a total of at least six time points via reverse transcription–PCR (RT–PCR) using the original Amplicor method, the high range method or the TaqMan RT–PCR test. The measurement ranges of these assays were 0.5–850 kIU/ml, 5–5000 kIU/ml and 1.2–7.8 log IU, respectively. Samples exceeding the measurement range were diluted with phosphate-buffered saline (PBS) and reanalysed. All values were reported as log IU/ml.

### ISDR and core amino acid substitutions

Amino acid substitutions in the HCV core and ISDRs were determined by direct sequencing of PCR products following extraction and reverse transcription of serum HCV RNA. Core amino acid substitutions at positions 70 and 91 (core70 and core91) were determined according to Akuta *et al*,[22 23] and the number of ISDR substitutions was established as in Enomoto *et al*.[19 21 24] Of the 817 patients in the study, substitutions for both ISDR and core70 could be determined for 379 patients.

### SNP genotyping

We genotyped each patient for two IL28B SNPs previously reported to be associated with treatment outcome, rs12979860 and rs8099917.[9–11] Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously.[25 26] The two SNPs are in strong linkage disequilibrium, with a correlation coefficient of 0.99. SNP genotypes for both rs12979860 and rs8099917 were determined for 815 patients (99.7%).

### Statistical analysis

All analyses were performed using the R statistical package (http://www.r-project.org). Non-parametric tests ($\chi^2$ and Mann–Whitney U tests) were used to detect significant associations. All statistical analyses were two sided, and $p<0.05$ was considered significant. Simple and multiple logistic regression analyses were used to examine the association between viral substitutions and clinical factors using $p<0.05$ as the criterion for inclusion in the initial multivariate model. Multivariate logistic regression analysis was performed using forward/backward stepwise selection based on Akaike Information Criterion (AIC) score and validated using the rms package in R. ORs and 95% CIs were calculated for each factor.

## RESULTS

### Patient characteristics

Patient profiles are shown in table 1. Forty-five per cent of patients achieved an SVR, 22% were transient responders and 33% failed to respond to treatment (NVR). Males were significantly more likely to achieve an SVR than females (50% and 38%, respectively; p=0.0011), and younger patients were more likely to achieve an SVR than older patients (59.2% and 40.9% above and below median age 58, respectively; p=1.57E-6). Patients who achieved an SVR also had lower γ-glutamyltranspeptidase (γGTP) levels (36 IU/l vs 45 IU/l; p= 0.008) and higher platelet counts (17.1 vs $15.3\times10^{10}$/L; p=3.649E-05) than those who did not.

### IL28B SNP genotypes

The genotypes of two IL28B SNPs were measured for each patient. Because of linkage disequilibrium, SNP results are nearly interchangeable. However, six patients showed an intermediate haplotype consisting of the favourable genotype for rs8099917 (TT) but an unfavourable genotype for rs12979860 (CT), whereas only one of the six patients achieved an SVR, suggesting that rs12979860 is a better predictor of SVR in this data set.

The frequency of the risk allele (T) for rs12979860 was 0.15 among all patients and 0.08 in SVR patients, 0.14 in TR patients and 0.27 in NVR patients. Patients homozygous for the rs12979860 favourable allele (CC) were significantly more likely to achieve an SVR compared with those with TC or TT genotypes (53% vs 24%, OR=3.55, p=3.95E-13). Conversely, patients with the risk allele (TC or TT) were significantly more likely to show an NVR (55% vs 25%; OR=0.265; p=4.4E-16). Patients with the rs12979860 CC genotype had a marginally lower baseline viral load (6.6 vs 6.4 log IU/ml; p=0.093), but showed significantly greater reduction in viral load by week 4 of treatment (−3.2 vs −0.8 log IU/ml; p<2.2E-16). The rs12979860 CC genotype was also associated with wild type core70 (78% vs 54%; p=1.6E-6) and non-wild type ISDR (67% vs 83%; p=0.007).

The frequency of the rs8099917 risk allele (G) was 0.15 among all patients, 0.08 in SVR patients, 0.13 in TR patients and 0.26 in NVR patients. Patients with the rs8099917 TT genotype were significantly more likely to achieve an SVR than patients with GT or GG genotypes (53% vs 24%, OR=3.43, p=2.18E-12), and GT/GG patients were significantly more likely to show an NVR

**Table 1** Patient profiles by response to treatment

| | All (813) | SVR (366) | TR (176) | NVR (271) |
|---|---|---|---|---|
| Sex (M/F) | 459/354 | 231/135 | 84/92 | 144/127 |
| Age | 58 (51–65) | 56 (47–63) | 60.5 (56–65.25) | 59 (52.5–66) |
| Body weight (kg) | 59 (52–67) | 60 (52–68.25) | 58 (51–66) | 60 (52–66.4) |
| BMI (kg/m$^2$) | 22.61 (20.81–24.65) | 22.44 (20.46–24.58) | 22.85 (20.85–24.89) | 22.76 (21.12–24.63) |
| Hypertension (yes/no) | 141/672 | 61/305 | 29/147 | 51/220 |
| Diabetes (yes/no) | 97/716 | 31/335 | 25/151 | 41/230 |
| Fibrosis (0–2/3–4) | 138/421 | 52/227 | 34/81 | 52/113 |
| Activity (0–1/2–3) | 274/272 | 136/138 | 53/56 | 85/78 |
| ISDR (0, 1/≥2) | 78/298 | 43/128 | 15/71 | 20/99 |
| Amino acid 70 (wild-type/mutant) | 256/139 | 137/45 | 54/35 | 65/59 |
| Amino acid 91 (wild-type/mutant) | 221/178 | 112/72 | 51/40 | 58/66 |
| WBC (/L) | $4.71\times10^9$ ($3.9\times10^9$–$5.7\times10^9$) | $4.9\times10^9$ ($4.0\times10^9$–$6.0\times10^9$) | $4.6\times10^9$ ($3.8\times10^9$–$5.4\times10^9$) | $4.6\times10^9$ ($3.7\times10^9$–$5.5\times10^9$) |
| Haemoglobin (g/dl) | 14.1 (13.2–15) | 14.2 (13.3–15.22) | 13.9 (13.1–14.8) | 14.1 (13.05–14.9) |
| Platelets ($\times10^4$L) | $16.1\times10^6$ ($12.5\times10^6$–$19.9\times10^6$) | $17.1\times10^6$ ($13.7\times10^6$–$20.7\times10^6$) | $15.5\times10^6$ ($11.3\times10^6$–$18.8\times10^6$) | $15.1\times10^6$ ($12\times10^6$–$19.2\times10^6$) |
| AST (IU/l) | 45 (34–65.5) | 43 (32.25–64) | 43.5 (33.25–66) | 48 (37–66.5) |
| ALT (IU/l) | 55 (37–87) | 57 (37–92) | 50 (33–78) | 53 (39–82.5) |
| γGTP (IU/l) | 40 (25–72) | 36 (23–65.75) | 36 (23–69) | 52 (32–86)a |
| Albumin (g/dl) | 3.9 (3.7–4.1) | 3.9 (3.7–4.1) | 3.8 (3.7–4) | 3.8 (3.7–4.1) |
| Total cholesterol (mg/dl) | 171 (150–192) | 169 (149.2–192) | 175 (158–191) | 170 (148.5–192.5) |
| Viral load (log IU/ml) | 6.5 (6.1–6.9) | 6.4 (5.9–6.825) | 6.6 (6.3–7) | 6.6 (6.2–7) |
| PEG-IFN-α2b (μg) | 80 (80–100) | 80 (80–100) | 80 (75–100) | 80 (60–100) |
| PEG-IFN-α2b/kg (μg/kg) | 1.19 (1.19–1.48) | 1.36 (1.19–1.48) | 1.19 (1.19–1.48) | 1.19 (1.02–1.48) |
| Ribavirin (mg) | 600 (600–800) | 600 (600–800) | 600 (600–800) | 600 (400–800) |
| Ribavirin/kg (mg/kg) | 8.9 (8.9–11.87) | 10.29 (8.9–11.87) | 8.9 (8.9–11.87) | 8.9 (7.8–11.86) |
| rs12979860 (CC/CT/TT) | 582/203/27 | 311/51/4 | 128/43/4 | 143/109/19 |
| rs8099917 (TT/TG/GG) | 588/199/25 | 311/51/3 | 132/40/4 | 145/108/18 |

For categorical data, the number of patients in each category is shown. For continuous data, the median and range are displayed.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; M, male; NVR, non-virological response; PEG-IFN, pegylated interferon; SVR, sustained virological response; TR, transient response; WBC, white blood cells.

(56% vs 25%; OR=0.26; p=3.33E-16). Patients with the rs8099917 TT genotype had marginally higher baseline viral load (6.6 vs 6.4 log IU/ml; p=0.077) but showed a significantly greater drop in viral load by week 4 of treatment (−3.1 vs −0.8 log IU/ml; p<2.2E-16). The rs8099917 TT genotype was also associated with wild-type core70 (79% vs 56%; p= 3.1E-6) and non-wild-type ISDR (68% vs 83%; p=0.015).

### Viral substitutions

Patients who achieved an SVR had significantly lower initial HCV RNA levels than those who did not (6.4 vs 6.6 log IU/ml; p=2.1E-6). The 140 patients (17%) with a substitution at position 70 of the HCV core protein (core70) were significantly less likely to achieve an SVR than patients with wild type core70 (33% vs 53%; p=0.00019) and were significantly more likely to show an NVR (42% vs 25%; p=0.0013). The 179 (22%) of patients with a substitution at position 91 (core91) were marginally less likely to achieve an SVR (41% vs 50%; p=0.08) but were significantly more likely to show an NVR (37% vs 27%; p=0.039). The 78 (10%) of patients who had two or more substitutions in the ISDR of NS5A were only marginally less likely to achieve an SVR than those with wild-type ISDR (43% vs 55%; p=0.066) and were not more likely to show an NVR (33% vs 26%; p=0.24).

### Predictive factors for an SVR

Significant univariate predictors for an SVR included patient clinical factors (age, sex, diabetes, platelet count, white blood cell count, haemoglobin level, γGTP level); SNP genotype (rs12979860 and rs8099917); and viral factors (baseline viral load and core70, core91 and ISDR substitutions) (table 2). Following multivariate analysis, only age, rs12979860 genotype, core70 substitution and baseline viral load were significant independent predictors (figure 1A). The joint effects of rs12979860 and core70 on response to treatments are illustrated in figure 2.

### Predictive factors for an NVR

Significant univariate predictors for an NVR included age, rs12979860 and rs8099917 genotypes, core70 and core91 substitutions, diabetes, aspartate aminotransferase (AST), baseline viral load, platelet count, white blood cell count and γGTP levels (table 3). Following multivariate analysis only age and rs12979860 genotype remained as independent predictors (figure 1B).

### Predictive factors for change in viral load by week 4 of treatment

Factors influencing virological response were assessed by examining change in viral load between the start of treatment and week 4. Using linear regression, sex, rs12979860, rs8099917, core70, core91, ISDR, baseline viral load, alanine aminotransferase (ALT), platelet count, white blood cell count, haemoglobin level and γGTP were found to be significant univariate predictors of change in viral load by week 4 (table 4). Independent factors included rs12979860, core70, ISDR, ALT, platelet count and γGTP. We also found a significant positive linear relationship between the total number of ISDR substitutions and change in viral load between week 0 and week 4 (slope=0.2; p=0.0047).

In patients with the favourable rs12979860 CC genotype, core70 wild type was a significant predictor of viral decline (p=0.007; figures 3A,B), but in patients with the CT or TT genotypes, viral decline did not vary with respect to core70 substitutions (p=0.18; figures 3C,D). Conversely, ISDR was not

**Table 2** Predictors for a sustained virological response

| | Simple | | | Multiple | | | |
|---|---|---|---|---|---|---|---|
| **Variable** | **n** | **OR** | **p Value** | **n** | **OR** | **95% CI** | **p Value** |
| Age | 813 | 0.58 | 1.22E-08*** | 362 | 0.432 | 0.31 to 0.60 | 6.61E-07*** |
| Sex (male vs female) | 813 | 1.28 | 0.0006*** | 362 | 1.2 | 0.95 to 1.54 | 0.133 |
| BMI (kg/m$^2$) | 800 | 0.87 | 0.1286 | | | | |
| rs12979860 (CC vs TC/TT) | 812 | 3.65 | 2.67E-14*** | 362 | 4.98 | 2.81 to 8.82 | 4.00E-08*** |
| rs8099917 (TT vs GT/GG) | 812 | 3.53 | 1.77E-13*** | | | | |
| Hypertension | 813 | 0.92 | 0.6452 | | | | |
| Diabetes | 813 | 0.53 | 0.005907** | | | | |
| Core amino acid 70 (wild type vs mutant) | 395 | 0.42 | 5.82E-05*** | 362 | 0.527 | 0.31 to 0.89 | 0.01575* |
| Core amino acid 91 (wild type vs mutant) | 399 | 0.66 | 0.0419* | | | | |
| ISDR | 376 | 1.12 | 0.1627 | | | | |
| Viral load (log IU/ml) | 695 | 0.68 | 2.09E-06*** | 362 | 0.77 | 0.62 to 0.96 | 0.02249* |
| Fibrosis (F0–1 vs F2–4) | 559 | 0.74 | 0.0817 | | | | |
| Activity (A0–1 vs A2–4) | 546 | 0.96 | 0.7975 | | | | |
| Total cholesterol (mg/dl) | 663 | 0.86 | 0.2151 | | | | |
| AST (IU/l) | 687 | 1.03 | 0.1069 | | | | |
| ALT (IU/l) | 692 | 1.26 | 0.0920 | | | | |
| Platelets ($\times 10^4$/L) | 694 | 1.49 | 3.57E-05*** | 362 | 1.39 | 0.97 to 1.99 | 0.073 |
| WBC (/L) | 693 | 1.31 | 0.0014** | | | | |
| Haemoglobin (g/dl) | 693 | 1.28 | 0.0043** | | | | |
| γGTP (IU/l) | 646 | 0.96 | 0.0052** | | | | |

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

a significant predictor of viral decline in patients with the rs12979860 CC genotype (p=0.078; figures 4A,B), but patients with the CT or TT genotypes and two or more substitutions in the ISDR showed significantly greater viral decline by week 4 than patients with zero or one ISDR substitution (p=0.007; figures 4C,D).

## DISCUSSION

In this study we showed that host factors (younger age, male sex, favourable IL28B SNP genotypes) as well as viral factors (baseline viral load, wild-type core70 and two or more substitutions in the ISDR) contribute to the successful outcome of PEG-RBV combination therapy. Although some of these factors independently predict an SVR or NVR in multivariate analysis, collectively they reflect a complex genotype-by-environment interaction involving common polymorphisms in both the virus and the human host.

Genetic variation within the human IL28 locus has been reported as the strongest pretreatment predictor of an SVR,[15] and the results of this study support this finding. Several tightly linked SNPs in the non-coding region of *IL28A* and *IL28B* have been shown to be associated with spontaneous viral clearance, rapid and early virological response and/or SVR following treatment with interferon and ribavirin for HCV genotype 1b.[9–15] *IL28A*, *IL28B* and *IL29* code for type III (λ) interferons, which are similar to type I interferons but use a different receptor and show high tissue specificity.[27 28] It has not been determined which, if any, of the reported SNPs directly affects function, but the functional SNP probably affects gene expression. IRF3- and IRF7-binding sites near the transcription start

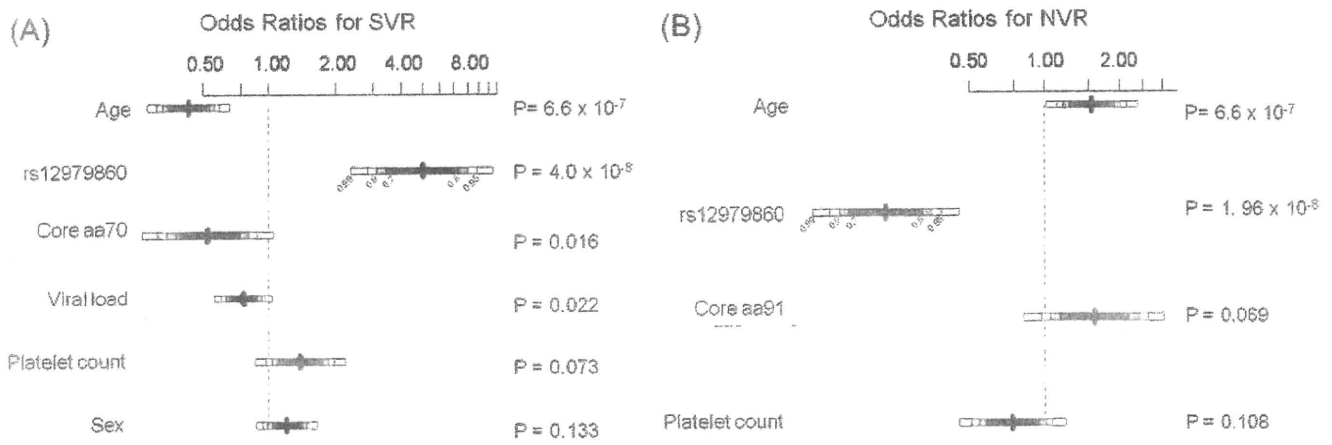


**Figure 1** ORs for predictive factors response to treatment. ORs and 95% CIs are shown for predictive factors for (A) sustained virological response (SVR) and (B) non-virological response (NVR) based on multiple logistic regression with stepwise selection.

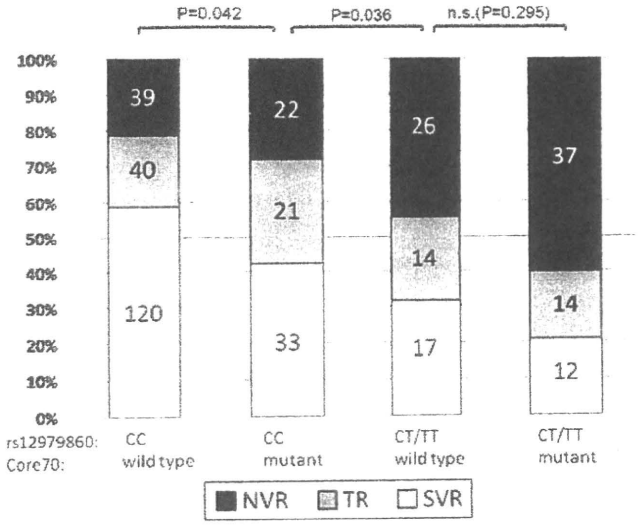


**Figure 2** Cumulative effects of rs12979860 genotype and core protein amino acid 70 substitutions. The relative effects of rs12979860 genotype (favourable CC vs non-favourable CT/CC) and core amino acid 70 substitutions (favourable wild type vs unfavourable substitutions) on response to treatment are shown. NVR, non-virological response; TR, transient response/relapser; SVR, sustained virological response.

site of *IL28B* are essential for gene expression, but distal clusters of nuclear factor-κB (NF-κB)-binding sites are necessary for maximal expression,[29 30] suggesting that upstream polymorphisms may potentially disrupt transcription factor-binding sites within a distal promoter or enhancer. Unintuitively, interferon-stimulated genes are downregulated in patients with the favourable rs8099917 TT genotype,[31] implying that responders have a lower baseline expression of immune response genes.[32] This might serve to prevent desensitisation and promote maximal induction of interferon-stimulated genes, but detailed gene regulation studies are needed to resolve the role of IL28B polymorphisms in antiviral defence.

In addition to effects of human genetic polymorphisms, a number of studies have reported significant association between HCV core70/core91 substitutions and treatment outcome.[20 33 34] We found significant independent associations between core70 substitutions and an SVR, as well as change in viral load by week 4, but the association was not significant for an NVR under multivariate analysis despite being highly significant in univariate analysis. Although the role of core70 substitutions is unclear, the core protein interacts with a number of viral and host proteins and disrupts the interferon signalling pathway.[35–37] The proportion of core70 substitutions in the host viral population has been reported to increase during treatment with PEG-RBV therapy, which may indicate positive selection at this position in response to treatment.[38] Substitutions at these positions appear to affect the antiviral response during the early stages of treatment, as wild-type core70 and core91 are associated with a rapid decrease in HCV RNA levels during the first 4 weeks of treatment.[39 40] Because a rapid virological response is also a strong predictor of SVR and NVR, core70 and core91 substitutions may affect treatment outcome either directly or indirectly.[40 41]

Unlike HCV core70 substitutions, we found only a marginal association between ISDR substitutions and SVR, and no association with NVR. However, ISDR substitution was a significant independent predictor of change in viral load by week 4. The presence of two or more mutations in this 40 amino acid stretch of the NS5A protein is associated with an SVR.[24 42] Other studies have found no significant association between ISDR and SVR but have found a higher overall mutation rate in the NS5A protein among SVR patients,[43 44] and one study suggests that the association with ISDR varies by strain and is more pronounced in Japan than in Europe.[45] It is not clear whether mutations in ISDR directly affect function or whether they reflect the genetic distance from an interferon-resistant

**Table 3** Predictors for a non-virological response

| | Simple | | | Multiple | | | |
|---|---|---|---|---|---|---|---|
| **Variable** | **n** | **OR** | **p Value** | **n** | **OR** | **95% CI** | **p Value** |
| Age | 813 | 1.30 | 0.01306* | 370 | 1.55 | 1.12 to 2.15 | 0.008367** |
| Sex (male vs female) | 813 | 0.90 | 0.178 | | | | |
| BMI (kg/m$^2$) | 800 | 1.07 | 0.3899 | | | | |
| rs12979860 (CC vs TC/TT) | 812 | 0.26 | 2.73E-17*** | 370 | 0.231 | 0.14 to 0.39 | 1.96E-08*** |
| rs8099917 (TT vs GT/GG) | 812 | 0.26 | 1.51E-17*** | | | | |
| Hypertension | 813 | 1.16 | 0.4323 | | | | |
| Diabetes | 813 | 1.55 | 0.04685* | | | | |
| Core amino acid 70 (wild type vs mutant) | 395 | 2.17 | 0.000496*** | | | | |
| Core amino acid 91 (wild type vs mutant) | 399 | 1.66 | 0.02029* | 370 | 1.58 | 0.96 to 2.60 | 0.06943 |
| ISDR | 376 | 0.92 | 0.06197 | | | | |
| Viral load (log IU/ml) | 695 | 1.32 | 0.01716* | | | | |
| Fibrosis (F0–1 vs F2–4) | 559 | 1.24 | 0.2608 | | | | |
| Activity (A0–1 vs A2–4) | 546 | 1.12 | 0.5499 | | | | |
| Total cholesterol (mg/dl) | 663 | 0.98 | 0.5824 | | | | |
| AST (IU/l) | 687 | 1.02 | 0.03148* | | | | |
| ALT (IU/l) | 692 | 0.91 | 0.8772 | | | | |
| Platelets (×10$^4$/L) | 694 | 0.76 | 0.008222** | 370 | 0.739 | 0.51 to 1.07 | 0.1077 |
| WBC (/L) | 693 | 0.83 | 0.04617* | | | | |
| Haemoglobin (g/dl) | 693 | 0.84 | 0.1201 | | | | |
| γGTP (IU/l) | 646 | 1.15 | 1.23E-05*** | | | | |

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

**Table 4** Predictors for change in viral load by week 4 of treatment

| | Simple | | | Multiple | | |
|---|---|---|---|---|---|---|
| **Variable** | **n** | **Coefficient** | **p Value** | **n** | **Coefficient** | **p Value** |
| Age | 500 | −0.01 | 0.138 | | | |
| Sex (male vs female) | 500 | −0.23 | 0.005** | | | |
| BMI (kg/m$^2$) | 494 | 0.00 | 0.958 | | | |
| rs12979860 (CC vs TC/TT) | 500 | 2.11 | 5.18E-38*** | 221 | 1.37 | 1.35E-08*** |
| rs8099917 (TT vs GT/GG) | 499 | 2.10 | 1.40E-36*** | | | |
| Hypertension | 500 | −0.25 | 0.249 | | | |
| Diabetes | 500 | −0.31 | 0.19 | | | |
| Core amino acid 70 (wild type vs mutant) | 259 | −1.01 | 1.38E-05*** | 221 | −0.665 | 0.001328** |
| Core amino acid 91 (wild type vs mutant) | 262 | −0.77 | 0.000*** | | | |
| ISDR | 247 | 0.20 | 0.006** | 221 | 0.186 | 0.001878** |
| Viral load (log IU/ml) | 500 | 0.37 | 0.000*** | 221 | 0.414 | 0.00012*** |
| Fibrosis (F0−1 vs F2−4) | 397 | −0.22 | 0.217 | | | |
| Activity (A0−1 vs A2−4) | 389 | −0.10 | 0.578 | | | |
| Total cholesterol (mg/dl) | 472 | 0.00 | 0.064 | | | |
| AST (IU/l) | 490 | 0.00 | 0.442 | | | |
| ALT (IU/l) | 493 | 0.00 | 0.005** | 221 | 0.00606 | 0.008895** |
| Platelets (×10$^4$/L) | 495 | 0.03 | 0.048* | 221 | 0.0701 | 7.24E-05*** |
| WBC (/L) | 495 | 0.00 | 0.027* | | | |
| Haemoglobin (g/dl) | 495 | 0.13 | 0.013* | | | |
| γGTP (IU/l) | 460 | 0.00 | 0.001*** | 221 | −0.00634 | 0.002095** |

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

strain. Nonetheless, the NS5A protein has been shown to be under purifying selection[44] and plays a critical role in both viral replication[46 47] and modulation of the immune response.[48] Therefore, the number of substitutions in one or more variable regions of the NS5A may be a useful predictor of early viral dynamics and an indirect predictor of SVR, although in this study we found a significant effect only for change in viral load by week 4 of treatment.

A number of factors have now been reported to influence outcome of PEG-RBV therapy, and it is important to determine which of these factors represent independent, clinically useful predictors. Because of the expense and occasionally severe side effects of the current standard of care, reliable pretreatment indicators, especially of poor response, will help guide treatment decisions and steer difficult-to-treat patients towards more

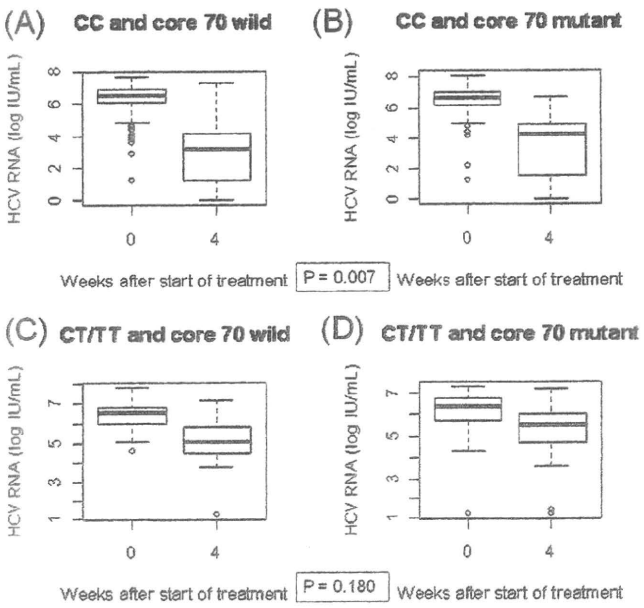


**Figure 3** Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and hepapitis C virus (HCV) core protein substitutions. The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and wild/mutant amino acid at core70 is shown.

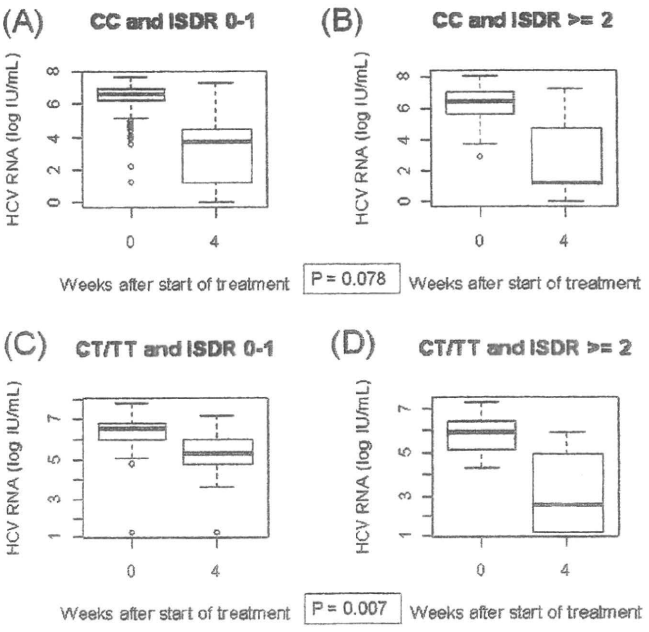


**Figure 4** Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and substitutions in the interferon sensitivity-determining region (ISDR). The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and the number of substitutions in the ISDR is shown.

effective treatments or enrolment in clinical trials. In order to identify the most important independent predictors, it will be necessary to disentangle the intriguing interactions between human and viral polymorphisms as well as gain better understanding of the role of type III interferon in the immune response against HCV.

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＜短　報＞

# C型慢性肝炎に対するペグインターフェロンとリバビリン併用療法における NS3-4Aプロテアーゼ阻害剤（Telaprevir）併用12週間治療のウイルス学的効果の検討

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緒言：現在，C型慢性肝炎に対する治療はペグインターフェロン（PEG-IFN）とリバビリンの併用療法が標準治療法となっているが，海外においては新規の抗HCV薬であるNS3-4A protease inhibitor（Telaprevir）の強力なHCV増殖抑制作用が報告され[1]，PEG-IFNとリバビリンとの3者併用療法により治療効果が飛躍的に改善することが明らかにされてきている．そこで今回我々は，genotype 1型，高ウイルス量のC型慢性肝炎患者に対してPEG-IFNα-2bとリバビリンの併用療法にTelaprevirを併用した3者併用12週間治療のウイルス学的効果を検討した．

対象と方法：対象は，genotype 1b，高ウイルス量の症例で，当院において2008年5月から2008年7月までにPEG-IFNα-2bとリバビリン治療にTelaprevirを併用する3者併用12週間治療を施行することに同意した初回治療例の10例である．男性4例，女性6例，年齢は36-64歳（中央値51歳）であった．Telaprevirは無作為に2群に分類され，A群は1回750 mg，B群は1回500 mgで8時間ごとに3回投与された．投与中のHCV RNAの陰性化をTaqMan PCR法にて評価し，さらに12週併用療法終了後24週経過観察した時点での完全著効（SVR）率を評価した．

結果：治療中および治療終了後の経過をFig. 1に示す．12週間の治療を完遂できたのは5例（50%）であった．4例はヘモグロビン値の低下，1例は倦怠感により治療中止となった．しかしながら，HCV RNAは全例で治療中に陰性化を認め，陰性化時期は2～5週（中央値2週）と非常に早期であった．Case 1～5は12週までに中止となったが，このうち2週目で陰性化した3例は5週目，7週目，10週目に治療を中止したにもかかわらずSVRとなった．Case 6～10は12週間投与を完遂した症例であるが，5週目で陰性化した1例を除き，4例がSVRに至った．最終的なSVR率は全体で7/10例（70%）と高率であった．

Telaprevirの用量はA群6例，B群4例に割り付けられた．中止率は両群とも50%であり，SVR率はA群4/6例（66.7%），B群3/4例（75%）と両群間で治療効果，副作用に差は認めなかった．

男女別にみると，男性3/4例（75.0%），女性4/6例（66.7%）であり，50歳以上の女性のみでみても，3/3例（100%）と高率にSVRを得られた．

HCV core領域70番目のアミノ酸変異の有無から治療効果をみると，wild typeの症例は5/6例（83.3%），mutant typeでは2/4例（50%）がSVRに至った．

考察：NS3-4A protease inhibitor（Telaprevir）を用いたPEG-IFNとリバビリンとの3者併用療法は非常に抗ウイルス効果が高く，以前我々は，genotype 1b型の慢性肝炎症例に対する3者併用12週間投与における治療中のHCV RNA動態を検討し，2週目で50%，4週目で79%，8週目で94%，12週目で100%にHCV RNAの陰性化を認めたことを報告した[2]．今回は，この症例のうち初回治療例について24週間の経過観察終了後の最終的な治療成績を検討した．その結果，初回治療例に対しては12週間の治療でもSVRに至る症例が70%に達し，ウイルス排除を目的とした治療として有用であることが判明した．これは欧米のPROVE1[3]および

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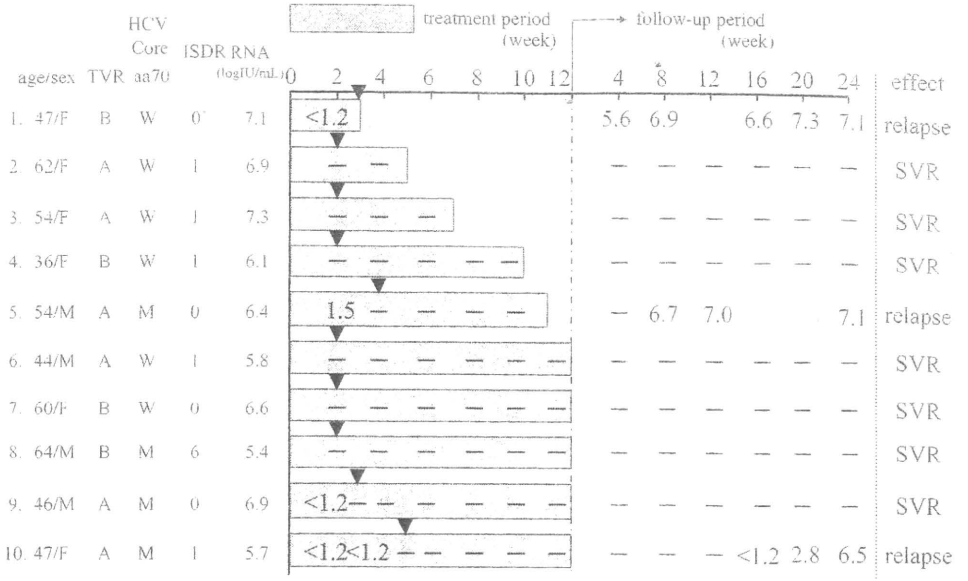


**Fig. 1** Clinical course and dynamics of HCV RNA during and after 24 weeks of the triple treatment with telaprevir, pegylated interferon and ribavirin. TVR, telaprevir; A, 2250 mg/day; B, 1500 mg/day; W, wild type; M, mutant type; ISDR, interferon sensitivity determining resion; * Numbers of amino acids substitutions in ISDR is shown. Arrowheads show the time of HCV RNA loss from the serum.

PROVE2[4]における3剤12週間併用療法の成績（35%と60%）と比較しても良好な成績であるといえる．欧米ではgenotype 1a型の割合が高く，それぞれの対象症例はgenotype 1a型が53%と45%を占めているのが特徴であり，この点が当院のgenotype 1b型の成績と比較しSVR率が低い原因であると考えられる．また，今回の検討ではPEG-IFNとリバビリンの2剤併用療法では治療効果が低いとされる50歳以上の女性においても，全例がSVRに至っており，こういった難治と考えられる症例に対しても治療効果を改善できるものと期待される．

一方，HCV core領域の70番目のアミノ酸がmutant typeの場合，治療中のHCV RNAの陰性化率は良好であるが，12週間の治療ではwild typeに比較してSVR率が低い可能性が示唆された．以前の我々の検討より，3剤併用療法時の治療早期のHCV RNAの低下にcore領域のアミノ酸変異が関与していることを報告しており[5]，今回の検討から最終的な治療成績にもHCV core領域の変異の有無が関与する可能性が考えられる．

Telaprevirを併用した3剤併用療法中における注意点としては，海外からの報告[3][4]にもあるように，掻痒，皮疹の出現頻度が約40-50%と高い点である．当院でも，1例全身性の皮疹の出現により治療中止となった症例を経験した[2]．また，貧血の出現も2剤の併用療法時に比し多いと報告されており，当院でも今回の検討症例を含めた5例がヘモグロビン値8.5 g/dL以下となり，治療中止となったことを報告した．その内3例は5週以内と早期に中止となっており，3剤併用療法時にはヘモグロビンの低下についてより厳重な経過観察が必要であり，早期にリバビリンの減量を考慮する必要があると考えられる．

今回の検討により，genotype 1b型の初回治療例に対しては，Telaprevirを併用した3者併用療法は12週間でも治療効果が高いことが示唆された．Telaprevirの用量については，1日2250 mg群と1500 mg群とで治療効果に差を認めなかったが，最終的な適正用量については現在進行中の12週間の3者併用療法後さらに12週間PEG-IFNとリバビリンを投与する24週間治療の有効性，安全性の結果をもとに検討されるべきである．また24週併用することにより，50歳以上の女性，HCV core領域70番目のアミノ酸がmutant typeの症例あるいは前治療で無効であった症例など難治と考えられる症例でも治療効果を改善しうるか，さらに詳細な検討が必要であると思われる．

索引用語：C型慢性肝炎，リバビリン併用療法，
NS3-4Aプロテアーゼ阻害剤

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## 英文要旨

## The efficacy of virological response in treatment-naïve patients with chronic hepatitis C treated by NS3-4A protease inhibitor (telaprevir), pegylated interferon and ribavirin for 12 weeks

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We investigated the efficacy of the triple treatment with telaprevir, pegylated interferon (PEG-IFN) and ribavirin for 12 weeks in treatment-naïve patients infected with hepatitis C virus (HCV) genotype 1b and high baseline viral loads. All of 10 cases became HCV-RNA negative during treatment. SVR rate attained to a high rate, 70% (7/10). Especially, SVR rate of females over 50 years old attained 100% (3/3). HCV RNA was lost from serum rapidly in patients infected with HCV-1b in high viral loads, and SVR rate of the triple treatment for 12weeks was high. Our results suggested that triple treatment with telaprevir, PEG-IFN and ribavirin could improve the efficacy in treatment-naïve patients.

**Key words**: chronic hepatitis C,
interferon plus ribavirin
combination therapy,
NS3-4A protease inhibitor

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＜短　報＞

# 核酸アナログ未使用のB型慢性肝炎症例へのエンテカビル治療中にrtA181T変異ウイルスが増殖した1症例

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**緒言**：核酸アナログ未使用のB型慢性肝炎患者へのエンテカビル治療中に，既報のエンテカビル耐性ウイルスが出現していないにもかかわらず，viral reboundを生じた症例を経験したため，報告する．

**症例**：51歳女性．1978年にB型慢性肝炎と診断され，2008年6月よりエンテカビル（0.5 mg/日）治療を開始した．治療開始時HBV-DNA 7.2 log copies/m*l*，HBeAg陽性，genotype Cであった．2009年2月HBV-DNA 2.5 log copies/m*l*まで下がるも，その後2009年4月HBV-DNA 6.0 log copies/m*l*，8月8.2 log copies/m*l*とviral reboundが出現し，トランスアミナーゼの上昇も認めた（Fig. 1）．

**治療開始時および治療中のHBV-DNA polymerase RT領域のアミノ酸配列の比較検討**：患者血清から抽出されたHBV-DNAはPCR法にて増幅したのち，direct sequence法にて塩基配列を決定した．クローニング解析もあわせて行った．ダイレクトシークエンスでは核酸アナログ未使用であるにもかかわらず，エンテカビル開始時にrtA181T変異のわずかな混在を認め，クローニング解析では8.5%（3/35クローン）にrtA181T変異を確認した．また治療開始後15カ月ではダイレクトシークエンスにてrtA181T変異の混在の割合が増加しており，クローニング解析にてrtA181T変異は39.5%（17/43クローン）に増加していた．尚，エンテカビル開始時および治療中にrtA181以外の既報のエンテカビル耐性に関与するアミノ酸（rtL180，T184，S202，M204，M250）に変異は認められなかった（Fig. 1）．

**考察**：今回我々は，エンテカビル投与にてrtA181T変異が増殖した症例を経験した．本症例はエンテカビル投与中にviral reboundを生じ，その際既報のエンテカビル耐性ウイルスは出現せず，治療開始時よりわずかに認められていたrtA181T変異ウイルスが増殖していた．クローニング解析にてrtA181T変異ウイルスは治療開始時8.5%から治療開始15カ月後に39.5%に増加し，他に有意なアミノ酸変異を認めないことから，rtA181T変異がエンテカビル耐性に関与している可能性が考えられた．しかし本症例で出現したrtA181T変異ウイルスのエンテカビル耐性への関与を証明するためには，今後本症例の血清を使用したin vitroの実験にて評価する必要があると考える．また本症例ではviral reboundと同時にトランスアミナーゼ上昇も認めたが，軽度上昇にとどまっているため，現在もエンテカビル治療を継続し厳重にフォローしている．

本症例は，核酸アナログ未使用のB型慢性肝炎症例であったにもかかわらず，エンテカビル治療開始前よりrtA181T変異が存在していた．核酸アナログ未使用症例にラミブジン耐性に関与するrtL180M，rtM204V変異が存在するという報告はあるが，本症例のようにrtA181T変異が核酸アナログ使用前に存在したという報告は過去になく，初めての報告である．

rtA181T変異は以前よりアデホビル耐性に関与するアミノ酸変異として知られていたが，最近ではラミブジンとアデホビルの交差耐性のある変異であることがわかっている[1]．このためrtA181T変異に対してエンテカビルの効果が期待されている．しかし海外からは，ラミブジン耐性ウイルスに対するアデホビル単独治療

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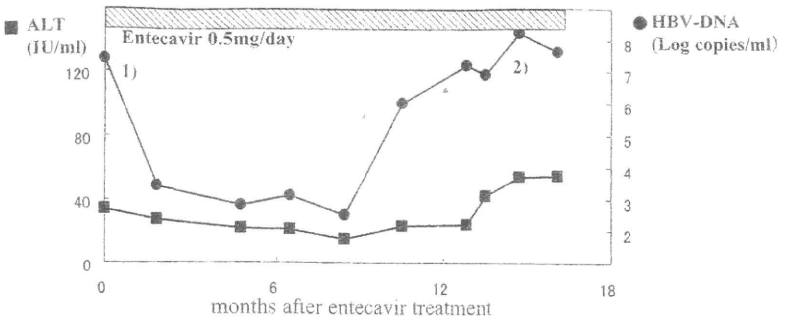


| | rt181 rt184 | rt202 rt204 | rt250 | No. of clones (Relative rate (%) of clones) |
|---|---|---|---|---|
| Amino acid(AB033550) | MGVGLSPFLLAQFTSAI | AFSYMDDL | NFMG | |
| 1) At the beginning of ETV | | | | |
| a) wild | ---------- | -------- | ---- | 32 (91.5%) |
| b) mutant | ----------T------ | -------- | ---- | 3 (8.5%) |
| 2) 15 months after ETV | | | | |
| a) wild | ---------- | -------- | ---- | 26 (60.5%) |
| b) mutant | ----------T------ | -------- | ---- | 17 (39.5%) |

**Fig. 1**　Clinical course and clonal analysis of samples from patient with viral rebound during entecavir therapy

中に耐性ウイルス(rtA181T/VまたはN236T変異ウイルス)が出現した症例は，ラミブジン耐性ウイルスのみの症例に比べ，エンテカビル治療におけるウイルス抑制効果が低いという報告があり[2]，また本症例のようにエンテカビル治療にてrtA181T変異ウイルスが増加する症例も存在することから，今後rtA181T変異ウイルスに対する治療として，エンテカビル以外の核酸アナログ（テノフォビル，その他新規薬剤等）の有効性も検討していく必要があると考えられる．

索引用語：エンテカビル，耐性ウイルス，
rtA181T

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## 英文要旨

## Increase of rtA181T mutant strains during entecavir therapy for a patient with chronic hepatitis B virus infection

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A 51-year-old Japanese woman with chronic hepatitis B who had never treated with nucleotide analogues was admitted to our hospital and treated with entecavir. In this patient, entecavir successfully reduced the HBV level, but viral and biochemical breakthrough was observed at 10 months after the beginning of therapy. The HBV viral load reached up to 8.2 log copies/ml, but direct sequence analysis showed no LAM and ETV resistant-related mutation (rtT184, S202, M204, M250). Comparison by clonal analysis of samples obtained before and after the viral breakthrough showed the increase of the rtA181T mutant strains (8.5% versus 39.5%). It was considered that the rtA181T mutant

strain in this case might be related to entecavir resistance.

**Key words**: entecavir, drug-resistant mutant, rtA181T

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＜速　報＞

# *IL28B* と HCV Core aa70 置換との関連

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はじめに：C型慢性肝炎の治療法であるPEG-IFN/Rivabirin併用療法のHCV genotype 1bで高ウイルス量症例では，その排除率が50％台である．この難治症例の治療効果予測因子としてHepatitis C virus NS5A領域のInterferon sensitivity-determining regionやCore領域の70番目，91番目のアミノ酸置換が有用であることは周知のごとくであったが，近年アメリカ・日本から宿主側因子として*IL28B*のSNPsがPEG-IFN/Rivabirin併用療法の治療効果予測として有用であると報告[1)~5)]されている．今回我々は，C型慢性肝疾患患者のHCV Core aa70と*IL28B*を測定し性差との関連性を検討した．

対象と方法：1997年から2005年までに虎の門病院倫理委員会及びヒトゲノム委員会で承認された同意書を得た患者291人のchromosome 19上の*IL28B*近傍の2つのSNPs（rs8099917（T/G），rs12979860（C/T））とHCV Core領域aa70を測定したHCV genotype 1bとした．内訳は，男性177人（年齢：21-82（中央値56歳），女性114人（年齢：37-82（中央値61）歳）であった．

*IL28B*のSNPs（rs8099917，rs12979860）のタイピングはInvador assay，Taqman assayまたはdirect sequencing法にて決定した．rs8099917は290例，rs12979860は289例のタイピング可能であった．HCV Core領域aa70の測定は，PCR-direct sequence法にて測定した．性別とSNPの遺伝子型を検討した．

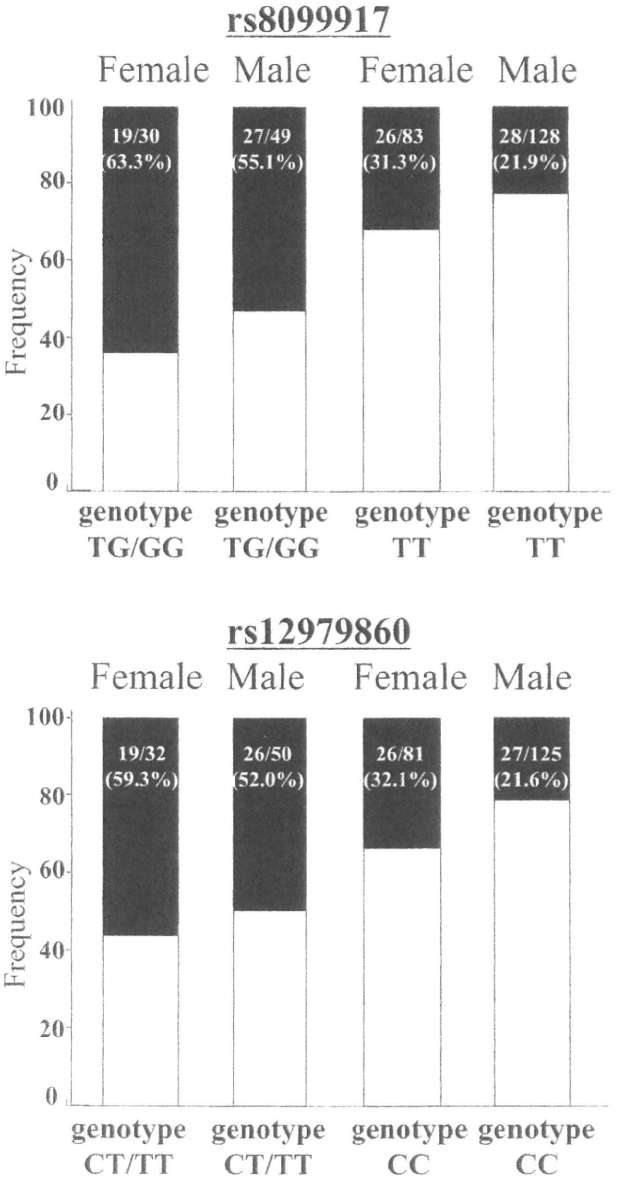


**Fig. 1**　Relationship between *IL28B* SNPs and amino acid substitution in hepatitis C virus core region in patients with chronic hepatitis C. Black bars represent aa70 mutant (Gln) while white bars represent aa70 wild (Arg)

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**結果**：Core aa70 置換からみた *IL28B* の SNP と性差の頻度

rs8099917 に関しては，Core aa70 の Mutant(Gln)がもっとも高頻度にみられたのは genotype TG/GG の女性で 19/30 例(63.3%)，次いで男性の genotype TG/GG で 27/49 例（55.1%），女性の genotype TT で 26/83 例(31.3%)であり，最も低率であったのが男性の genotype TT で 28/128 例（21.9%）であった（Fig. 1）．

rs12979860においても同様の傾向を認め，女性の genotype CT/TT で 19/32 例（59.3%），男性の genotype CT/TT で 26/50 例(52.0%)であり，女性の genotype CC で 26/81 例(32.1%)，男性の genotype CC で 27/125 例（21.6%）であった（Fig. 1）．

**考案**：近年，*IL28B* 領域の SNPs が C 型肝炎ウイルスの自然排除[4]および慢性肝炎の PEG-IFN/Rivabirin 併用療法の治療効果と関連があることが報告された[1)~3)]．我々は，ウイルス側の予測因子である Core aa70 置換について性差を加味して SNP の遺伝子型別にその頻度を解析したところ 2 つの SNP で女性のマイナーアレルホモ接合体及びヘテロ接合体群において Core aa70(Gln) Mutant の頻度がいずれも 50% 台であった．このことは，高齢の女性は PEG-IFN/Rivabirin 併用療法の治療効果が低い傾向を示すことなんらかの関連が推測され，女性において Core aa70 は，経過観察中にメジャークローンとマイナークローンが入れ代わる可能性が示唆された．今後，治療効果予測として宿主側因子の一つである *IL28B* の SNPs と Core aa70 置換の組み合わせにより，より有効な治療効果予測が可能になると思われた．

索引用語：C 型慢性肝疾患，*IL28B*，コア領域

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## 英文要旨

### Relationship between SNPs in the *IL28B* region and amino acid substitutions in HCV core region in Japanese patients with chronic hepatitis C

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*IL28* locus polymorphisms have been reported to affect PEG-IFN plus ribavirin combination therapy for patients with genotype 1b hepatitis C virus (HCV) infection. We examined a relationship between *IL28B* SNPs (rs8099917 and rs12979860) and amino acid substitutions in core region of HCV in patients with genotype 1b chronic hepatitis C. In each SNP, frequency of core aa 70 mutation was higher rate in female patients carrying minor allele than in male or female patients carrying no minor allele. Measurement of *IL28B* and Core aa70 before treatment is useful in PEG-IFN plus ribavirin therapy.

**Key words**: *IL28B*, HCV, core region

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

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

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

**Abbreviations**

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

M. Nakagawa and N. Sakamoto contributed equally to this work.

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

## Introduction

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

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

## Methods

### Patients and methods

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

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

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

### Nucleotide sequencing of the *NS5A* gene

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

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

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

### Nucleotide sequencing of the core gene

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

### Study design and treatment regimens

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

### Outcomes

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

### Statistical analysis

SPSS software package (SPSS 12J for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis, which was carried out using the $\chi^2$ 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 ($\times 10^3$/μl)[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