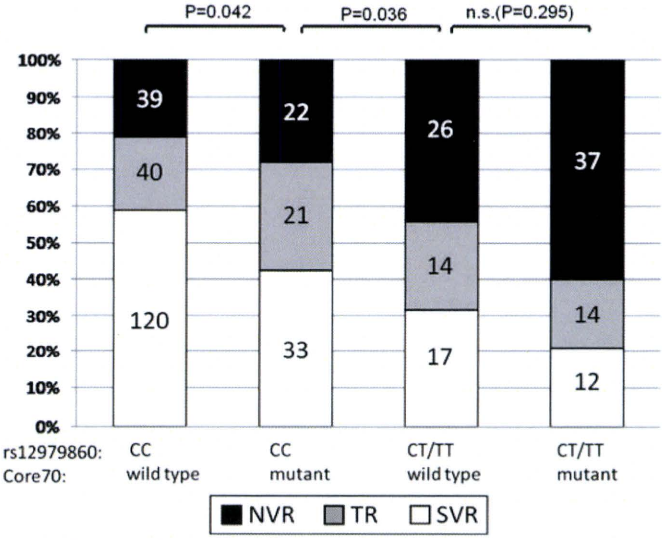

**Figure 2** Cumulative effects of rs12979860 genotype and core protein amino acid 70 substitutions. The relative effects of rs12979860 genotype (favourable CC vs non-favourable CT/CC) and core amino acid 70 substitutions (favourable wild type vs unfavourable substitutions) on response to treatment are 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

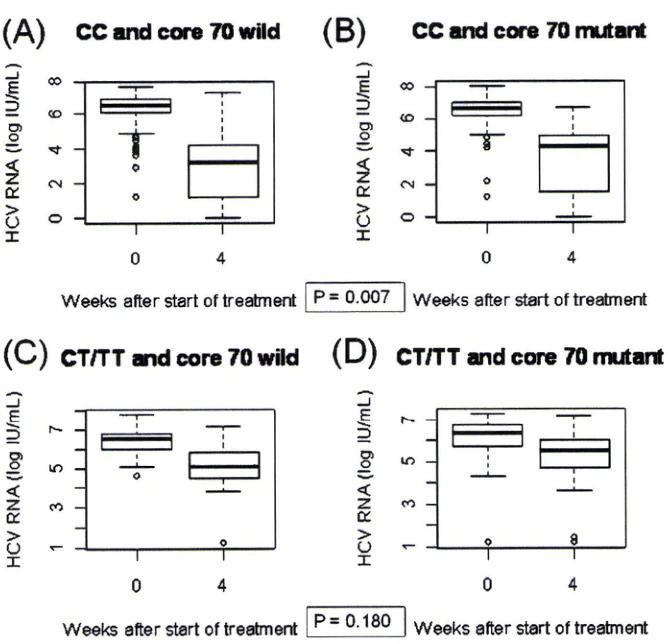

**Figure 3** Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and hepapitis C virus (HCV) core protein substitutions. The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and wild/mutant amino acid at core70 is shown.

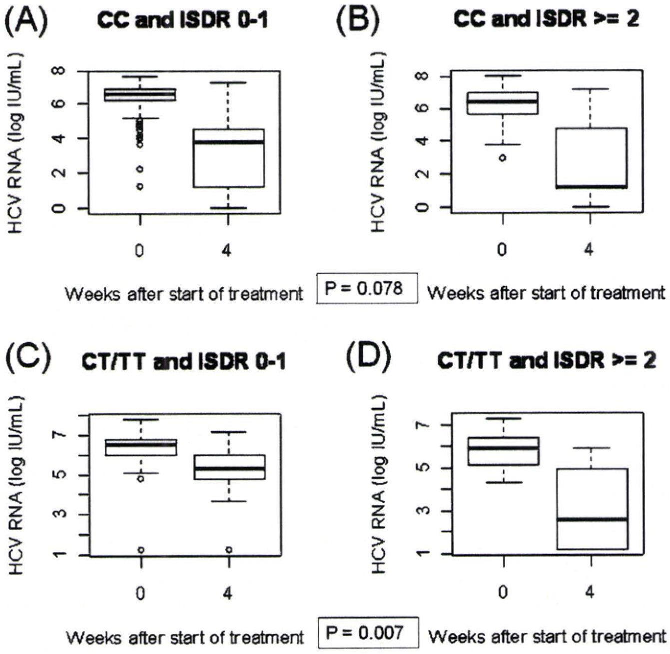

**Figure 4** Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and substitutions in the interferon sensitivity-determining region (ISDR). The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and the number of substitutions 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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**Competing interests** None.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

# Animal model for study of human hepatitis viruses

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**Key words**
hepatitis B virus, hepatitis C virus, uPA/scid mouse model.

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## Abstract

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infect only chimpanzees and humans. Analysis of both viruses has long been hampered by the absence of a small animal model. The recent development of human hepatocyte chimeric mice has enabled us to carry out studies on viral replication and cellular changes induced by replication of human hepatitis viruses. Various therapeutic agents have also been tested using this model. In the present review, we summarize published studies using chimeric mice and discuss the merits and shortcomings of this model.

## Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are pathogens that cause chronic infection in humans. There are 360 million and 170 million people infected worldwide with HBV or HCV, respectively.[1,2] Infected individuals develop acute hepatitis, chronic hepatitis and liver cirrhosis. The viruses are also important causative agents of hepatocellular carcinoma, especially in the Asia–Pacific region.[3] Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model that supports hepatitis virus infection. This is probably as a result of the lack of receptor molecules necessary for viral infection in animal liver cells.

Transgenic mice that express over-length HBV-DNA export viral particles into the serum,[4] and such animals can be used to evaluate antiviral agents,[5–7] as well as HBV-targeted siRNA[8]. However, the virus life cycle is not established in this model, and it is inappropriate for studying drug-resistant HBV strains. Accordingly, researchers attempted to transplant human hepatocytes into mice. The development of the trimera mouse was one such attempt, in which human hepatocytes were transplanted under the kidney capsule of immune-deficient mice after lethal irradiation.[9,10] However, the number of hepatocytes that could survive on the kidney capsule was small, and normal liver architecture was not present. Although 85% of HBV-inoculated animals developed HBV viremia, the titer was less than $10^5$ virus particles or IU/mL.[9] Similarly, 85% of HCV-inoculated animals also developed viremia,[10] but the level of the viremia only reached $10^5$/mL. Thus, the advent of human hepatocyte transplanted uPA/scid mice has provided the first really useful model for acute and chronic infections of human hepatitis virus.

## Human liver cell transplanted uPA/scid mice

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer were developed and shown to have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth.[11] Transplanted rat hepatocytes proliferated and repopulated injured livers in immunodeficient uPA mice, which were produced by mating uPA transgenic mice with scid mice.[12] Human hepatocytes were then transplanted into uPA/scid mice; these cells proliferated and replaced the apoptotic mice liver cells (Fig. 1).

Such human hepatocyte chimeric mice have been shown to be susceptible to both HBV[16] and HCV[17] infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV[13] and HCV[17] were higher in mice in which the repopulation index was higher. A unique attempt to remove mouse residual liver cells with the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system failed to result in a higher repopulation rate as a result of damage to the transplanted human hepatocyte caused by bystander effects.[18] Despite this, mice with livers that have been highly repopulated with human hepatocytes

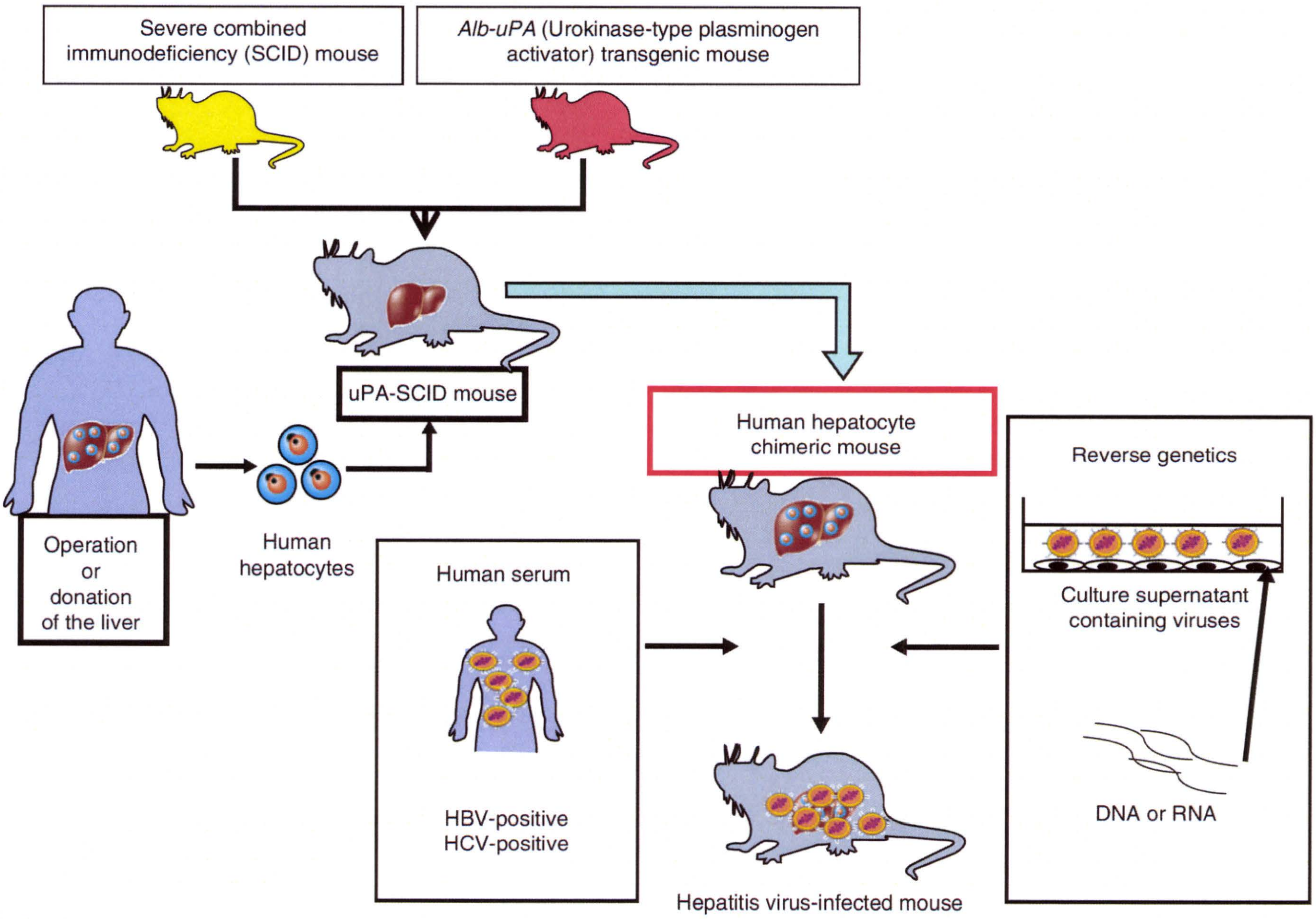

**Figure 1** Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HBC) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV[13] or HCV[14] created in cell culture or by injecting HCV RNA into the mouse liver.[15]

are susceptible to infection with both HBV and HCV, and as such comprised the most effective small animal model for chronic hepatitis so far developed.[19,20] An example of a highly repopulated mouse liver that we are using in experiments is shown in Figure 2.

Highly repopulated mice have been shown to be a valuable model for the study of drug metabolism.[21–29] Advances in technology for human hepatocyte transplantation have enabled serial passage of human hepatocytes in uPA/scid mice and have been shown to retain infectivity for HBV.[30]

This mouse model and other animal models for the study of hepatitis viruses have been summarized in reviews by Meuleman and Leroux-Roels,[31] Dandri *et al.*,[32,33] Barth *et al.*,[34] and Kneteman and Toso.[35] The present review will focus on key issues and updated information.

## Study of hepatitis B virus infection using human hepatocyte chimeric mice

Since the initial reports of successful transmission of HBV to human hepatocyte chimeric mice in 2001 and 2004,[16,27] several researchers have reported transmission of HBV into similar mice.[13,36,37] In these studies, passage experiments studies show that HBV replicating in mice retain infectivity.[13,36] Further, the presence of viral proteins has been shown immunohistochemically in human hepatocytes transplanted into mouse livers, but these are not present in mouse hepatocytes.[13,36,37] Formation of viral particles in infected mouse livers can be shown by electron microscopy.[36,37] Genetically engineered viruses lacking HBe-antigen have also been shown to infect chimeric mice, proving that e antigen is dispensable for viral infection and replication.[13] In contrast, HBx protein has been shown to be indispensable for viral replication.[38] Transcomplementation of HBx protein with hydrodynamic injection restored HBV infectivity in mice. Interestingly, all revertant viruses show a restored ability to express HBx.[38]

By infecting chimeric mice with genotype A, B and C, differing proliferative capacity has been shown between HBV genotypes.[37] In mice infected for a relatively short time, there are no morphological changes in HBV infected mice livers in studies.[13,36] In contrast, the occurrence of liver cell damage has been reported after long-term infection of chimeric mice with HBV[39] or with specific strains of HBV;[40] these findings are consistent with direct cytopathic effects of HBV under certain conditions.

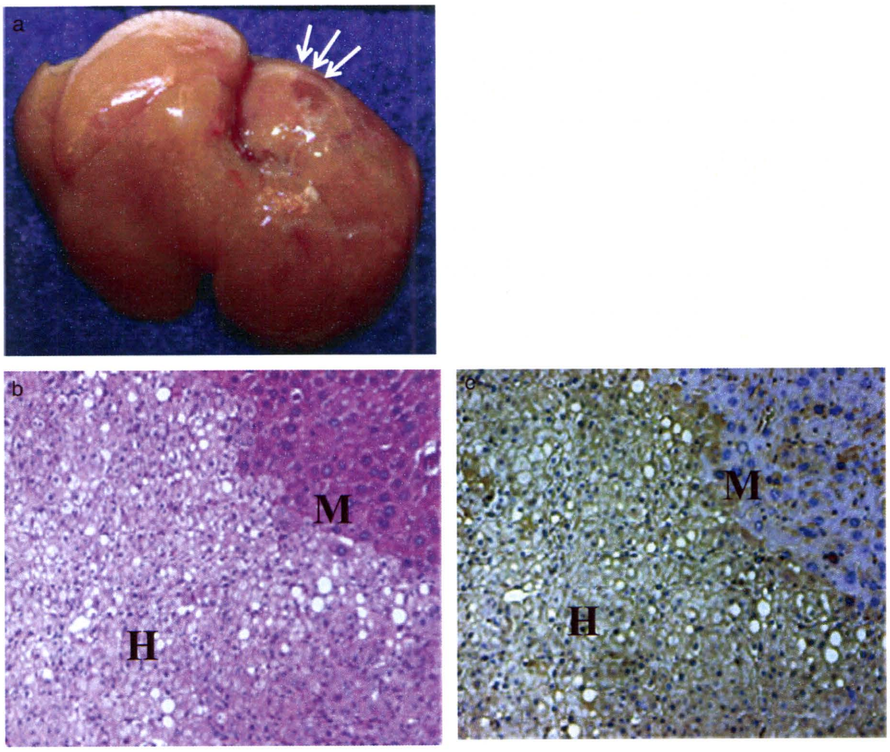

**Figure 2** Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: ×100). (c) Microscopic figure of the mouse liver stained with antibody directed against human serum albumin.

The biological properties of a newly identified unique strain of HBV, genotype G, which replicates only in the presence of another genotype, were confirmed using the chimeric mouse.[41] Infectivity of another novel HBV strain, identified from a Japanese patient, that is divergent from known human and ape HBV has also been confirmed.[42] Titration of HBV infectivity, which previously could only be carried out using chimpanzees, can be carried out effectively using chimeric mice.[43]

Taking advantage of the absence of human immune cells in the chimeric mice, Noguchi *et al.*[44] showed that hypermutation of HBV increases in human hepatocytes under interferon treatment. Dandri *et al.* measured viral half-life in human and chimeric mice repopulated with wooly monkey hepatocytes.[45] The results clearly showed that viral half-life is shortened by immunological mechanisms in humans with low viral levels, but not in chimeric mice where functional immunity is absent. Hiraga *et al.*[46] showed an absence of interference between HBV and HCV.

Evaluation of therapeutic agents is the most important role for this mouse model. Tsuge *et al.*[13] assessed the effect of interferon and lamivudine using chimeric mice. Similarly, Dandri *et al.*[47] showed the effects of adefovir using uPA/scid mice repopulated with tupaia hepatocytes, which also support replication of human HBV. Oga *et al.*[48] identified a novel lamivudine-resistant variant that has an amino acid substitution outside of the YMDD motif. They showed that lamivudine was ineffective against the novel mutant strain. It is thus apparent that this mouse/human liver chimeric model is ideal to study the susceptibility of mutant strains to various drugs, because mutant viruses can easily be made and infected into chimeric mice.[13] The model has also been utilized to evaluate viral entry inhibitors derived from the large envelope protein.[49]

## Study of hepatitis C virus using human hepatocyte chimeric mice

As observed in studies on HBV, HCV infection efficiency was poor and levels of viremia were low in mice where the repopulation rate of the mouse liver with human hepatocyte was low.[17,50] As shown in Figure 3, human albumin levels in mouse serum were significantly higher in mice in which measurable viremia developed (Hiraga *et al.* unpublished data). Recent studies have therefore been carried out using highly repopulated mice. The usefulness of a newly developed HCV assay,[51] and infectivity of a newly identified intergenotypic recombinant strain,[52] have been reported using the chimeric mice.

Using the remarkable replication ability of the JFH1 genotype 2a strain,[53] infectivity of JFH1 or intergenotypic chimeric viral particles, previously shown in cell culture, has now been shown to be infectious in chimeric mice.[54–56] Infectivity of viruses that were replicated in chimeric mice in cell culture has also been shown, and virus fitness has been studied.[55,56] The role of the HCV core+1 open reading frame and core *cis*-acting RNA elements has also been examined using the chimeric virus.[57] These elegant studies have the limitation that the non-structural part of the virus is limited to that of JFH1. Hiraga *et al.*[14] have shown that infectious clones of genotype 1a and JFH1 can be infected with direct injection of *in vitro* transcribed RNA into the mouse liver.[14] Similarly, Kimura *et al.*[15] reported the establishment of infectious clones of genotype 1b and ablation of RNA polymerase by site-directed mutagenesis abolish infectivity. These infectious clones will be useful for the study of drug-resistant strains.

The model of HCV infection has also been used to show that infection of the virus can be prevented by antibodies against

**Table 1** New therapeutic strategies tested by human hepatocyte chimeric mice

| *n* | Drug or cell | Strategy | Reference |
|---|---|---|---|
| 1 | Interferon alpha 2b<br>BILN-2061<br>HCV371 | Activation of antiviral genes<br>NS3-4A protease inhibition<br>NS5B polymerase inhibition | Kneteman *et al.*[65] |
| 2 | Modified BID | Induction of apoptosis | Hsu *et al.*[66] |
| 3 | Serine palmitoyltransferase inhibitor | Disruption of lipid raft | Umehara *et al.*[67] |
| 4 | Lymphoblastoid interferon alpha | Activation of antiviral genes | Hiraga *et al.*[14] |
| 5 | Amphipathic DNA polymers | Blocking viral entry | Matsumura *et al.*[60] |
| 6 | Sec-butyl-analogue of HCV-371 | NS5B polymerase inhibition | LaPorte *et al.*[68] |
| 7 | HCV796 | NS5B polymerase inhibition | Kneteman *et al.*[69] |
| 8 | Liver allograft-derived lymphocyte | Adoptive immunotherapy | Ohira *et al.*[70] |
| 9 | Telaprevir | NS3-4A protease inhibition | Kamiya *et al.*[71] |

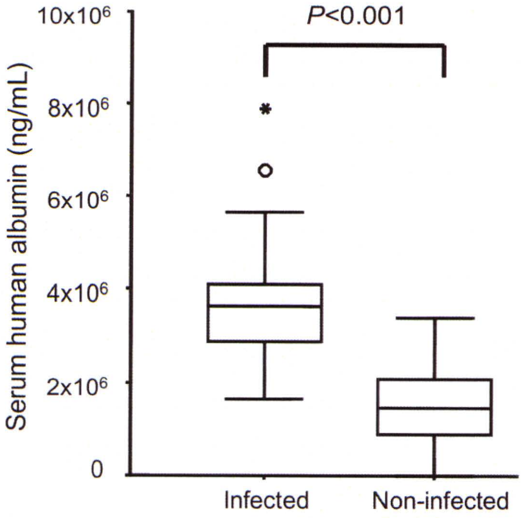

**Figure 3** Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing $5 \times 10^5$ virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 2 weeks after human hepatocyte transplantation were compared between infected and non-infected mice.

CD81,[58] polyclonal human immunoglobulin directed to a similar strain,[59] and amphipathic DNA polymers.[60] Notably, the presence of broadly neutralizing antibodies to HCV that protect against heterologous viral infection has been reported, suggesting the possibility of a prophylactic vaccine against HCV.[61]

With respect to evasion of the virus against the innate immune response, altered intrahepatic expression profiles in the early phase of infection is of particular interest. The chimeric mice model is ideal for such studies; cross-hybridization of mouse and human can be avoided by careful experimental procedures.[62] Microarray analysis of livers of HCV infected and non-infected mice showed transcriptional activation of genes related to innate immune response, lipid metabolism, endoplasmic reticulum (ER) stress and apoptosis in HCV-infected mice.[63,64] The HCV infected mouse model is particularly useful for the study of newly developed HCV agents. The effect of recently developed chemicals and a unique therapy using intrahepatic lymphocytes have been shown using this model (Table 1). However, none of these therapies have yet been able to completely eradicate HCV from mice. It is noteworthy that ultra-rapid cardiotoxicity has been reported with the protease inhibitor BILN 2061 in the uPA/scid mice, but not in scid mice, implicating involvement of the uPA transgene.[72] Care should therefore be taken in interpreting the results obtained by this model.

## Conclusion

Development of a small animal model using human hepatocyte chimeric mice has enabled us to study key aspects of HBV and HCV biology. The characteristic feature of the absence of human immune cells is suitable for studying viral replication and observing changes occurring in liver cells during viral infection, such as the innate immune response and cellular stress and metabolic responses. The model is also useful for studying the effect of drugs without the influence of cytokines and cytotoxic T lymphocytes. Nonetheless, the model is insufficient to study carcinogenesis of hepatitis viruses, because non-parenchymal cells in mouse liver are of mouse origin and do not support inflammation and fibrosis, which are probably closely related to carcinogenesis. The lack of human immune cells also limits the study of inflammation and immunity. Furthermore, the availability of human hepatocytes is limited. Despite these limitations, the current model shows great potential as a mouse model for the study of hepatitis viruses. Development of a small animal model with or without human immunity using stem cells or iPS cells would be an ideal model in the future.

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# Original article

# Amino acid substitutions in core and NS5A regions of the HCV genome can predict virological decrease with pegylated interferon plus ribavirin therapy

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**Background:** The current standard therapy for chronic hepatitis C is pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. Recently, it has been reported that amino acid (aa) substitutions in the core region, as well as the IFN-sensitivity-determining region (ISDR), were predictive of non-virological response (NVR), sustained virological response (SVR) and early virological response. Despite the importance of these two predictive factors for combination therapy, their interaction is poorly understood.

**Methods:** A total of 117 patients who were treated with PEG-IFN-α2b plus RBV combination therapy were selected for participation in this study. We determined the aa sequences in the core region and ISDR by direct sequencing and analysed them along with clinical data to indentify predictive factors for therapeutic response.

**Results:** The aa sequences in the core region and γ-glutamyl transpeptidase (GTP) levels were associated with SVR and NVR, but aa sequences in the ISDR were not. However, substitutions at both aa 70 and aa 91 in the core region without substitutions in the ISDR and higher levels of γ-GTP were independent predictive factors for NVR. Wild-type aa 70 and aa 91 in the core region, higher platelet counts and lower levels of γ-GTP were independent predictive factors for SVR.

**Conclusions:** These results indicate that analyses of aa substitutions in both the core region and the ISDR are useful for predicting the effectiveness of combination therapy, and could help to avoid therapy exposure for patients who have a low probability of SVR.

## Introduction

HCV is one of the most serious global health problems, affecting >170 million people worldwide [1–3]. Chronic HCV infection leads to development of chronic hepatitis, cirrhosis and hepatocellular carcinoma [4–8]. In an attempt to eradicate the virus and prevent the development of advanced liver diseases and hepatocellular carcinoma, interferon (IFN) is administered to patients with chronic HCV infection, with success in a subset of patients in whom marked biochemical and histological improvements can be obtained [9,10]. However, patients with high virus titres who are infected with genotype 1b, which is the major genotype, affecting approximately 70% of Japanese HCV patients, show poor response to IFN monotherapy. Less than 20% of patients treated with IFN monotherapy show sustained virological response (SVR) [11–14]. With the advent of pegylated interferon (PEG-IFN) and a newer antiviral agent, ribavirin (RBV), the eradication rate of the virus has improved overall. However, the eradication rate of

**Table 1.** Clinical characteristics of 117 patients before pegylated interferon plus ribavirin combination therapy

| Characteristic | Value |
|---|---|
| Male/female gender, *n* | 60/57 |
| Age, years | 62 (15–82) |
| BMI, kg/m$^2$ | 22.7 (17.4–31.8) |
| WBC count, cells/µl | 5,100 (2,030–9,610) |
| Neutrophil count, cells/µl | 2,831 (830–7,145) |
| Haemoglobin, g/dl | 13.9 (9.6–17.3) |
| Platelet count, ×10$^4$ cells/mm$^3$ | 14.0 (5.0–75.9) |
| AST, IU/l | 46 (10–209) |
| ALT, IU/l | 48 (5–214) |
| γ-GTP, IU/l | 39 (10–371) |
| Total cholesterol, mg/dl | 174 (107–248) |
| HCV RNA, $\log_{10}$ IU/ml | 6.34 (5.2–7.1) |
| SVR/non-SVR, *n* | 46/71 |

Data are median (range) unless indicated otherwise. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

genotype 1b with high viral load still remains only 40–50% [15–17].

Many host and viral factors that are predictive of the response to PEG-IFN plus RBV combination therapy have been reported to date. The SVR rate of the combination therapy has been reported to be significantly lower in older patients and in female patients [18–22]. The SVR rate was only 30% in female genotype 1b patients older than 65 years who have high viral loads [23]. Recently, sequencing analysis of HCV genomes has been actively performed to predict the effects of PEG-IFN plus RBV combination therapy. According to sequence analysis of HCV hypervariable region 1, quasispecies in this region at baseline were related to early virological response (EVR) and virological rebounds following combination therapy [24]. It has also been reported that the number of amino acid (aa) substitutions in the IFN-sensitivity-determining region (ISDR; nucleotides 2209–2248 or aa positions 237–276 of the NS5A region) correlates with SVR rate for combination therapy in HCV genotype 1b patients [25]. Recently, Akuta *et al.* [18,19,26,27] reported that the presence of aa 70 and/or aa 91 substitutions in the HCV core region is an independent and significant predictor of virological response (VR; for example, EVR, SVR and non-virological response [NVR]) to combination therapy. Okanoue *et al.* [28] also reported that the wild-type HCV core aa 70 and ≥2 aa substitutions in the ISDR are useful predictive factors for SVR. These reports, however, did not analyse the interaction of aa substitutions in the core region and the ISDR.

We recently analysed aa substitutions in the HCV core region and ISDR from patients treated with conventional IFN-α2b and RBV combination therapy and showed that substitutions in the core region and ISDR are useful predictive factors of response to therapy [29]. To date, no report has precisely analysed the relationship between both of these viral and clinical factors in patients who received PEG-IFN plus RBV combination therapy. In the current study, we analysed the relationship between clinical background and aa substitutions in the core region and ISDR to clarify the predictive factors of VR to combination therapy.

## Methods

### Patients

A total of 353 adult Japanese patients infected with HCV genotype 1b provided written informed consent to participate in the present study at Hiroshima University Hospital (Hiroshima, Japan). Among these patients, 117 who were treated with the PEG-IFN-α2b plus RBV combination therapy from August 2004 to June 2008, were selected as study participants based on the following criteria: serum HCV RNA titre >5.0 $\log_{10}$ IU/ml using HCV RNA quantitative analysis with Amplicor™ PCR analysis (Roche Diagnostics, Indianapolis, IN, USA; HCV RNA>5.0 $\log_{10}$ IU/ml was included in the category of high viral load based on guidelines by the Ministry of Health, Labor and Welfare [MHLW] of Japan [30]); no coinfection with other viruses, such as HIV or HBV; no other liver diseases, such as alcoholic liver disease, autoimmune hepatitis or decompensated cirrhosis (alcoholic liver disease was defined as patients with a lifetime total ethanol intake >100 kg [31]); and no coexisting conditions, such as poorly controlled diabetes mellitus, decompensated renal disease, preexisting psychiatric disease, seizure disorders, cardiovascular disease, haemophilia, autoimmune diseases or post-transplantation. All selected patients were followed until 6 months after the completion of combination therapy. Overall, 56 of 117 patients had received prior antiviral treatments, such as IFN monotherapy or IFN plus RBV combination therapy, but they had never previously received PEG-IFN-α2b monotherapy or PEG-IFN-α2b plus RBV combination therapy. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee. Baseline clinical characteristics of the 117 patients are shown in Table 1.

Patients were injected weekly with 1.5 µg/kg of PEG-IFN-α2b (PegIntron®; Schering–Plough, Kenilworth, NJ, USA) subcutaneously and were administered oral RBV (Rebetol®; Schering–Plough) daily for 48 weeks. The initial dose of RBV was adjusted according to body weight following the standard Japanese regimen (600 mg/day for ≤60 kg, 800 mg/day for 60–80 kg and 1,000 mg/day for >80 kg) [30]. Adverse events were monitored clinically by careful interview and haematological examination throughout the study. Blood samples were taken before the beginning of therapy and every 4 weeks thereafter. Biochemical and haematological tests were performed at the Hiroshima University Hospital laboratory. The remaining sera were stored at -80°C for further virological analysis. HCV genotypes were determined by phylogenetic analysis after reverse transcription (RT)-PCR and direct sequencing.

### Assessment of efficacy

Serum HCV RNA was detected by RT-nested PCR assay (limit of detection 50 IU/ml; Cobas Amplicor HCV Test version 2.0; Roche Diagnostics) every 4 weeks during treatment and 24 weeks after cessation of therapy. Positive samples were analysed further by quantitative assay (limit of detection 500 IU/ml; Cobas Amplicor HCV Monitor version 2.0; Roche Diagnostics).

The following are definitions of the VR to PEG-IFN-α2b plus RBV therapy. Rapid virological response (RVR) and EVR were defined as undetectable HCV RNA by qualitative PCR test at 4 and 12 weeks after the start of the treatment, respectively. SVR was defined as continuously undetectable HCV RNA by qualitative PCR assay and normalized alanine aminotransferase (ALT) levels for 24 weeks after the end of treatment. VR was defined as negative HCV RNA by qualitative PCR assay at least once during the treatment. NVR was defined as HCV RNA that was never detected as negative by qualitative PCR assay in all examinations.

### Determination of aa sequences in the HCV core region and ISDR

HCV RNA was extracted from 100 μl of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan) and dissolved in 20 μl of $H_2O$ and converted to complementary DNA by RT with random primers and Moloney murine leukaemia virus RT (Takara Shuzo, Tokyo, Japan). The complementary DNA was then amplified by nested-PCR to determine the nucleotide sequences in the HCV core region. The PCR protocol involved initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 30 s at 94°C, annealing of primers for 1 min at 57°C and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The primers for the first-round PCR were cc11 (forward 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the primers for the second-round PCR were cc9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as previously described [18,27,32,33]. To determine the nucleotide sequences of the HCV ISDR, we also performed RT nested-PCR with the same protocol as in the HCV core region. The primers used were IM11 (forward 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5A02KI (reverse 5′-CCC GTC CAT GTG TAG GAC AT-3′) for the first-round PCR and 5A05KI (forward 5′-GGG TCA CAG CTC CCA TGT GAG CC-3′) and IM10 (reverse 5′-GAG GGT TGT AAT CCG GGC GTG C-3′) for the second-round PCR. After amplification, the final PCR products were separated in 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Sequence analysis was performed using the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared with the nucleotide sequences of genotype 1b HCV-J (GeneBank accession number D90208) [34].

To analyse the relationship between the aa substitutions in the core regions, we divided the study participants into four groups on the basis of the presence of mutant (M) and wild-type (W) substitutions as follows: coreMM included patients with both aa 70 and aa 91 substitutions in the core region; coreMW included patients with only an aa 70 substitution in the core region; coreWM included patients with an aa 91 substitution in the core region; and coreWW included patients with neither aa 70 nor aa 91 substitutions in the core region. To analyse the effect of aa substitutions in the ISDR, we divided the patients into two groups as follows: ISDR≥1 included patients with ≥1 aa substitutions in the ISDR and ISDR0 included patients with no aa substitutions in the ISDR.

### Statistical analyses

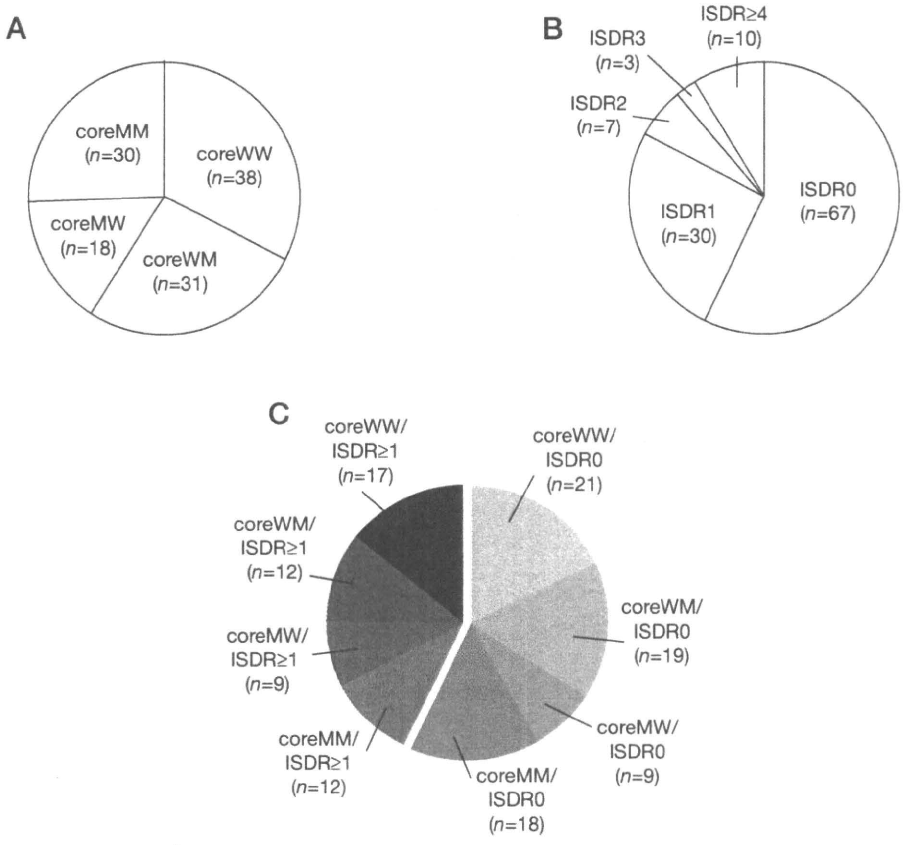
The baseline characteristics of the patients in the two groups were compared and the differences were assessed by $\chi^2$ test with Yate's correction, Fisher's exact probability test and the Mann–Whitney U test. All $P$-values $<0.05$ by two-tailed test were considered statistically significant. To determine the predictors of VR, univariate and multiple logistic regression analysis was performed. Variables with statistical significance ($P<0.05$) or marginal significance ($P<0.10$) in univariate analysis were retained for multiple logistic regression to identify significant independent predictive factors. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Core region and ISDR aa sequences, and their relationship with clinical parameters

To analyse the relationship between clinical background and aa sequences of the core region and ISDR, we compiled the clinical backgrounds of 117 patients who were treated with PEG-IFN-α2b plus RBV combination therapy for 48 weeks (Table 1). The amount of PEG-IFN-α2b injection was reduced in 48 of 117

**Figure 1.** Frequency of HCV amino acid substitutions in the core region and ISDR



**(A)** A total of 117 study patients were partitioned into four groups based on HCV core amino acid (aa) 70 and aa 91 substitution patterns. **(B)** Patients were grouped based on the number of HCV interferon-sensitivity-determining region (ISDR) substitutions. **(C)** Patients were divided into eight groups by the presence of HCV core substitutions and the number of ISDR substitutions. coreMM, substitutions in both core aa 70 and aa 91; coreMW, substitutions in core aa 70 only; coreWM, substitutions in core aa 91 only; coreWW, no substitutions in either core aa 70 or aa 91; ISDR0, absence of substitutions in the ISDR; ISDR≥1, subsitutions in ≥1 region of the ISDR.

patients because of moderate or severe thrombocytopaenia and/or neutropaenia during the course of combination therapy. The dose of RBV was also reduced in 55 of 117 patients because of progression of anaemia, but none of the study patients received erythropoietin or blood transfusions during treatment. In total, 19 patients failed to complete the 48-week treatment because of very poor reduction of HCV RNA, and these patients were categorized as NVR; however, none of the study patients discontinued treatment because of adverse effects. We analysed the nucleotide and aa sequences of the core region and ISDR. In these 117 patients, core aa 70 (R70Q/Y/H) and core aa 91 (L91M) substitutions were observed in 48 (41.0%) and 61 (52.1%) patients, respectively. A marginal association was observed between the sequences of core aa 70 and core aa 91 ($P$=0.0920; Figure 1A). For the aa sequence of the ISDR, the wild-type was the most frequently detected sequence, and the frequency decreased as the number of aa substitutions increased (Figure 1B). We then evaluated the relationship between the aa substitutions of the core region and the ISDR (Figure 1C). The Breslow–Day test showed no heterogeneity in core mutation patterns between patients with or without aa substitutions in the ISDR ($P$=0.84). We then compared the clinical parameters and core region aa substitution patterns. There was no relationship between age or gender and aa patterns of the core region (SK *et al.*, data not shown).

### Clinical and virological predictive factors for VR and NVR to the combination therapy

Statistical analysis was used to compare VR and NVR patients in order to identify predictive factors

**Table 2.** Comparison of baseline characteristics between patients with virological response and non-virological response

| Variable | Virological response (*n*=88) | Non-virological response (*n*=29) | *P*-value |
|---|---|---|---|
| Male/female gender, *n* | 42/46 | 18/11 | 0.204[a] |
| Age, years | 62 (15–77) | 64 (44–82) | 0.467 |
| BMI, kg/m$^2$ | 22.4 (17.4–29.9) | 23.6 (20.4–31.8) | 0.170 |
| WBC count, cells/μl | 5,135 (2,030–9,610) | 4,750 (2,630–9,490) | 0.293 |
| Neutrophil count, cells/μl | 2,840 (830–7,145) | 2,657 (1,143–5,409) | 0.357 |
| Haemoglobin, g/dl | 13.9 (9.6–17.0) | 14.1 (10.1–17.3) | 0.455 |
| Platelet count, ×10$^4$ cells/mm$^3$ | 14.3 (5.0–75.9) | 12.8 (6.7–38.0) | 0.103 |
| AST, IU/l | 45 (10–209) | 48 (15–149) | 0.779 |
| ALT, IU/l | 49 (5–214) | 40 (20–163) | 0.383 |
| γ-GTP, IU/l | 34 (10–226) | 64 (19–371) | 0.003 |
| Total cholesterol, mg/dl | 175 (107–248) | 173 (129–226) | 0.483 |
| HCV RNA, log$_{10}$ IU/ml | 6.34 (5.2–7.1) | 6.34 (5.2–6.7) | 0.522 |
| Core aa 70 W/M substitution, *n* | 60/28 | 9/20 | <0.001[a] |
| Core aa 91 W/M substitution, *n* | 47/41 | 9/20 | 0.053[a] |
| ISDR0/ISDR≥1, *n* | 47/41 | 20/9 | 0.194[a] |

Data are median (range) unless indicated otherwise. Statistical analyses were performed with the Mann–Whitney U test and [a]the $\chi^2$ test. aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; ISDR0, absence of substitutions in the interferon-sensitivity-determining region (ISDR); ISDR≥1, subsitutions in ≥1 region of the ISDR; M, mutant; SVR, sustained virological response; W, wild-type; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

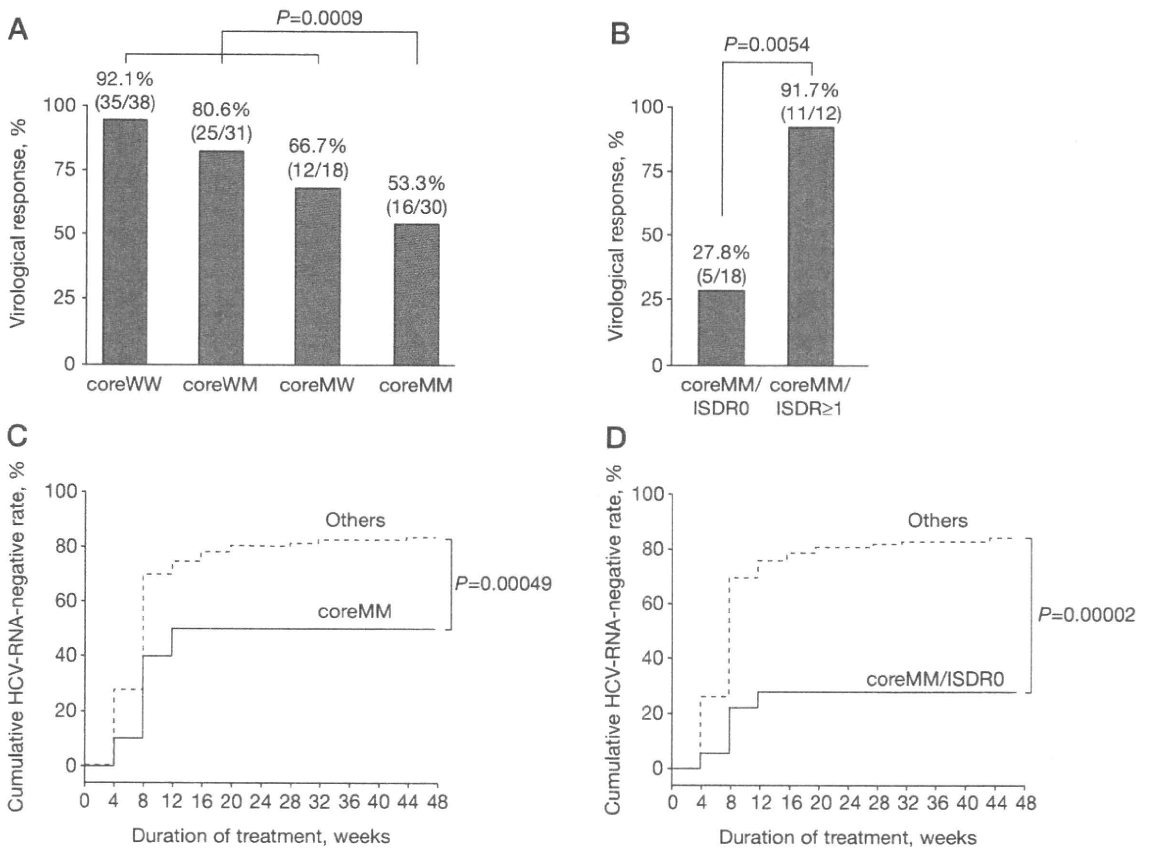
for response to PEG-IFN-α2b plus RBV combination therapy. Among the clinical background factors, only serum γ-glutamyl transpeptidase (GTP) levels differed significantly (*P*=0.0032), and of the virological factors, core aa 70 and aa 91 substitutions were significantly (*P*<0.001) and marginally significantly (*P*=0.053) associated with VR (Table 2), respectively. When core region substitution patterns were compared, the rate of achieving VR decreased in the following order: coreWW, coreWM, coreMW and coreMM (Figure 2A). By contrast, no association was found between ISDR substitutions and VR (Table 2); however, a significantly lower number of patients with core aa pattern MM and no ISDR substitutions achieved VR (Figure 2B). In addition, the patients who had core aa pattern MM with ≥1 aa substitutions in the ISDR achieved VR with higher frequency (Figure 2B). Similar tendencies were also evident when the clearance rate of the virus over time was compared among coreMM patients versus others (Figure 2C) and coreMM/ISDR0 patients versus others (Figure 2D). These results showed that the aa sequence of ISDR is useful to discriminate poor responders among coreMM patients.

We performed a logistic regression analysis for NVR using γ-GTP levels and substitutions in the core region and ISDR as potential predictive factors. As shown in Table 3, core aa 70 substitutions (*P*=0.002) and high γ-GTP levels (*P*=0.049) were independent predictive factors of NVR. Additionally, logistic regression for NVR was performed using the serum γ-GTP level as a continuous variable. ISDR0 (*P*=0.038), core aa 70 substitutions (*P*=0.008) and high γ-GTP levels (*P*=0.003) were independent predictive factors of NVR. However, when combined with core region aa substitutions, the core aa pattern MM did not increase the strength of association for NVR (SK *et al.*, data not shown). To identify more effective predictors, we analysed the haplotype of the core region and the ISDR. In multivariate analyses of core region and ISDR substitutions, coreMM/ISDR0 was the most significant predictive factor for NVR (Table 4). These results suggest that the evaluation of aa substitutions in both the core region and the ISDR are useful for predicting poor responders to combination therapy.

### Clinical and virological predictive factors for SVR to the combination therapy

Next, we analysed predictive factors for SVR in patients treated with PEG-IFN-α2b plus RBV combination therapy. As shown in Table 5, some clinical background data, including age, white blood cell count, platelet count and γ-GTP levels, were significantly different between SVR and non-SVR patients in univariate analyses, and neutrophil count was marginally significantly different. Among the viral factors, core substitutions at aa 70 and aa 91 were significantly associated with SVR, but HCV RNA titres and the number of ISDR substitutions were not significantly associated with SVR (Table 5). RVR and EVR were also significantly associated with SVR. Liver biopsy was performed in 94 of 117 patients, but no significant association was found between SVR and liver fibrosis (Table 5). When the pattern of aa substitutions of the core region was compared with SVR, the rate of achieving SVR was significantly higher for coreWW than the other substitution patterns (Figure 3A). Remarkably, none of the

**Figure 2.** Analysis of factors associated with virological response



**(A)** Virological response rates were compared between the combinations of the HCV core substitutions. Statistical analysis was performed using the $\chi^2$ test with Yate's correction. A significant difference was found between participants with substitutions in both core amino acid (aa) 70 and aa 91 (coreMM) and each of the other groups. **(B)** Analysis of participants with coreMM by HCV core plus interferon-sensitivity-determining region (ISDR) haplotypes. Statistical analyses was performed using Fisher's exact probability test. **(C&D)** The cumulative HCV-RNA-negative rate during 48 weeks of combination therapy was analysed by the log-rank test. Groups were classified based on the presence of HCV core double substitutions (coreMM) and ISDR substitutions. coreMW, substitutions in core aa 70 only; coreWM, substitutions in core aa 91 only; coreWW, no substitutions in either core aa 70 or aa 91; ISDR0, absence of substitutions in the ISDR; ISDR≥1, subsitutions in ≥1 region of the ISDR.

**Table 3.** Predictive factors for non-virological response in 117 patients by multivariate analyses

| Factor | OR (95% CI) | *P*-value |
|---|---|---|
| Core aa 70 substitution | – | 0.002 |
| Wild-type | 1 | – |
| Mutant | 4.398 (1.749–11.055) | – |
| γ-GTP | – | 0.049 |
| <60 IU/l | 1 | – |
| ≥60 IU/l | 2.506 (1.005–6.248) | – |
| ISDR | – | 0.083 |
| 0 | – | – |
| ≥1 | – | – |
| Core aa 91 substitution | – | 0.165 |
| Wild-type | – | – |
| Mutant | – | – |

aa, amino acid; CI, confidence interval; ISDR, interferon-sensitivity-determining region; OR, odds ratio; γ-GTP, γ-glutamyl transpeptidase.

**Table 4.** Predictive factors for non-virological response in 117 patients by multivariate analysis

| Factor | OR (95% CI) | *P*-value |
|---|---|---|
| Core aa 70/91 and ISDR substitutions | – | <0.001 |
| Other | 1 | – |
| coreMM/ISDR0 | 16.098 (4.447–61.266) | – |
| γ-GTP | – | 0.018 |
| <60 IU/l | 1 | – |
| ≥60 IU/l | 3.366 (1.236–9.167) | – |

aa, amino acid; CI, confidence interval; coreMM, substitutions in both core aa 70 and aa 91; ISDR, interferon-sensitivity-determining region; ISDR0, absence of substitutions in the ISDR; OR, odds ratio; γ-GTP, γ-glutamyl transpeptidase.

**Table 5.** Comparison of baseline characteristics between SVR and non-SVR patients

| Characteristic | SVR (*n*=46) | non-SVR (*n*=71) | *P*-value |
|---|---|---|---|
| Male/female gender, *n* | 23/23 | 37/34 | 0.852[a] |
| Age, years | 57 (15–77) | 64 (29–82) | 0.012 |
| BMI, kg/m$^2$ | 22.4 (17.3–31.8) | 23.2 (17.4–31.8) | 0.659 |
| WBC count, cells/μl | 5,200 (2,590–9,340) | 4,880 (2,030–9,610) | 0.038 |
| Neutrophil count, cells/μl | 3,073 (951–7,145) | 2,657 (1,143–5,409) | 0.058 |
| Haemoglobin, g/dl | 14.0 (9.6–16.8) | 13.8 (10.1–17.3) | 0.983 |
| Platelet count, ×10$^4$ cells/mm$^3$ | 15.4 (5.0–75.9) | 12.3 (6.7–38.0) | 0.005 |
| AST, IU/l | 43 (18–113) | 47 (10–209) | 0.293 |
| ALT, IU/l | 49 (22–137) | 46 (5–214) | 0.789 |
| γ-GTP, IU/l | 33 (14–180) | 53 (10–371) | 0.003 |
| Total cholesterol, mg/dl | 180 (113–236) | 169 (107–248) | 0.150 |
| HCV RNA, log$_{10}$ IU/ml | 6.32 (5.2–7.1) | 6.36 (5.2–6.9) | 0.592 |
| Liver fibrosis F0–F1/F2–F3, *n* | 17/20 | 19/38 | 0.279[a] |
| Core aa 70 W/M substitution, *n* | 34/12 | 35/36 | 0.012[a] |
| Core aa 91 W/M substitution, *n* | 29/17 | 27/44 | 0.013[a] |
| ISDR0/ISDR≥1, *n* | 24/22 | 43/28 | 0.445[a] |
| RVR/non-RVR, *n* | 23/23 | 5/66 | <0.001[a] |
| EVR/non-EVR, *n* | 43/3 | 35/36 | <0.001[a] |

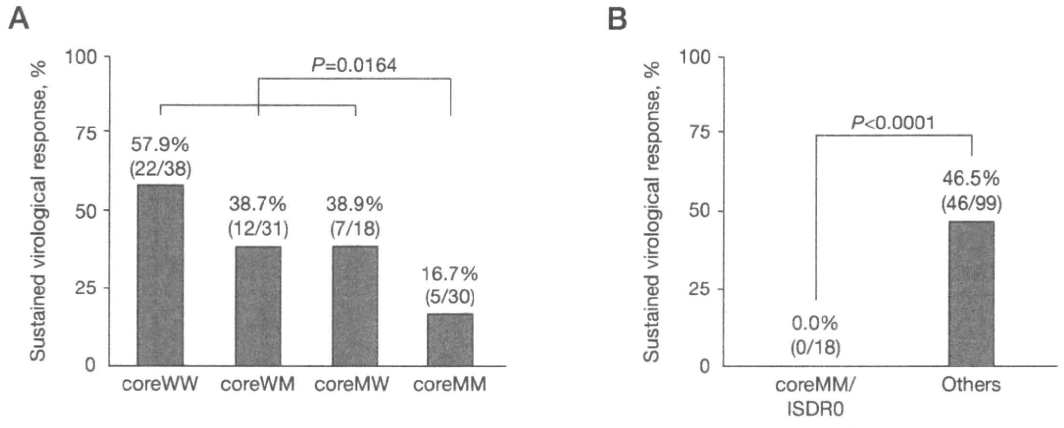
Data are median (range) unless indicated otherwise. Statistical analyses were performed with the Mann-Whitney U test and [a]the $\chi^2$ test. aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; EVR, early virological response; ISDR0, absence of substitutions in the interferon-sensitivity-determining region (ISDR); ISDR≥1, substitutions in ≥1 region of the ISDR; M, mutant; RVR, rapid virological response; SVR, sustained virological response; W, wild-type; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

patients with coreMM or ISDR wild-type showed SVR (Figure 3B). We thus performed a logistic regression analysis for SVR using the following potential predictive factors: age, white blood cell count, platelet count, γ-GTP and aa substitutions in the core region and ISDR (Table 6). In this multivariate analysis, we excluded factors such as RVR and EVR to assess the predictive factors before the initiation of combination therapy. As shown in Table 6, higher platelet count (*P*=0.002), the core aa pattern WW (*P*=0.008) and low γ-GTP levels (*P*=0.013) were independent predictive factors of SVR, but the combination of coreWW and the ISDR≥1 did not increase the odds ratio for SVR (SK *et al.*, data not shown). Additionally, logistic regression analysis for SVR was performed using the continuous values for age, white blood cell count, platelet count and serum γ-GTP level. Younger age (*P*=0.003), as well as core aa pattern WW (*P*=0.013) and lower level of γ-GTP (*P*=0.017), were independent predictive factors of SVR, although the effect of platelet count on SVR decreased (*P*=0.136).

## Discussion

Recently, combination therapy with PEG-IFN and RBV has been introduced for clinical applications, and the efficacy of IFN therapy for chronic HCV infection drastically improved. The SVR rate with PEG-IFN plus RBV combination therapy increased up to 40–50% in patients infected with high viral titre of HCV genotype 1b [15,17,35]. However, more than one-half of these patients have not yet been able to achieve SVR with combination therapy. Recently, the relationship between HCV aa substitutions and IFN responses has been extensively analysed in laboratories worldwide. In 1996, Enomoto *et al.* [36] reported that the number of substitutions in the HCV ISDR was associated with response to IFN monotherapy. They analysed ISDR sequences from 84 Japanese patients chronically infected with HCV genotype 1b and revealed that HCV-infected patients with >4 ISDR substitutions were more sensitive to IFN therapy than those with ≤4 ISDR substitutions. Subsequent supportive studies were reported by several laboratories [37–40]. Thereafter, with advances in IFN treatment, new evidence on the relationship between HCV aa sequences and IFN response was demonstrated. Akuta *et al.* [32] reported that the existence of substitutions in the HCV core aa 70 and aa 91 regions was one

**Figure 3.** Association between the HCV amino acid substitutions and sustained virological response



(A) Sustained virological response rates were compared between the HCV core substitution haplotypes. Statistical analysis was performed using the $\chi^2$ test with Yate's correction. A significant difference was found between participants with substitutions in both core amino acid (aa) 70 and aa 91 (coreMM) and each of the other groups. (B) All patients were partitioned into two groups: those with double mutants plus the wild-type interferon-sensitivity-determining region (ISDR; coreMM/ISDR0) and others. Statistical analysis was performed using Fisher's exact probability test. coreMW, substitutions in core aa 70 only; coreWM, substitutions in core aa 91 only; coreWW, no substitutions in either core aa 70 or aa 91.

**Table 6.** Predictive factors for sustained virological response in 117 patients by multivariate analysis

| Factor | OR (95% CI) | *P*-value |
|---|---|---|
| Platelet count | – | 0.002 |
| <12×10$^4$ cells/mm$^3$ | 1 | – |
| ≥12×10$^4$ cells/mm$^3$ | 4.698 (1.748–12.629) | – |
| Core aa 70/91 substitutions | – | 0.008 |
| Other | 1 | – |
| WW | 3.410 (1.386–8.387) | – |
| γ-GTP | 1 | 0.013 |
| <60 IU/l | – | – |
| ≥60 IU/l | 3.374 (1.294–8.793) | – |
| Age | – | 0.200 |
| <65 years | – | – |
| ≥65 years | – | – |
| ISDR substitutions | – | 0.345 |
| ISDR0 | – | – |
| ISDR≥1 | – | – |
| WBC count | – | 0.545 |
| <4,500 cells/μl | – | – |
| ≥4,500 cells/μl | – | – |

aa, amino acid; CI, confidence interval; ISDR, interferon-sensitivity-determining region; ISDR0, absence of substitutions in the ISDR; ISDR≥1, substitutions in ≥1 region of the ISDR; OR, odds ratio; W, wild-type; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

of the predictive factors of NVR with IFN plus RBV combination therapy. Okanoue *et al.* [28] reported that wild-type core aa 70 and the presence of >2 aa substitutions in ISDR were predictive factors for SVR with PEG-IFN-α2b plus RBV combination therapy, but that the core aa 91 substitution was not. In the present study, we analysed the relationship between the HCV aa substitutions (core aa 70, aa 91 and ISDR) and IFN responses among patients who were infected with HCV genotype 1b with a high viral load and who were treated with PEG-IFN plus RBV combination therapy. The early-phase VR, such as RVR and EVR, were significantly associated with clinical background factors, such as age, platelet count and γ-GTP levels, but HCV aa sequences were not (SK *et al.*, data not shown). These results suggest that HCV aa substitutions do not affect the antiviral effect of IFN directly. Our previous report using human hepatocyte chimeric mice might be relevant to this supposition [41]. When the mice infected with our original infectious HCV clone, which was generated from serum infected with HCV genotype 1b [42], were treated with IFN for 4 weeks, there was no significant difference in HCV reduction between HCV coreWW clone and coreMM clone (NH *et al.*, unpublished observations). Our clinical data also suggested by sequence analysis that HCV could not develop IFN resistance by aa substitutions in the core regions (SK *et al.*, data not shown). We speculate from these results that HCV aa substitutions in the core and NS5A regions might affect host immunity by modulating the expression of IFN-stimulated genes.

By contrast, the long-term responses, SVR and VR, were significantly associated with HCV aa substitutions. In the analysis of SVR, both the aa 70 and aa 91 core substitutions significantly reduced the SVR rate (SK *et al.*, data not shown), and MM viruses, having both core substitutions, were the most tolerant to combination therapy (Figure 3A). In addition, no patient infected with HCV coreMM/ISDR0 achieved SVR (Figure 3B); however, we could not assess the association between the number of ISDR aa substitutions and HCV RNA in the present study. In the analysis of VR, 72% of the patients infected with HCV core MM/ISDR0 failed to achieve VR (SK *et al.*, data not shown). Given the varying degrees of viral reduction within patients with the same core and ISDR mutation patterns, we speculated that differences among the VRs were partly dictated by differences in the personal immune response. Recently, the relationship between HCV-related proteins and the IFN signalling pathway has been further elucidated. IFN signalling is activated through the Jak-STAT pathway, inducing the transcription of IFN-stimulated genes, such as double-stranded RNA-dependent protein kinase R, myxovirus resistance 1 and 2′,5′-oligoadenylate synthetase (OAS) [43–50]. However, in the presence of the HCV core protein and the non-structural protein NS5A, the IFN signal cascade has been known to be suppressed by the interaction with protein kinase R or 2′,5′-OAS and the induction of IFN signal inhibitors, suppressor of cytokine signalling-3 and interleukin-8 [51–55]. Based on our results and those of other groups, we speculated that the patients who were infected with HCV coreMM/ISDR0 might be more immunologically suppressed by HCV related proteins. If this speculation is correct, a compound working as a protease inhibitor against HCV non-structural proteins could reduce immune suppression caused by the HCV non-structural proteins. Thus, HCV non-structural proteins might be important potential targets for the development of new therapies.

Among the host factors, γ-GTP level was identified as a predictive factor for both SVR and NVR. Okanoue *et al.* [28] demonstrated that low-density lipoprotein cholesterol, homeostasis model assessment for insulin resistance and fatty change of liver tissues were significantly associated with SVR. This finding might support our present results because hepatic fatty change is clinically correlated with γ-GTP increase. However, the molecular mechanisms of the relationship between hepatic fatty change and IFN response are still unclear. Further study is needed to clarify this issue.

Our study was performed based on the guidelines of the MHLW of Japan [30]. In the previous studies, substitutions in the HCV core regions and ISDR were analysed separately [18,19,28], and associations were demonstrated between HCV substitutions and IFN response. In the present study, we clarified the association between the IFN response and the combined information of both core and ISDR substitutions. Our study suggests that the combined information of both core and ISDR substitutions might be very useful for predicting the effect of PEG-IFN-α2b plus RBV combination therapy.

The main limitation of our study is that some selection biases and confounders, such as serum HCV RNA titres, alcohol consumption, prior antiviral therapy in some patients, and variation in RBV dose, might affect the internal validity of the study. We identified predictive factors for the effect of PEG-IFN-α2b plus RBV combination therapy, especially among patients with high viral loads, whereas the patients with HCV RNA<5.0 $\log_{10}$ IU/ml were excluded from the study. A previous report showed that ethanol intake of >24 g/day decreased the SVR rate of IFN treatment [31]; therefore, we excluded patients with a lifetime total ethanol intake of >100 kg to reduce the effect of heavy drinking. We consider that selection of the study participants based on HCV RNA titre and total ethanol intake in our study is valid and appropriate, although we might be unable to disregard the effects of selection bias. A total of 48% of study participants received prior IFN monotherapy or IFN plus RBV combination therapy, but all study participants were naive to PEG-IFN-α2b plus RBV combination therapy. In the present study, SVR rates did not differ significantly ($P$=0.136) with or without prior antiviral therapy. According to the guidelines of the MHLW of Japan [30], reduced RBV doses were recommended relative to the universal guidelines. Although the study participants were treated with lower RBV doses, the SVR rate was comparable to the expected rate in Japan and other countries. Thus, we consider potential confounders, such as prior antiviral therapy and RBV dose, might not have a strong influence on our study results.

In conclusion, we analysed the relationship between HCV aa sequence substitutions and the effectiveness of PEG-IFN-α2b plus RBV combination therapy. We identified significant associations among SVR and NVR, HCV core and ISDR substitutions, γ-GTP levels and platelet counts. The poor response of patients infected with HCV coreMM/ISDR0 should be taken into account when considering treatment options.

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## Disclosure statement

The authors declare no competing interests.

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