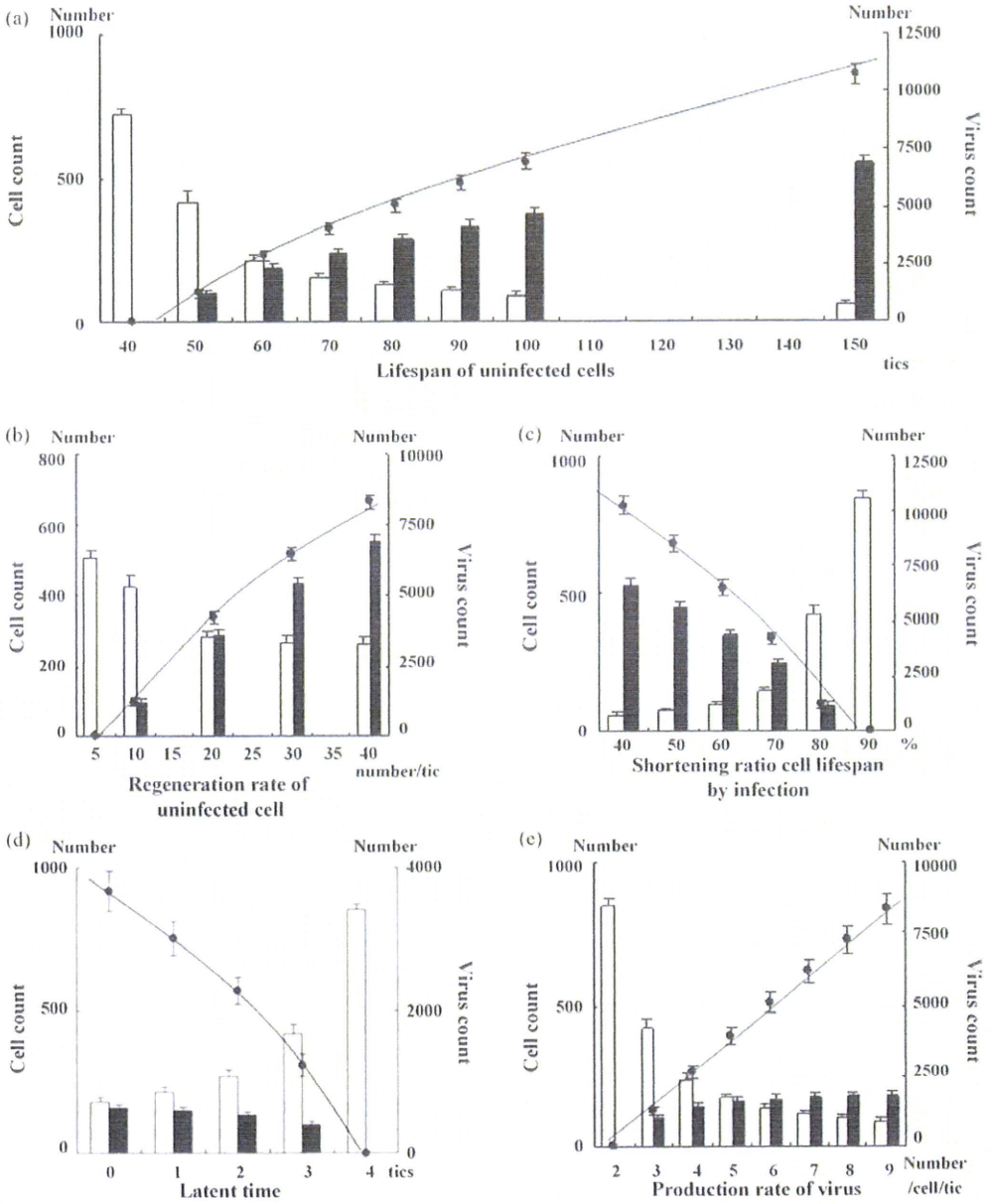

Fig. 5. Effects of changes in cell parameters. (a) Extending the uninfected cell lifespan and (b) increasing the uninfected cell regeneration rate increased the virus count. (c) Raising the lifespan-shortening ratio as a result of infection shortened the lifespan of infected cells, thereby decreasing the virus count. (d) Extending the latent period shortened the period of virus production from infected cells, thereby decreasing the virus count. (e) Increasing the virus production count resulted in a linear increase in equilibrium-phase virus count. Black circles: virus count; line: virus count approximation curve; white bars: uninfected cell count; black bars: infected cell count.

phase itself (Fig. 4b). Extending the lifespan of viruses resulted in a linear increase in equilibrium-phase virus count (Fig. 4c). Although the infected cell count increased, the rate of increase gradually declined. Changing the speed of viral movement resulted in the equilibrium-phase virus count to eventually decline after 100 grids/tic was reached, allowing movement over an area twice the size of the simulation space (Fig. 4d).

3.5. Uninfected Cell Parameters

Extending the lifespan of uninfected cells led to an increased virus count during the equilibrium phase (Fig. 5a). Increasing the uninfected cell regeneration rate also contributed to increased equilibrium-phase virus count (Fig. 5b). In both the cases, the increases in virus count and infected cell count were not linear, but showed a tendency for the rate of increase to decline gradually.

3.6. Infected Cell Parameters

We carried out an investigation of the effects of variation in the lifespan-shortening ratio on the virus count on the assumption that cell lifespan is shortened by infection. When this ratio was increased, the virus count decreased (Fig. 5c). An extended latent period was also related to a decreased virus count (Fig. 5d). However, the virus production from infected cells led to a linear increase in the virus count (Fig. 5e).

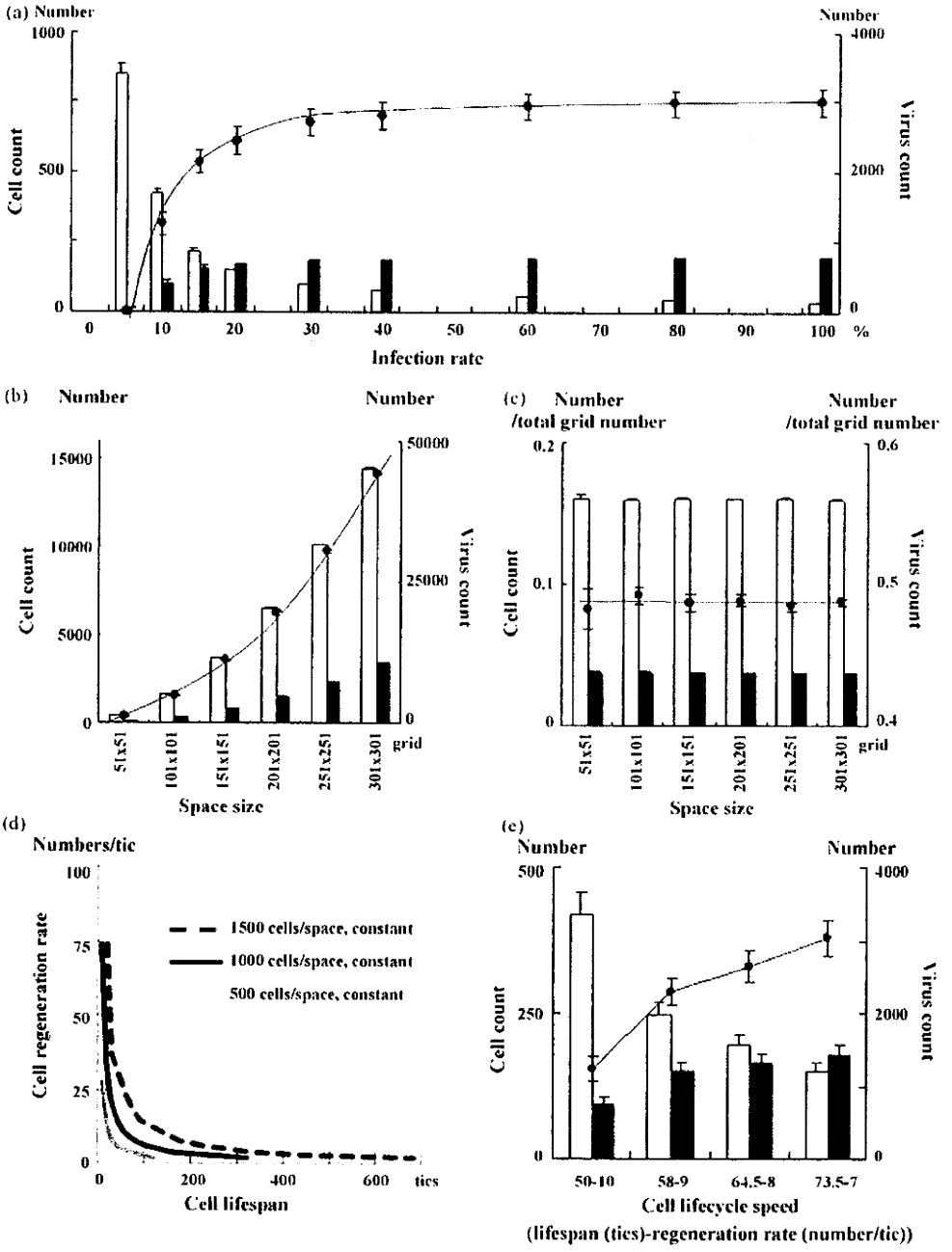

Fig. 6. (a) Increasing the infection rate increased the virus count in equilibrium periods, but the virus count did not change at infection rates of 30% or more. (b) The size of the simulation space increased not only virus count but also the cell count; however, (c) when virus and cell counts were divided by the total number of grids in the space, they were constant for all space sizes. (d) Changing the lifespan and regeneration rate of uninfected cells in opposite directions at the same time makes it possible to change only the cell cycle speed without altering the uninfected cell count. (e) When the cell cycle speed was reduced, the virus count increased toward the right of the graph. This may be because the effect of extending the lifespan of cells exceeds that of reducing their regeneration rate. (a–c and e) Black circles: virus count; line: virus count approximation curve; white bars: uninfected cell count; black bars: infected cell count.

3.7. Infection Rate and Space Size

Increasing the infection rate caused an increase in the virus count, but the change was minimal at an infection rate of 30% or more. The same results were seen for infected cell count, but a decrease in uninfected cell count resulted in a tendency for the infection rate to decrease by up to 60% (Fig. 6a).

The larger the space, higher the increase in both virus and cell counts (Fig. 6b). This increase was proportional to space size, however, when virus and cell counts were divided by the total number of grids in the space they were all constant (Fig. 6c).

3.8. Cell Cycle Speeds

Running a simulation with the initial virus count set to zero enables only the equilibrium condition for uninfected cells to be simulated. Changing the lifespan and regeneration rate of uninfected cells in opposite directions at the same time makes it possible

to change the cell cycle speed without altering the uninfected cell count (Fig. 6d). We used this technique to investigate how changing the cell cycle speed affected the equilibrium phase. Fig. 6e shows the results. Cell lifespan increases while the cell cycle speed declines. The equilibrium virus count increased in accordance with slower cell cycle speeds.

4. Discussion

In this study, we investigated the models using two agent-based simulation methods to program a simple virus–host chronic infection model. The same model written in two different programming language systems displayed the same results. The transient phase was unlike that seen in a mathematical simulation with no overshoot in virus count, but rather a smooth transition to the equilibrium phase. The virus count at the start of the simulation only had effect on the rate of infection development. Increases in virus lifespan, uninfected cell lifespan, uninfected cell regeneration rate, virus production count from infected cells, and infection rate all led to increased equilibrium-phase virus count. Rises in the infected cell lifespan-shortening ratio, latent period, and cell cycle speed decreased the equilibrium-phase virus count. The size of the space itself had no innate effect on the equilibrium phase, but a speed of movement of the virus that was twice the size of the space produced the maximum virus count.

Reproducibility is the basis for all scientific study, but there are many problems to prove it in computer simulations, such as programming bugs. As agent-based simulation deals with numerous agents individually, it requires vast amounts of calculations. Accumulation of very small change of values leads to large differences of results. In this study, we investigated two programs based on two programming languages to confirm the reproducibility of our simulation results in different programming languages. The results of two simulations were consistent, but in StarLogo, the lifespan parameters had a tendency to be lower than when they were set while simulations were actually in progress. This may be because the number of digits used in calculations was different between the two programs. RePast performs calculations to at least eight decimal places. In StarLogo, the library settings only enable settings to be made up to five decimal places. It is probable that these small differences accumulate during repeated calculations and are reflected in the simulation. Ultimately, we confirmed that the differences in results obtained by using different libraries and programming languages were not innate and by making the parameters consistent during simulation, consistent results were obtained.

Mathematical models using formulae for HIV therapy was published in 1994, the method has since been applied to HBV and HCV (Ho et al., 1995; Nowak et al., 1996; Neumann et al., 1998), and they were thought to be good reflections of the reality. In the mathematical model, viruses and cells are conceived as individuals in the concept itself, but both of them are perceived *en masse* when calculations are performed. However a feature of the agent-based simulation is that it deals with individual viruses and cells as separate agents. By moving each agent individually, it probes the factors influencing overall shifts from the micro viewpoint. When the space is viewed as a whole, it is possible to watch on the screen the collective movement of groups of agents. Recently, models that provide a visual representation of Epstein-Barr virus and HIV infection have been reported, both of which are useful for an instinctive and intuitive understanding (Duca et al., 2007; Shapiro et al., 2008; Castiglione et al., 2007).

In agent-based simulation model, virus count transit smoothly to the equilibrium phase. On the other hand, virus counts overshoot during transient phase in mathematical model. We think this difference is derived from technicality of different modeling. The difference in concepts between mathematical models and agent-based models is the space. The mathematical model has no space in concept, but agents move across the space in the agent-based model. In agent-based models, the densities of virus and cells change overtime especially in the transition phase because of the limited space. These changes of the densities of virus and cells lead to the dynamic change of the encounter rate of viruses and cells. The mathematical model does not make such concept of the density; the encounter rate is constant. This may be the reason for the difference between two models in the transition phase. Since no overshoot of virus counts in transient phase had been reported from in vivo studies of hepatitis C virus and simian immunodeficiency virus (Dahari et al., 2005; Nowak et al., 1997), agent-based model correlates with actual biology in vivo at least for these viruses. The increase of initial virus count at the start of simulation correlates with higher encounter rate of viruses and cells which make the linear increasing of infection forming rate. Mathematical model can only express the infection formation rate as "infected or not".

The importance of viral passing speed in the agent-based model is also explained by the "space". Although the virus actually moves through the blood stream in our body and virus could not decide their moving speeds by themselves, there is most appropriate speed for virus to meet the cells on the simulation space by the highest probability. The effect of cell cycle speed should be mentioned by another affection of the space. A fast cell cycle speed means that the lifespan of uninfected cells is short. Then fast cell cycle speed leads to the short lifespan of infected cells. A higher regeneration rate for uninfected cells results in a higher rate of infection among uninfected cells by viruses, but in situations where viruses and cells are dispersed around the space this is ineffective in increasing the infection rate, as the latter depends on the probability that they will encounter one another. As a result, the infected cell count decreases during the equilibrium phase, as does the virus count.

In this study, we confirmed the reproducibility and usability of agent-based models in expressing the interaction between viruses and cells. A feature of this simulation system is that it uses the concept of space as actual space, which means that the existence of the space becomes an additional controlling factor on the simulation results. This is a concept that is absent from mathematical models. The reality is that we have a spatial existence, and an advantage of the agent-based simulation system is the fact that it accounts for the space. Another feature of the simulation system is that it enables the condition to be perceived in visual terms, making it easy to understand. However it may be affected by computer performance and by the limitations of programming languages or the program itself, this system may offer a powerful tool for the future analysis of real virus–host interaction disease.

Conflict of interest

No conflicts of interest exist for all authors.

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Research Article

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Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

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

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

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Patients

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

Laboratory and histological tests

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

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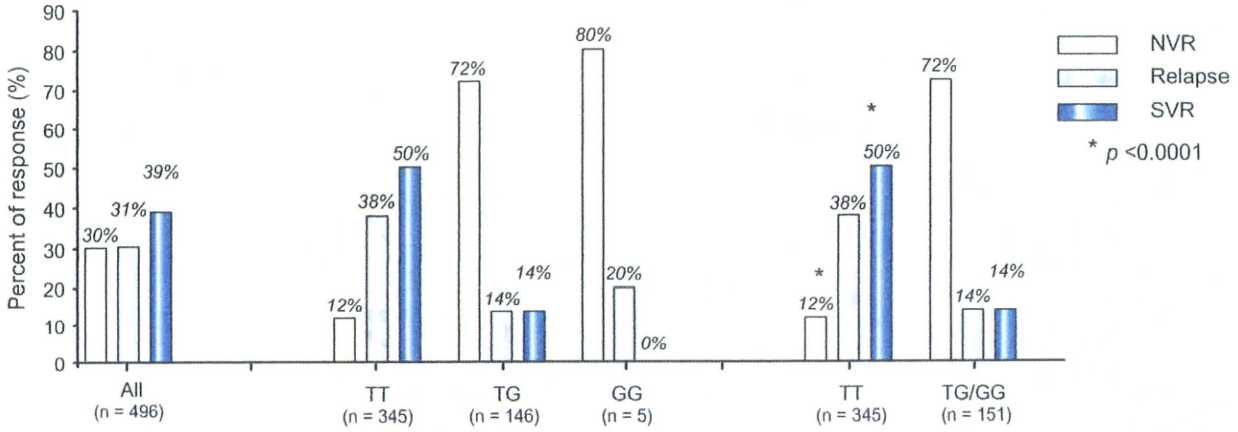

Fig. 1. Association between the IL28B genotype (rs8099917) and treatment response. The rates of response to treatment are shown for each rs8099917 genotype. The rate of null virological response (NVR), relapse, and sustained virological response (SVR) is shown. The *p* values are from Fisher's exact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

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

Statistical analysis

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

Results

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

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

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

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

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

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

Factors associated with response by multivariate logistic regression analysis

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

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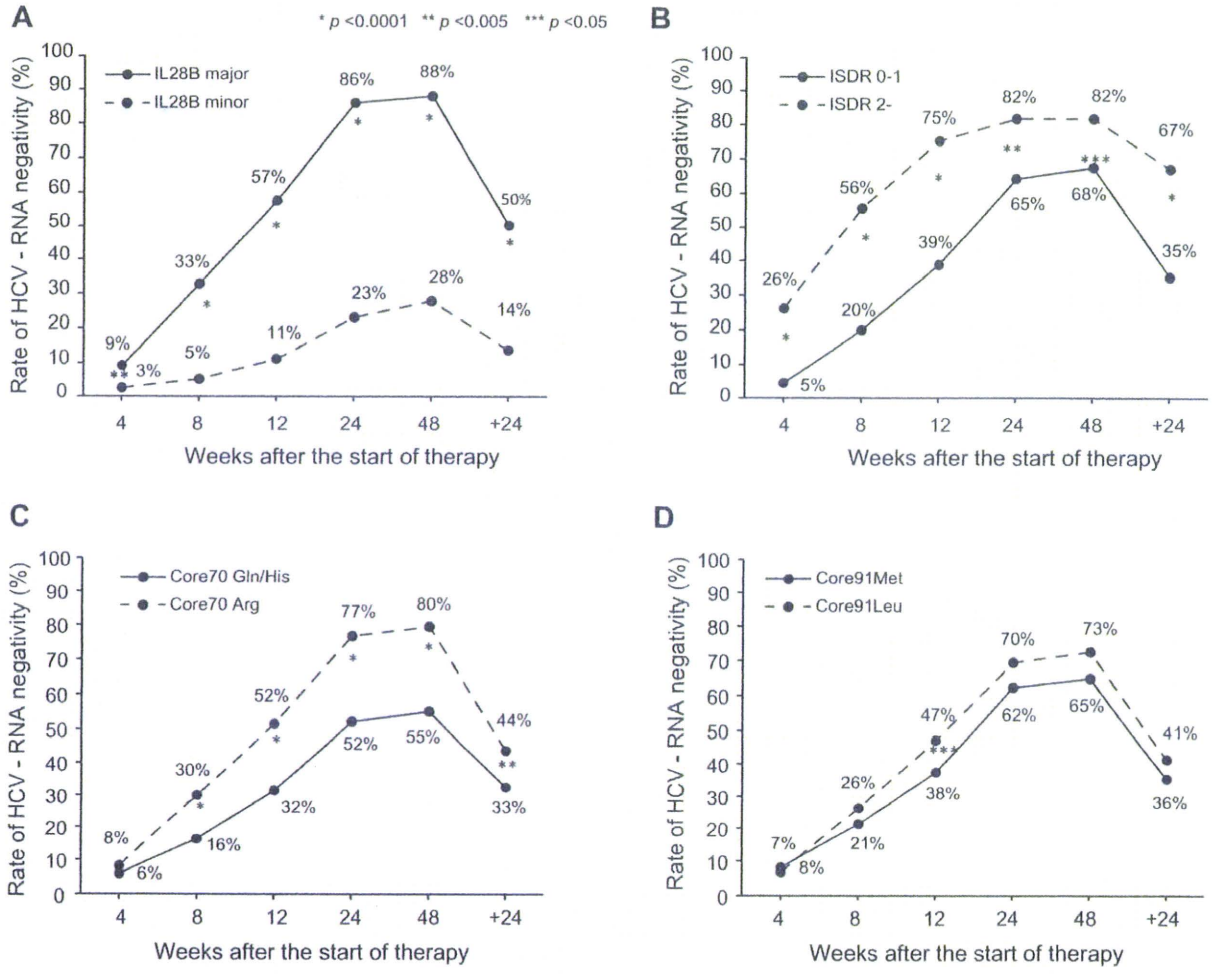

Fig. 2. Effect of *IL28B* mutations in the ISDR, Core70 and Core91 of HCV on time-dependent clearance of HCV. The rate of undetectable HCV-RNA was plotted for serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks) and for 24 weeks after the completion of therapy. Patients were stratified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

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

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

Factors associated with the IL28B polymorphism

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

Data mining analysis

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

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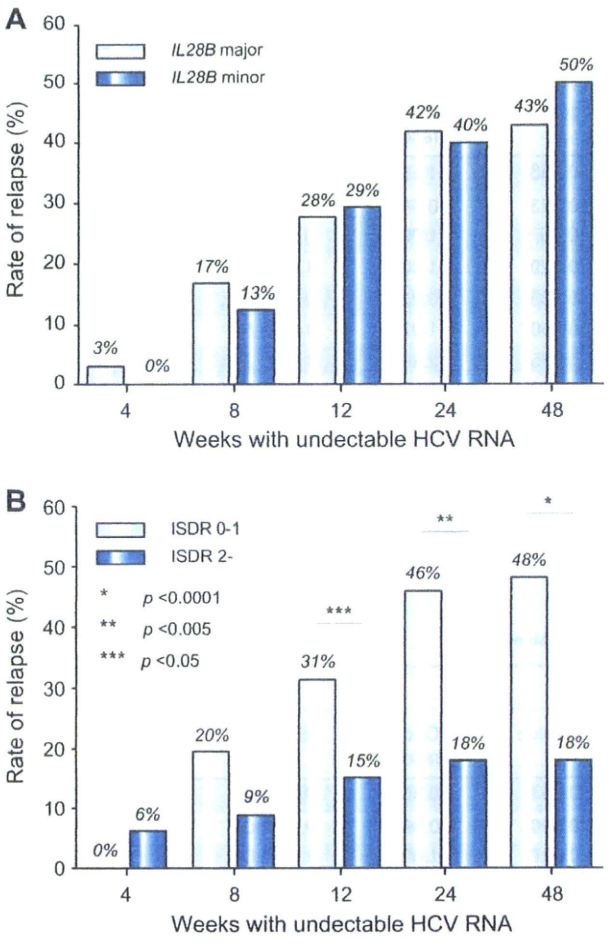

Fig. 3. Association between relapse and the *IL28B* allele or mutations in the ISDR. The rate of relapse was calculated for patients who had undetectable HCV-RNA at serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks). Patients were stratified according to (A) the *IL28B* allele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

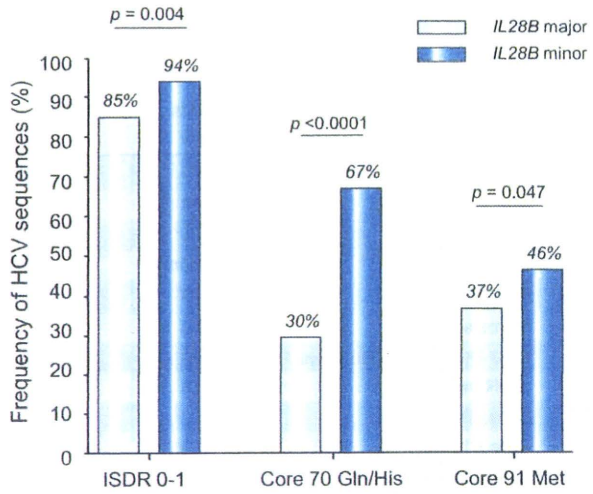

Fig. 4. Associations between the *IL28B* allele and HCV sequences. The prevalence of HCV sequences predicting a resistant phenotype to IFN was higher in patients with the minor *IL28B* allele than those with major allele. (A) 0 or 1 mutation in the ISDR of NS5A, (B) Gln or His at Core70, and (C) Met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

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

Validation of the data mining analysis

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

Discussion

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

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Table 2. Factors associated with NVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	*p* value	Odds ratio	95%CI	*p* value
Gender: female	0.98	0.67-1.45	0.938	1.29	0.75-2.23	0.363
Age	1.01	0.97-1.01	0.223	0.99	0.97-1.02	0.679
ALT	1.00	1.00-1.00	0.867	1.00	0.99-1.00	0.580
GGT	1.004	1.00-1.01	0.029	1.00	1.00-1.00	0.715
Platelets	0.95	0.91-0.99	0.009	0.92	0.87-0.98	0.006
Fibrosis: F3-4	2.23	1.46-3.42	0.0002	1.97	1.09-3.57	0.025
HCV-RNA: ≥600,000 IU/ml	1.83	1.05-3.19	0.035	2.49	1.17-5.29	0.018
ISDR mutation: ≤1	2.14	1.08-4.22	0.030	0.96	0.78-1.18	0.707
Core 70 (Gln/His)	3.23	2.16-4.78	<0.0001	1.41	0.83-2.42	0.207
Core 91 (Met)	1.39	0.95-2.06	0.093	1.21	0.72-2.04	0.462
IL28B: Minor allele	19.24	11.87-31.18	<0.0001	20.83	11.63-37.04	<0.0001

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Gln, glutamine; His, histidine; Met, methionine; Minor allele, heterozygote or homozygote of minor allele.

Table 3. Factors associated with SVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	*p* value	Odds ratio	95%CI	*p* value
Gender: female	0.81	0.56-1.16	0.253	0.86	0.55-1.35	0.508
Age	0.97	0.95-0.99	0.0003	0.99	0.96-1.01	0.199
ALT	1.00	1.00-1.00	0.337	1.00	1.00-1.01	0.108
GGT	1.00	1.00-1.00	0.273	1.00	1.00-1.00	0.797
Platelets	1.12	1.01-116	<0.0001	1.13	1.08-1.19	<0.0001
Fibrosis: F0-2	2.64	1.65-4.22	<0.0001	1.87	1.07-3.28	0.029
HCV-RNA: <600,000 IU/ml	2.49	1.55-3.98	0.0001	2.75	1.55-4.90	0.001
ISDR mutation: 2≤	3.78	2.14-6.68	<0.0001	2.11	1.06-4.18	0.033
Core 70 (Arg)	1.61	1.11-2.28	0.012	0.84	0.52-1.35	0.470
Core 91 (Leu)	1.28	0.88-1.85	0.185	1.26	0.81-1.96	0.300
IL28B: Major allele	6.21	3.75-10.31	<0.0001	7.41	4.05-13.57	<0.0001

ALT, alanine aminotransferase; GGT, Gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Leu, leucine; Major allele, homozygote of major allele.

Among baseline factors, IL28B was the most significant predictor of NVR and SVR. Moreover, the IL28B allele type was also correlated with early virological response: the rate of RVR and cEVR was significantly high for the IL28B major allele compared to the IL28B minor allele: 9% vs. 3% for RVR and 57% vs. 11% for cEVR (Fig. 2). On the other hand, the relapse rate was not different between the IL28B genotypes within patients who achieved RVR or cEVR (Fig. 3). We believe that optimal therapy should be based on baseline features and a response-guided approach. Our findings suggest that the IL28B genotype is a useful baseline predictor of virological response which should be used for selecting the treatment regimen: whether to treat patients with PEG-IFN and RBV or to wait for more effective future therapy including direct acting antiviral drugs. On the other hand, baseline IL28B genotype might not be suitable for determining the treatment duration in patients who started PEG-IFN/RBV therapy and whose virological response is determined because the IL28B genotype is not useful for the prediction of relapse. The duration of therapy should be personalized based on the virological response. Future studies need to explore whether the combination of baseline IL28B genotype and response-guided approach further improves the optimization of treatment duration.

The SVR rate in patients having the IL28B minor allele was 14% in the present study while it was 23% in Caucasians and 9% in African Americans in a study by McCarthy et al. [33]. On the other hand, the SVR rate in patients having the IL28B minor allele was 28% in genotypes 1/4 compared to 80% in genotypes 2/3 in a study by Rauch et al. [9]. These data imply that the impact of the IL28B polymorphism on response to therapy may be different in terms of race, geographical areas, or HCV genotypes, and that our data need to be validated in future studies including different populations and geographical areas before generalization.

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Table 4. Factors associated with IL28B genotype.

	IL28B major allele n = 345	*IL28B* minor allele n = 151	*p* value
Gender: male	166 (48%)	84 (56%)	0.143
Age (years)	57 ± 10	57 ± 10	0.585
ALT (IU/L)	79 ± 60	78 ± 62	0.842
Platelets (10^9/L)	153 ± 54	155 ± 52	0.761
GGT (IU/L)	51 ± 45	78 ± 91	0.001
Fibrosis: F3-4	76 (22%)	45 (30%)	0.063
Steatosis:			
>10%	16/88 (18%)	13/23 (57%)	0.024
>30%	6/88 (7%)	6/23 (26%)	0.017
HCV-RNA: >600,000 IU/ml	284 (82%)	125 (83%)	1.000

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

Four GWAS studies have shown the association between a genetic polymorphism near the IL28B gene and response to PEG-IFN plus RBV therapy. The SNPs that showed significant association with response were rs12979860 [8] and rs8099917 [6,7,9]. There is a strong linkage-disequilibrium (LD) between these two SNPs as well as several other SNPs near the IL28B gene in Japanese patients [34] but the degree of LD was weaker in Caucasians and Hispanics [8]. Thus, the combination of SNPs is not useful for predicting response in Japanese patients but may improve the predictive value in patients other than Japanese who have weaker LD between SNPs.

Other significant predictors of response independent of IL28B genotype were platelet counts, stage of fibrosis, and HCV RVA load. A previous study reported that platelet count is a predictor of response to therapy [35], and the lower platelet count was related with advanced liver fibrosis in the present study. The association between response to therapy and advanced fibrosis independent of the IL28B polymorphism is consistent with a recent study by Rauch et al. [9].

There is agreement that the viral genotype is significantly associated with the treatment outcome. Moreover, viral factors such as substitutions in the ISDR of the NS5A region [10] or in the amino acid sequence of the HCV core [4] have been studied in relation to the response to IFN treatment. The amino acid Gln or His at Core70 and Met at Core91 are repeatedly reported to be associated with resistance to therapy [4,14,15] in Japanese patients but these data wait to be validated in different populations or other geographical areas. In this study, we confirmed that patients with two or more mutations in the ISDR had a higher rate of undetectable HCV-RNA at each time point during therapy. In addition, the rate of relapse among patients who achieved cEVR was significantly lower in patients with two or more mutations in ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.05$). Thus, the ISDR sequence may be used to predict a relapse among patients who achieved virological response during therapy, while the IL28B polymorphism may be used to predict the virological response before therapy. A higher number of mutations in the ISDR are reported to have close association with SVR in Japanese [11–13,15,36] or Asian [37,38] populations but data from Western countries have been controversial [39–42]. A meta-analysis of 1230 patients including 525 patients from Europe has shown that there was a positive correlation between the SVR and the number of mutations in the ISDR in Japanese as well as in European patients [43] but this correlation was more pronounced in Japanese patients. Thus, geographical factors may account for the different impact of ISDR on treatment response, which may be a potential limitation of our study.

To our surprise, these HCV sequences were associated with the IL28B genotype: HCV sequences with an IFN resistant phenotype were more prevalent in patients with the minor *IL28B* allele than those with the major allele. This was an unexpected finding, as we initially thought that host genetics and viral sequences were completely independent. A recent study reported that the IL28B polymorphism (rs12979860) was significantly associated with HCV genotype: the IL28B minor allele was more frequent in HCV genotype 1-infected patients compared to patients infected with HCV genotype 2 or 3 [33]. Again, patients with the IL28B minor allele (IFN resistant genotype) were infected with HCV sequences that are linked to an IFN resistant phenotype. The mechanism for this association is unclear, but may be related to an interaction between the IL28B genotype and HCV sequences in the development of chronic HCV infection as discussed by McCarthy et al., since the *IL28B* polymorphism was associated with the natural clearance of HCV [44]. Alternatively, the HCV sequence within the patient may be selected during the course of chronic infection [45,46]. These hypotheses should be explored through prospective studies of spontaneous HCV clearance or by testing the time-dependent changes in the HCV sequence during the course of chronic infection.

How these host and viral factors can be integrated to predict the response to therapy in future clinical practice is an important question. Because various host and viral factors interact in the same patient, predictive analysis should consider these factors in combination. Using the data mining analysis, we constructed a simple decision tree model for the pre-treatment prediction of SVR and NVR to PEG-IFN/RBV therapy. The classification of patients based on the genetic polymorphism of *IL28B*, mutation in the ISDR, serum levels of HCV-RNA, and platelet counts, identified subgroups of patients who have the lowest probabilities of NVR (0%) with the highest probabilities of SVR (90%) as well as those who have the highest probabilities of NVR (84%) with the lowest probability of SVR (7%). The reproducibility of the model was confirmed by the independent validation based on a second

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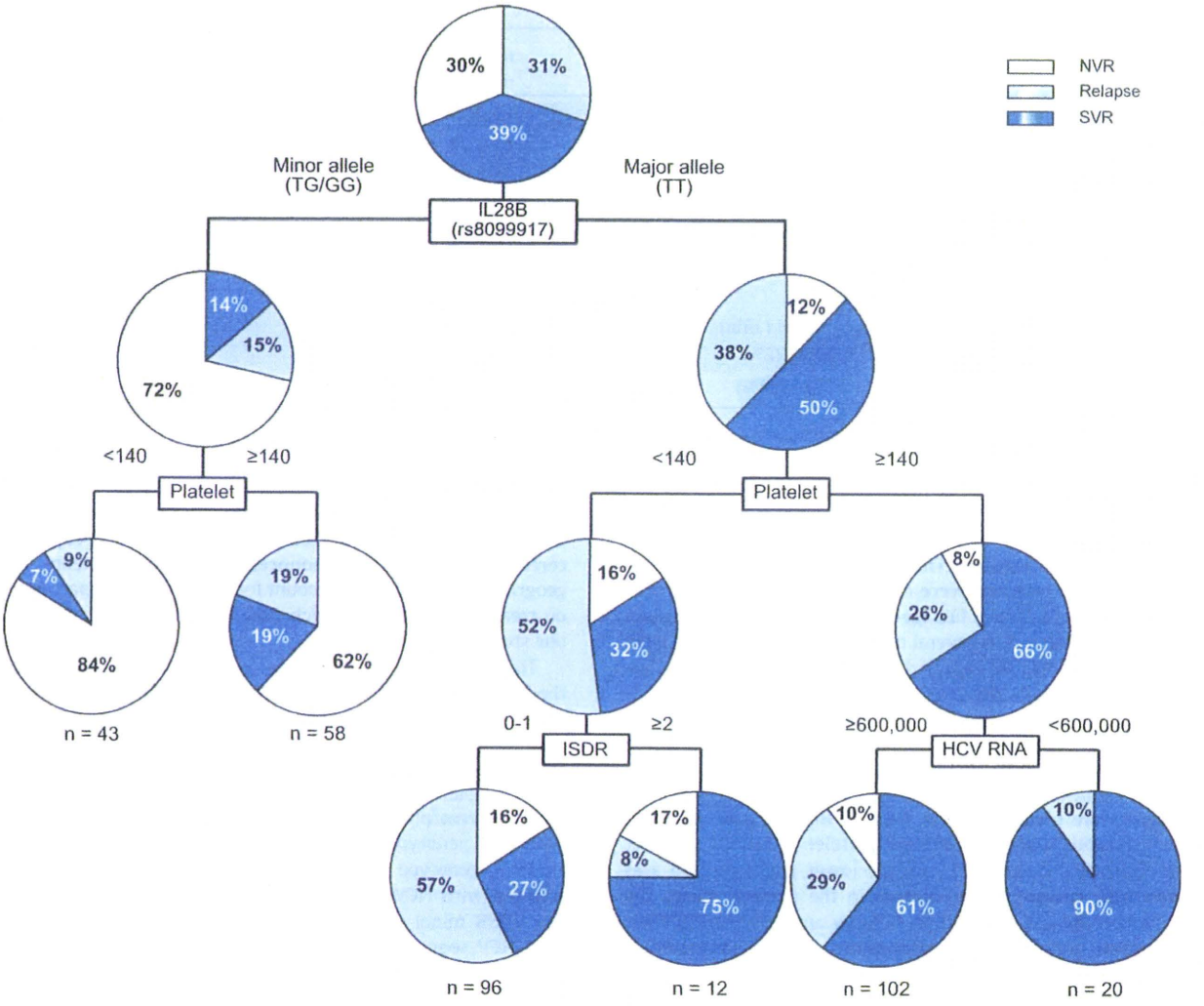

Fig. 5. Decision tree for the prediction of response to therapy. The boxes indicate the factors used for splitting. Pie charts indicate the rate of response for each group of patients after splitting. The rate of null virological response, relapse, and sustained virological response is shown. [This figure appears in colour on the web.]

group of patients. Using this model, we can rapidly develop an estimate of the response before treatment, by simply allocating patients to subgroups by following the flow-chart form, which may facilitate clinical decision making. This is in contrast to the calculating formula, which was constructed by the traditional logistic regression model. This was not widely used in clinical practice as it is abstruse and inconvenient. These results support the evidence based approach of selecting the optimum treatment strategy for individual patients, such as treating patients with a low probability of NVR with current PEG-IFN/RBV combination therapy or advising those with a high probability of NVR to wait for more effective future therapies. Patients with a high probability of relapse may be treated for a longer duration to avoid a relapse. Decisions may be based on the possibility of a response against a potential risk of adverse events and the cost of the therapy, or disease progression while waiting for future therapy.

We have previously reported the predictive model of early virological response to PEG-IFN and RBV in chronic hepatitis C [26]. The top factor selected as significant was the grade of steatosis, followed by serum level of LDL cholesterol, age, GGT, and blood sugar. The mechanism of association between these factors and treatment response was not clear at that time. To our interest, a recent study by Li et al. [47] has shown that high serum level of LDL cholesterol was linked to the IL28B major allele (CC in rs12979860). High serum level of LDL cholesterol was associated with SVR but it was no longer significant when analyzed together with the IL28B genotype in multivariate analysis. Thus, the association between treatment response and LDL cholesterol levels may reflect the underlining link of LDL cholesterol levels to IL28B genotype. Steatosis is reported to be correlated with low lipid levels [48] which suggest that IL28B genotypes may be also associated with steatosis. In fact, there were significant correlations between the IL28B genotype and the presence of steatosis in the present study (Table 4). In addition, the serum level of GGT, another predictive factor in our previous study, was significantly associated with IL28B genotype in the present study

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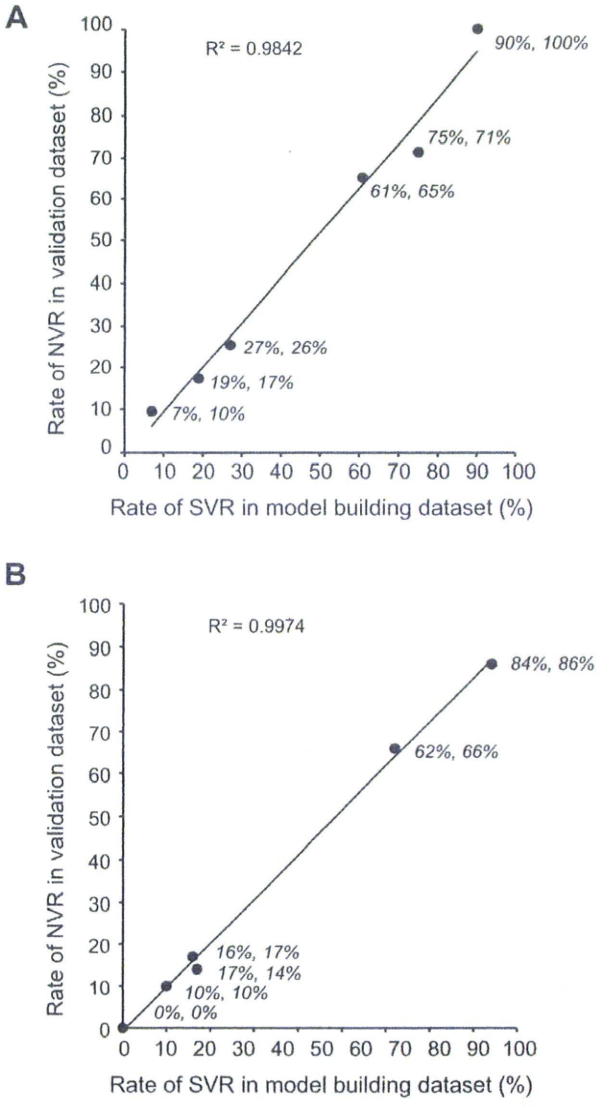

Fig. 6. Validation of the CART analysis. Each patient in the validation group was allocated to one of the six subgroups by following the flow-chart form of the decision tree. The rate of (A) sustained virological response (SVR) and (B) null virological response (NVR) in each subgroup was calculated and plotted. The *X*-axis represents the rate of SVR or NVR in the model building patients and the *Y*-axis represents those in the validation patients. The rate of SVR and NVR in each subgroup of patients is closely correlated between the model building and the validation patients (correlation coefficient: $r^2 = 0.98$–0.99).

(Table 4). The serum level of GGT was significantly associated with NVR when examined independently but was no longer significant when analyzed together with the IL28B genotype. These observations indicate that some of the factors that we have previously identified may be associated with virological response to therapy through the underlining link to the IL28B genotype.

In conclusion, the present study highlighted the impact of the *IL28B* polymorphism and mutation in the ISDR on the pre-treatment prediction of response to PEG-IFN/RBV therapy. A decision model including these host and viral factors has the potential to support selection of the optimum treatment strategy for individual patients, which may enable personalized treatment.

Conflicts of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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REVIEW

Viral factors influencing the response to the combination therapy of peginterferon plus ribavirin in chronic hepatitis C

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Abstract Hepatitis C virus (HCV) is a single-stranded RNA virus known for its high genetic variability owing to the lack of a proofreading mechanism of its RNA dependent RNA polymerase. Until now, numerous studies have been undertaken to clarify the correlation between pretreatment HCV genetic variability and the therapeutic response. Even with the recent combination therapy of peginterferon plus ribavirin for chronic hepatitis C, viral response is variable, and only half of treated patients could clear the virus [sustained viral response (SVR)]. In this review, the contribution of viral genetic variability affecting the treatment outcome is discussed according to each HCV genomic region.

Keywords Hepatitis C virus · Peginterferon plus ribavirin therapy · Viral predictive factor

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases worldwide; 180 million people, or some 3% of the world's population, are infected with HCV. Seventy percent of acute infections become persistent, and 50–75% of patients with chronic HCV infection progress to hepatocellular carcinoma. Though interferon-based therapy for HCV has been greatly advanced, half of patients still cannot eradicate the virus [sustained virological response (SVR)] even with the most recent combination therapy of peginterferon plus ribavirin [1].

HCV has a 9.5 kb single positive stranded RNA genome, and contains a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). HCV is classified as hepacivirus, a family of flaviridae. HCV is known for its high mutation rate owing to the lack of proofreading activity of its RNA dependent RNA polymerase (1.4×10^{-3} to 1.9×10^{-3} substitutions/nucleotide/year [2, 3]). In accord with this mechanism, HCV presents a high degree of genetic variability, and the resultant molecular polymorphisms of HCV are suspected as one of the major causes determining the treatment responses.

HCV genotypes

By phylogenetic analysis, HCV is classified into six major genotypes, and then further classified into subtypes in each genotype determined by their genetic distances [4]. Among all the viral factors investigated, viral genotypes are the most important, and a well-established predictive factor determining the treatment outcome. Geographically, genotypes 1–3 are associated with worldwide epidemic, while genotypes 4–6 are endemic. In comparison among major genotypes 1–3, a high SVR rate (~84%) was observed in patients with genotype 2 or 3, while a low SVR rate(~42%) was observed in genotype 1 [5–7]. Comparing between genotypes 2 and 3, genotype 2 could have more favorable outcomes [8, 9]. The study of genotype 4 was mainly from Egypt, and the SVR rate was reported to be intermediate (55–69%) [10, 11]. In genotypes 5 and 6, the SVR rate has been considered to be intermediate between the SVR of genotype 1 and genotypes 2–3, but studies

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focusing on the response of genotype 5 and 6 are limited because of their minor distribution [12].

Genomic regions and the treatment response

5′UTR

The 5′UTR of the HCV genome is 341 nucleotides long, and is the most conserved region throughout the HCV genome among different HCV genotypes. The 5′UTR together with the first 30 nucleotides of the core region acts as an internal ribosome entry site (IRES) regulating the cap-independent translation of HCV RNA to polyprotein. Secondary and tertiary conformation of IRES has the critical role in the initiation of polyprotein translation, and an IRES contains four highly structured domains (Domain I–IV). Since the structures play a pivotal role in HCV replication, changes in the conformation of an IRES, as well as changes in primary nucleotide sequence, result in a decrease of efficiency of protein translation. Therefore, it was suspected that IRES heterogeneity might correlate with the response to interferon-based therapy clinically. Several studies for interferon-based therapy, including peginterferon plus ribavirin, have been undertaken to date, however, its clinical value as a predictive factor for therapy is still in question, since most of these studies showed conflicting results of the relationship between 5′UTR variability and treatment response [13–18].

Core

The core protein is considered to form the viral nucleocapsid of HCV, and its mature form consists of a secondary structure made of a large folded multimer of ~24 monomers. It is 21 kDa in size, and is separated into two domains, an N-terminal two-thirds hydrophilic domain (D1, residues 1–117) and a C-terminal one-third hydrophobic domain (D2, residues 118–170), respectively. The D1 domain contains many positively charged amino acids, and is implicated to bind RNA. The D2 domain is required for proper folding of domain D1. The mature core protein shares high homology among HCV genotypes. The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions, such as its proapoptotic or antiapoptotic actions [19], immunomodulatory roles [20], or oxidative stress [21]. Recently, much attention has been paid to its relationships with liver steatosis, insulin resistance and hepatocellular carcinoma [22, 23]. HCV core proteins of genotype 3a and 1b were reported to interfere with the insulin signaling pathway in different ways depending on genotype-specific mechanisms [24].

As its contribution to the clinical treatment response, Akuta et al. first reported that substitutions of the amino acid 70 and 91 in the core protein were significantly related to the final outcome in the 48 weeks of interferon plus ribavirin combination therapy in 50 Japanese patients infected with genotype 1b HCV [25]. In successive studies, they reported that substitutions in those core regions were related to the final outcome, viral kinetics, early viral response, and extended 72 weeks of therapy [26–29]. They also reported substitution of core protein was associated with elevated alpha-fetoprotein, and hepatocarcinogenesis [29, 30]. Correlation of substitutions in the core protein in the treatment of interferon-based therapy was also reported in several other studies [31–33].

E2

E2 is a type I transmembrane protein 70 kDa in size which assembles with E1 protein forming a heterodimer to become the mature viral envelope. It is a glycoprotein possessing several potential conserved glycosylation sites. Because the protein is essential for the virus's entry into hepatocytes, E2 interacts with potential HCV receptors, CD81, SR-BI [34] and occludin [35]. In E2, hypervariable region 1 (HVR1) was identified in the first 27 amino acids of the E2 ectodomain. HVR1 is known for its significant genomic variability and is suspected to be the target of antibodies. Its significant genetic variability could be induced by antibody selection.

PePHD

A region between amino acid residues of E2 659–670 is well-conserved, and is known as the phosphorylation site of PKR/eIF-2α phosphorylation homology domain (PePHD). The PePHD motif is similar to the phosphorylation sites of PKR and eIF2alpha. The PePHD has been shown to interact with PKR, one of the important antiviral proteins of the host cell, and inhibit antiviral action of PKR in vitro, suggesting a possible mechanism of HCV for countering the antiviral effects of interferon [36–38]. According to those observations, mutations in this PePHD were suspected to influence the clinical response to interferon-based therapy. However, the results of those studies are conflicting, and its clinical importance as the predictive value for treatment outcome has been controversial. Though some studies supported its significance [39–42], other recent studies could not find evident correlations [43–49].

NS5A

NS5A is phosphorylated on multiple serine and threonine residues, and forms two distinct molecules of basal

phosphorylated form (p56) and hyperphosphorylated form (p58), being 56 and 58 kDa in size, respectively. The protein has three distinct domains (domains I, II, and III) being separated by low complexity sequences (LCS I and II). The study of the X-ray crystal structure analysis of domain I suggested that the NS5A is a dimer, and it forms a large putative RNA binding groove. Recent genetic study has shown many residues in domain II are essential for RNA replication, while domain III is less conserved and might be dispensable. Though the true function of NS5A is still under investigation, the protein is considered as a component of the HCV replication complex, where it modulates HCV replication through interaction with other viral proteins. Among all HCV proteins, NS5A has been most extensively explored for its relationship to interferon-based therapy.

ISDR and PKR-BD

The interferon sensitivity determining region (ISDR), located in the C-terminal half of NS5A, was originally identified as the 40 amino acid region (aa2209–2248) significantly related to the treatment outcome in the monotherapy of interferon-alpha in Japanese patients infected with genotype-1b HCV [50, 51]. The "mutant-type," having 4 or more mutations in the region, was associated with a high SVR rate (16/16: 100%), while the SVR rate was low in the "intermediate-type" [1–3 mutations: SVR rate 5/38 (13%)], or the "wild-type" [no mutation: SVR rate 30/30 (0%)]. Following studies from Japan were also concordant with the initial study [52–54]. However, controversy occurred as to the predictive value of ISDR since studies from Europe and North America did not necessarily report evident correlations between ISDR and treatment outcomes [55–60]. However, a recent meta-analysis study clearly confirmed its value, even in the Western countries [61]. Different results observed in North America and Europe might have been been caused partly by smaller rates of mutant-type patients in Western countries, and by the different treatment regimen in Japan compared to Western countries [62–66]. Though ISDR was found in the era of interferon monotherapy, its predictive value in the treatment outcome of the recent peginterferon plus ribavirin regimen has continued to be reported in most large cohort studies [26, 33, 67–69]. In searching for the biological ISDR function, Gale et al. reported that NS5A represses PKR through a direct interaction with the PKR binding domain (PKR-BD, aa2209–2274) and that the PKR-BD contains the ISDR [70]. Thus, they insisted that inactivation of PKR may be one mechanism by which HCV avoids the antiviral effects of interferon.

V3 domain and IRRDR

The V3 domain located in the C-terminal region of NS5A (aa2356–2379) was originally identified as a genomic region of genotype-1b HCV showing a marked heterogeneity between Japanese and American isolates [71]. A correlation of its mutations and the response to interferon-based therapy was first reported by Duverlie et al., and they reported that sequences of the V3 domain were highly conserved in resistant strains, but were highly variable in sensitive strains [72]. Most following studies also reported concordant results [46, 47, 68, 73, 74]. El-Shamy et al. reported a high degree of sequence variations in the V3 and the flanking pre-V3 regions (aa2334–2355) of NS5A, and they designated the region as the interferon/ribavirin resistance-determining region (IRRDR) (aa2334–2379). They reported that substitution number in the IRRDR was closely correlated with early virological response (EVR) by week 16 in 47 HCV-1b-infected patients treated with peginterferon plus ribavirin [75]. In their follow up study for the same group of patients, sequence variation in the IRRDR was also significantly related to the final outcome. The positive predictive values of IRRDR of 6 or more for SVR was 89% (16/18), whereas negative predictive values of IRRDR of 5 or less for non-SVR was 81% (22/27) [76].

Other region in NS5A

Pfeiffer et al. reported that two responsible mutations resided in the C-terminal region of NS5A: G404S and E442G were considered as mechanisms accounting for ribavirin resistance during HCV RNA replication, using HCV replicon-containing cell lines in the presence of increasing concentrations of ribavirin [77]. However, the clinical importance of such mutations and their relevance to ribavirin-related therapy is not evident.

NS5B

NS5B is 68 kDa proteins in size, and known as an RNA-dependent RNA polymerase. The enzyme synthesizes HCV–RNA using HCV–RNA as a template. NS5B is considered as one component of the HCV–RNA replication complex, and its activity as an RNA polymerase is modulated by NS3 and NS5A. Since this enzymatic activity is critical for HCV replication, the correlation between its mutations and treatment response has been explored, to date, in several studies.

Though the viral inhibitory mechanism of ribavirin in the treatment of HCV is unknown, its action as a mutagen is especially focused on the NS5B protein. During

ribavirin monotherapy, Young et al. reported that a specific mutation of NS5B amino acid 415 Phe-to-Tyr (F415Y) had emerged in five out of five patients infected with genotype-1a HCV [78]. To clarify the biological relevance of this mutation in ribavirin monotherapy, they introduced NS5B F415Y mutations into subgenomic HCV replicons, and reported that they observed different drug sensitivities in HCV replicons according to this NS5B polymorphism in a ribavirin dose-dependent manner. However, subsequent studies done in Japan and the UK could not find an evident relationship between specific selection of NS5B 415 mutations and the treatment of combination therapy of peginterferon and ribavirin. Sugihara et al. reported that they did not find specific mutations in NS5B 415 in the both serum obtained before and after therapy in 18 patients infected with genotype-1b HCV [79], and Ward et al. could not find evidence of a relationship of these mutations in the therapy of peginterferon and ribavirin in 15 patients infected with genotype-1a [80]. Hamano et al. explored genetic changes of genotype-1b HCV during the treatment of interferon-alpha and ribavirin, and reported that mutations at positions 300–358 of NS5B, including polymerase motif B–E, occurred more frequently in SVR patients or in end-of-treatment response patients when compared to null-response patients [81]. Mutation rate of NS5B in patients undergoing treatment with ribavirin monotherapy was also explored in patients treated with peginterferon/ribavirin therapy, since error catastrophe from an increase in mutation rate could be a possible mechanism of ribavirin in HCV infection [82]. Lutchman et al. reported that ribavirin was only associated with an early transient increase in the HCV mutation rate, but lethal mutagenesis and error catastrophe was unlikely to be the sole mechanism of ribavirin [83].

Conclusions

Viral genetic variability of HCV and its potential correlation to the interferon-based treatment response is briefly discussed here. Understanding the biological features of drug-resistant HCV, may helpl us to predict the treatment response in each patient in advance. Furthermore, though trials of HCV specific protease inhibitors are on-going, and are just about to be incorporated into the new standard therapy, understanding those biological features of HCV would further clarify and focus which patients will most benefit from being treated with the new treatment regimens. This viral genetic approach could be crucial even in the era of HCV protease inhibitors for achieving global eradication of HCV.

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