of IFN on HCC development among the studies, probably due to the large differences in the baseline rate of HCC development among the different trials [12]. Whether the incidence of HCC development could be reduced in all patients with chronic hepatitis C, especially in those without liver cirrhosis, remains to be elucidated.

Data mining analysis, unlike conventional statistical analysis, is performed in an exploratory manner without considering a predefined hypothesis. Decision tree analysis, the major component of data mining analysis, is used to extract relevant factors from among various factors. These relevant factors are then combined in an orderly sequence to identify rules for predicting the incidence of the target outcome [13]. Data mining analysis has been used to define prognostic factors in various diseases [14–20]. In the field of hepatic diseases, data mining analysis has proven to be a useful tool for predicting early response [21], sustained virological response (SVR) [22–25], relapse [26], and adverse events [27] in patients with chronic hepatitis C treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV). The findings of data mining analysis are expressed as flowcharts and are therefore easily understood [28] and readily available for clinical use, even by physicians without a detailed understanding of statistics.

In the present study, data mining analysis was used to identify risk factors for HCC development in a cohort of patients with chronic hepatitis C who had been followed for at least 5 years. An HCC risk prediction model was constructed on the basis of simple and generally available tests because the goal was to make the model easy to use in the clinic. The suitability, reproducibility, and generalizability of the results were validated using the data of an external cohort that was independent of the model derivation cohort.

Materials and methods

Patients

The model derivation cohort consisted of 1003 chronic hepatitis C patients without cirrhosis who had a non-sustained virological response (nonSVR) to previous IFN administered at the Musashino Red Cross Hospital and were followed for at least 5 years. Patients who had SVR or those who were followed for less than 5 years were not included. An analytical database on age, body mass index, albumin, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, γ-glutamyltransferase (GGT) levels, total bilirubin levels, total cholesterol levels, hemoglobin levels, and platelet count at the start of the observation was created. Histological data such as fibrosis stage, activity grade, or degree of steatosis was not included in the database because the goal of the present study was to make the model on the basis of simple and generally available tests. The patients who developed HCC more than 5 years after the start of the observation were considered not to have developed HCC by the 5-year point because the model was intended to predict HCC development within 5 years. The 1072 chronic hepatitis C patients included in the external validation cohort were treated with PEG-IFN and RBV at the University of Yamanashi, Tokyo Medical and Dental University, Osaka University, Osaka City University, Nagoya City University, or Toranomon Hospital and followed for at least 5 years. Among them, 600 had nonSVR and 472 had SVR. Data from nonSVR patients in this external cohort were used for external validation of the HCC prediction model. To assess the preventive effect of PEG-IFN plus RBV therapy on HCC development, the cumulative HCC development rate was compared between SVR and nonSVR patients in the external validation cohort after stratification by the risk of HCC development as determined by data mining analysis. Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committees of all concerned hospitals.

HCC surveillance and diagnosis

HCC surveillance was conducted by performing abdominal ultrasonography every 4–6 months. Contrast-enhanced computer tomography, magnetic resonance imaging, or angiography were performed when abdominal ultrasonography suggested a new lesion suspicious for HCC. Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

Statistical analysis

The IBM-SPSS Modeler 13 (IBM SPSS Inc., Chicago, IL, USA) was used for decision tree analysis. The statistical methods used have been described previously [21,22,24–27]. In brief, the software searched the analytical database for the factor that most effectively predicted HCC development and for its cutoff value. The patients were divided into two groups according to that predictor. Each divided group was repeatedly assessed and divided according to this 2-choice branching method. Branching was stopped when the number of patients decreased to $\leqslant$20 to avoid over fitting. Finally, an HCC risk prediction model was created through this analysis. The model classified patients into subgroups with different HCC development rates in a flowchart form. For model validation, nonSVR patients from an external cohort were individually fitted into the model and classified into the subgroups and the HCC development rates of those subgroups were then calculated. The suitability and reproducibility of the model were validated by comparing the subgroup HCC development rates of the model derivation group to those of the validation group.

On univariate analysis, Student's *t*-test was used for continuous variables and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. A log-rank test for Kaplan–Meier analysis was used to statistically test HCC development rates over time. *p*-Values of <0.05 were considered significant. SPSS Statistics 18 (IBM SPSS Inc.) was used for these analyses.

Results

Univariate and multivariate analysis of factors associated with HCC development

The baseline characteristics of patients are shown in Table 1. The 5-year HCC development rate in the model derivation group was 6.2%, which did not differ significantly from the rate of 6.0% in the nonSVR group of the external cohort, but the rate of 2.0% in the SVR group of the external cohort was significantly lower than that in the model derivation group ($p = 0.0003$) and the nonSVR group of the external cohort ($p = 0.0012$). On univariate analysis, the factors found to be associated with HCC development in the model derivation cohort were age, AST levels, albumin levels, total cholesterol levels, and platelet count. On multivariate analysis, age (odds ratio 1.086), albumin levels (odds ratio 0.248), and platelet count (odds ratio 0.842) were significant predictors of HCC development (Table 2).

HCC risk prediction model by data mining analysis

The results of decision tree analysis are presented in Fig. 1. Age was selected as the first predictor. The 5-year HCC development rate was 3.4% in younger patients (<60 years) and 8.6% in older patients ($\geqslant$60 years). The second predictor for younger patients (<60 years) was platelet count. The HCC development rate was 6.9% in patients with a lower platelet count ($<150 \times 10^9$/L) and 0.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The second predictor for older patients ($\geqslant$60 years) was also platelet count. The HCC development rate was 13.1% in patients with a lower platelet count ($<150 \times 10^9$/L) and 1.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The third predictor was albumin levels,

Table 1. Baseline characteristics of patients for model deviation and external validation.

	Model derivation (n = 1003)	External cohort, non-SVR (n = 600)	External cohort, SVR (n = 472)
Sex: Male/Female*	463 (46%)/540 (54%)	306 (51%)/294 (49%)	299 (63%)/173 (37%)
Age (yr)	57.3 (11.1)	55.9 (9.6)	51.4 (10.6)
Body mass index (kg/m^2)	23.5 (3.2)	23.4 (3.3)	23.3 (3.1)
Albumin (g/dl)	4.1 (0.3)	4.0 (0.4)	4.0 (0.3)
AST (IU/L)	64.2 (36.5)	67.3 (43.8)	62.5 (48.3)
ALT (IU/L)	80.6 (55.1)	81.2 (62.3)	88.6 (82.1)
GGT (IU/L)	59.3 (50.5)	67.6 (65.1)	55.7 (71.2)
Total cholesterol (mg/dl)	172.1 (31.5)	168.2 (31.0)	174.3 (33.7)
Platelet (10^9/L)	154.0 (53.0)	153.7 (53.2)	176.6 (49.7)
Hemoglobin (g/dl)	13.3 (1.5)	14.2 (1.5)	14.4 (1.4)
HCC development within 5 years: n (%)*	62 (6.2%)	36 (6.0%)	10 (2.0%)

Data expressed as mean (standard deviation) unless otherwise indicated.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.
*Data expressed as number of patients (percentage).

whose cutoff value was 3.75 g/dl in patients with a higher platelet count ($\geq 150 \times 10^9$/L). The HCC development rate was 6.3% when albumin levels were lower (<3.75 g/dl) and 1.5% when levels were higher ($\geq$3.75 g/dl). The cutoff value for albumin levels was 4.0 g/dl in patients with a lower platelet count ($<150 \times 10^9$/L). The HCC development rate was 20.9% when albumin levels were lower (<4.0 g/dl) and 6.4% when levels were higher ($\geq$4.0 g/dl). The fourth and final predictor was AST levels. The HCC development rate was 7.3% when AST levels were at least 40 IU/L and 0% when the levels were <40 IU/L. On the basis of this analysis, seven subgroups with a 5-year HCC development rate of 0–20.9% were identified. The area under the receiver operating characteristic curve according to the HCC risk prediction model was 0.817.

External validation of the HCC risk prediction model with an independent external cohort

Six hundred nonSVR patients from an external cohort were fitted into the HCC risk prediction model and classified into the seven subgroups. The 5-year HCC development rate of these subgroups was 0–17.9%. The HCC development rate in the individual subgroups of the model derivation group was closely correlated to that in the corresponding subgroups of the external validation group (Fig. 2; correlation coefficient $r^2 = 0.981$). The HCC development rate in the subgroup of patients with the highest risk of HCC development (high-risk group) according to the model older age ($\geq$60 years) with a lower platelet count ($<150 \times 10^9$/L) and lower albumin levels (<4.0 g/dl) was 20.9% in the model derivation group and 17.9% in the external validation group. The intermediate-risk group or the patients with an HCC development rate of at least 5% consisted of the following three subgroups: (1) older age ($\geq$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geq$4.0 g/dl), and higher AST levels ($\geq$40 IU/L); (2) older age ($\geq$60 years), higher platelet count ($\geq 150 \times 10^9$/L), and lower albumin levels (<3.75 g/dl); and (3) younger age (<60 years) and lower platelet count ($<150 \times 10^9$/L). In these intermediate-risk groups, the 5-year HCC development rate was 6.3–7.3% in the model derivation group and 5.3–7.9% in the external validation group. The low-risk group consisted of the following three subgroups: (1) younger age (<60 years) and higher platelet count ($\geq 150 \times 10^9$/L); (2) older age ($\geq$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geq$4.0 g/dl), and lower AST levels (<40 IU/L); and (3) older age ($\geq$60 years), higher platelet count ($\geq 150 \times 10^9$/L), and higher albumin levels ($\geq$3.75 g/dl). In these low-risk groups, the 5-year HCC development rate was 0–1.5% in the model derivation group and 0–2.9% in the external validation group.

Table 2. Multivariable analysis of factors associated with subsequent development of HCC within 5 years.

	Odds ratio	95% CI	*p* value
Age	1.086	1.029-1.146	0.003
Albumin	0.248	0.100-0.613	0.003
Platelet	0.842	0.769-0.921	<0.0001

CI, confidence interval.

Predictability of the HCC risk prediction model on HCC development rate beyond 5 years

Cumulative HCC development rates in the high-, intermediate-, and low-risk groups were compared over time using the Kaplan–Meier method. The 10-year rates were 28.9% in the high-risk group, 22.9% in the intermediate-risk group, and 4.8% in the low-risk group (Fig. 3A). The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups had a significantly higher cumulative HCC development rate than the low-risk group beyond 5 years (Fig. 3B; 5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; $p < 0.0001$).

Effect of response to PEG-IFN plus RBV therapy in the reduction of HCC development: analysis stratified by the HCC risk prediction model

The 600 nonSVR patients and 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and

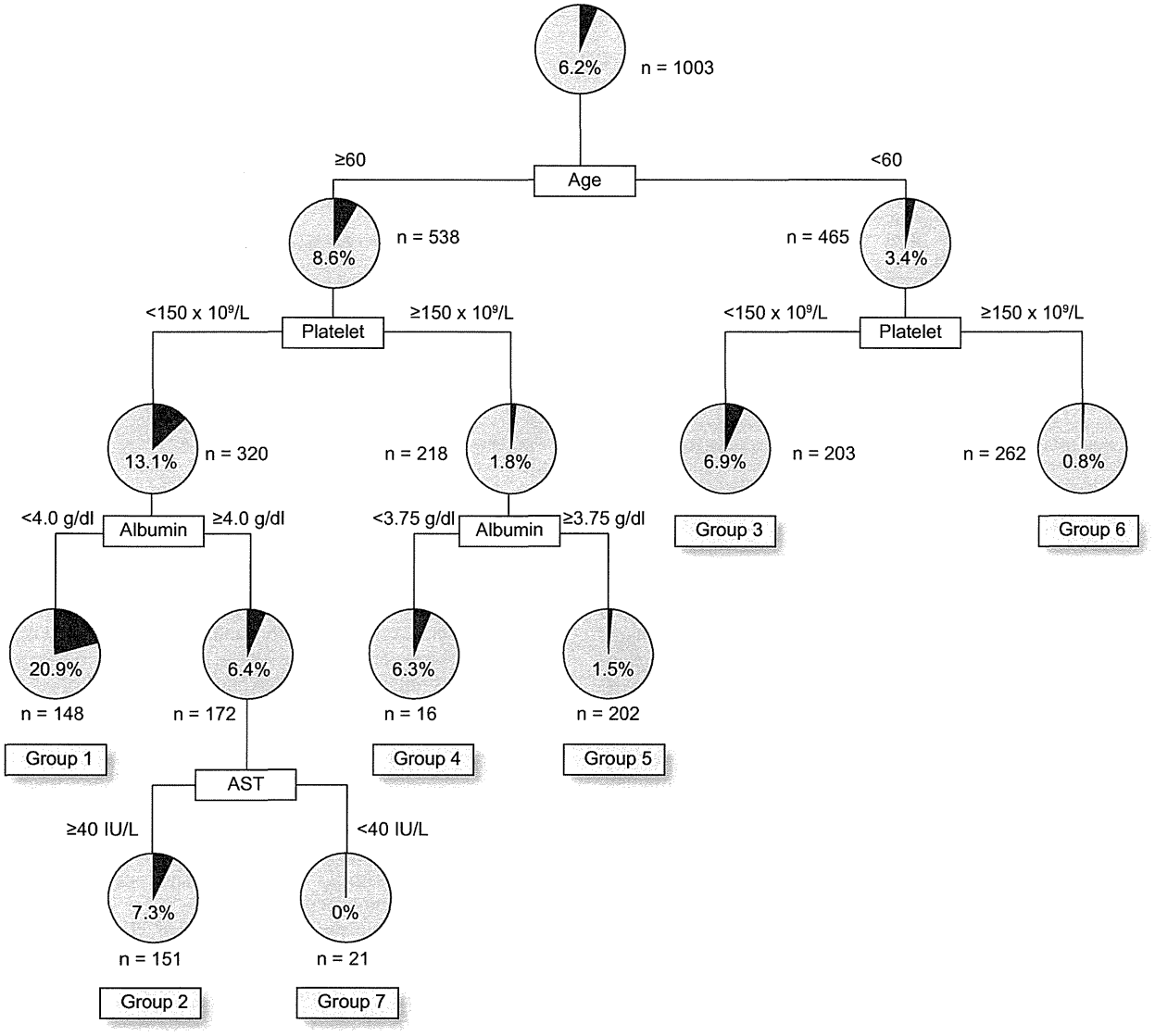

Fig. 1. The decision tree model of HCC development within 5 years. Boxes indicate the factors used to differentiate patients and the cutoff values for those different groups. Pie charts indicate the HCC development rate within 5 years for each group of patients after differentiation. Terminal groups of patients differentiated by analysis are numbered from 1 to 7.

classified into the high-and intermediate-risk group or the low-risk group, as defined above. The HCC development rate was significantly lower in SVR patients than in nonSVR patients in the high-and intermediate-risk group (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$, log-rank test). In the low-risk group, the 5-year rate was 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates were low and not significantly different ($p = 0.331$, log-rank test) (Fig. 4).

Discussion

An awareness of the risk of HCC development in the context of routine care for chronic hepatitis C is essential for formulating an HCC surveillance plan personalized for individual patients. The risk of developing HCC from chronic hepatitis is lower than that from cirrhosis [7]; therefore, across-the-board surveillance for chronic hepatitis C is not recommended [3]. A method to easily determine this risk, without performing serial liver biopsies, would be extremely significant clinically. In the present study, an HCC risk prediction model that included the factors such as age, platelet count, albumin levels, and AST levels was constructed. The model was found to have excellent reproducibility when validated with an external cohort. This model could identify subgroups of chronic hepatitis C patients at high risk of HCC development; the 5-year HCC development rate for the high- and intermediate-risk groups was 11.6%, yielding an annual incidence of 2.3%. This HCC risk prediction model requires only

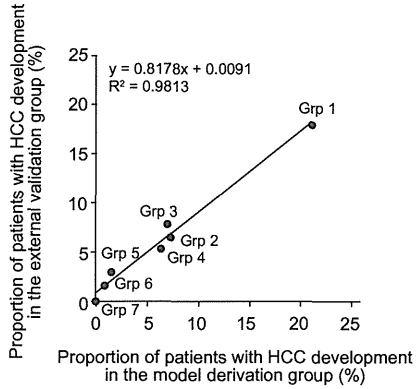

Fig. 2. External validation of the decision tree model with an independent cohort. Each patient in the external validation group was allocated to groups 1–7 following the flowchart of the decision tree. The HCC development rates were then calculated for each group and the graph plotted. The *x*-axis represents the HCC development rate in the model derivation group, and the *y*-axis represents the HCC development rate in the external validation group. The HCC development rates in each subgroup of patients are closely correlated between the model derivation group and the external validation group (correlation coefficient: $R^2 = 0.981$).

simple test values that are readily obtained in routine care and can therefore be easily used at the patient bedside. The model can be used to identify patients with a high risk of HCC development and therefore requiring surveillance, thereby allowing the formulation of surveillance plans personalized for individual patients.

Advanced fibrosis has been reported as independent risk factors for HCC development [7,8]. Platelet counts and albumin levels, which were factors selected for discrimination of the risk of HCC development, are closely related to the stage of fibrosis. Their correlation with the HCC risk has been repeatedly demonstrated [9–11,29–31]. The present study confirmed the impact of old age and advanced fibrosis, as reflected by low platelet counts and albumin levels. These results are consistent with our previous report [32]. What is unique to the present study was the study design to build a simple and reliable model for

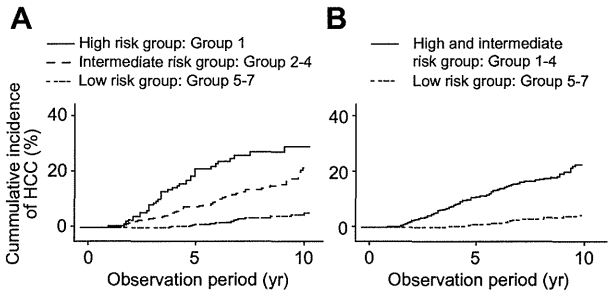

Fig. 3. Cumulative incidence of HCC development beyond 5 years in subgroups of patients defined by the decision tree model. Cumulative incidences of HCC in the groups classified by the decision tree model are compared. (A) The cumulative HCC development rate beyond 5 years is higher in the high- (group 1) and intermediate-risk (groups 2–4) groups compared to the low-risk group (groups 5–7). (B) The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups has a significantly higher cumulative HCC development rate than the low-risk group (5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; $p < 0.0001$).

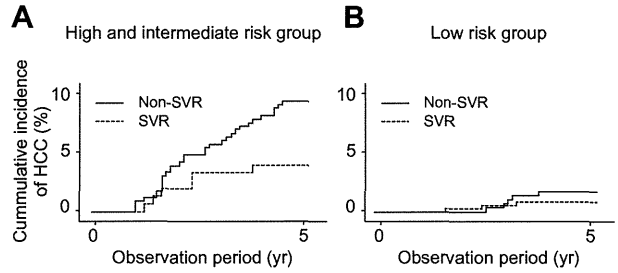

Fig. 4. Sustained virological response to PEG-IFN plus RBV therapy reduces the incidence of HCC development after stratification by the HCC risk. The 600 nonSVR patients and the 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and classified into the high and intermediate-risk group or the low-risk group. The HCC development rate is significantly lower in SVR patients than in nonSVR patients in the high and intermediate-risk group (groups 1–4) (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$). In the low-risk group (groups 5–7), the 5-year rate is 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates are low and not significantly different ($p = 0.331$).

the prediction of HCC development that could be easily used in the clinic. For this purpose, a novel statistical method was used, histological factors were excluded in the analysis, the model derivation cohort was restricted to those who had nonSVR and had a long follow-up period duration (5 years), and the reproducibility of the model was independently validated by an external cohort. These are the major differences of the present study compared to our previous report. Many researchers have put a lot of efforts to formulate regression models for HCC prediction [9,10,33]. These prediction models are useful for identifying high-risk patients but are somewhat complicated to use at the bedside because they require calculations to be performed. Our prediction model is used simply by incorporating patients' data obtained through simple tests into the decision tree and following the flowchart. These prediction models based on factors easily accessible in routine clinical settings help physicians identify high-risk patients out of chronic hepatitis.

Viral eradication is the short-term goal of IFN therapy, but the ultimate goal is the prevention of HCC occurrence. Previous reports have shown that SVR to IFN therapy suppresses HCC occurrence in patients with type C liver cirrhosis and chronic hepatitis [7,12,30,34,35]. However, there is a marked heterogeneity in the magnitude of the treatment effect on the risk of HCC among studies, probably due to differences in the baseline risk of HCC among different trials [12]. Thus, the question remains whether the preventive effect of IFN therapy on HCC development could apply to all patients with chronic hepatitis C, especially those without liver cirrhosis. The result of the present study indicated that among high- and intermediate-risk patients, as assessed with our HCC risk prediction model, the cumulative HCC development rate was significantly reduced in SVR patients compared with nonSVR patients. This finding suggests that patients with chronic hepatitis, in whom disease has not yet progressed to hepatic cirrhosis but who are at a high risk of HCC development, benefit from antiviral treatment. The preventive effect of IFN on HCC development was not evident in low-risk patients within 5 years of observation. A longer observation term may be required to analyze the possible effect of antiviral therapy in these patients. Application of the present model on treatment decision may have limitations in that effect to prevent HCC development may differ in newer therapeutic agents such as protease

inhibitors [36,37], and that low-risk patients may also benefit from therapy after a longer term observation period such as 15–20 years.

Patients with chronic hepatitis often have no subjective symptoms accompanying their disease and therefore have a low consciousness of the disease. The broad array of adverse reactions and the high cost of IFN therapy are frequent hurdles in motivating patients to undergo therapy. However, patients may be convinced to undergo therapy or remain motivated for continued therapy if they are made aware of their risk of HCC development and the preventive effect of IFN on HCC development.

In conclusion, a reproducible HCC risk prediction model, which includes the factors such as age, platelet count, albumin levels, and AST levels, was constructed to predict the 5-year HCC development rate in patients with chronic hepatitis C. The model requires only a combination of readily available test values and can therefore be easily used at the bedside. The information provided by the model allows the physician to identify patients requiring IFN therapy for the prevention of HCC and formulate plans for imaging HCC surveillance.

Conflict of interest

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

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Original article

Age and total ribavirin dose are independent predictors of relapse after interferon therapy in chronic hepatitis C revealed by data mining analysis

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Background: This study aimed to define factors associated with relapse among responders to pegylated interferon (PEG-IFN) plus ribavirin (RBV) therapy in chronic hepatitis C.

Methods: A cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN plus RBV and who had an undetectable HCV RNA by week 12 (n=951) were randomly assigned to model derivation (n=636) or internal validation (n=315) groups. An independent cohort (n=598) were used for an external validation. A decision tree model for relapse was explored using data mining analysis.

Results: The data mining analysis defined five subgroups of patients with variable rates of relapse ranging from 13% to 52%. The reproducibility of the model was confirmed by internal and external validations (r^2=0.79 and 0.83, respectively). Patients with undetectable HCV RNA at week 4 had the lowest risk of relapse (13%), followed by patients <60 years with undetectable HCV RNA at week 5–12 who received ≥3.0 g/kg of body weight of RBV (16%). Older patients with a total RBV dose <3.0 g/kg had the highest risk of relapse (52%). Higher RBV dose beyond 3.0 g/kg was associated with further decrease of relapse rate among patients <60 years (up to 11%) but not among older patients whose relapse rate remained stable around 30%.

Conclusions: Data mining analysis revealed that time to HCV RNA negativity, age and total RBV dose was associated with relapse. To prevent relapse, ≥3.0 g/kg of RBV should be administered. Higher dose of RBV may be beneficial in patients <60 years.

Introduction

The currently recommended therapy for chronic hepatitis C is a combination of pegylated interferon (PEG-IFN) plus ribavirin (RBV) [1]. This therapy is effective in 50% of patients with HCV genotype 1b [2,3]. The most reliable predictor of sustained virological response (SVR) is the response during early weeks of therapy. A satisfactory response to therapy in the early weeks is associated with a high rate of SVR [4–8]. A basic concept of response-guided therapy is to modify the duration of therapy according to the time to HCV RNA negativity. Extended therapy may be given to patients with delayed virological response [9–13]. Modification of duration of therapy or drug dose may also be necessary in patients with early virological

response (EVR), because approximately 20% of these patients experience relapse after the completion of 48 weeks of therapy. Recent reports have revealed that single nucleotide polymorphisms located near the *IL28B* gene are strongly associated with SVR or a null response to PEG-IFN plus RBV therapy [14–16]. However, single nucleotide polymorphisms located near the *IL28B* gene are not associated with relapse after EVR [17]. Identification of risk factors for relapse among patients with virological response may lead to more individualized therapy and improved SVR rate.

Decision tree analysis, a core component of data mining analysis, is a method that explores data to develop predictive models [18]. This method has been originally used in business and recently in medical fields [19–25]. Decision tree analysis was successfully used to build a predictive model of EVR [26] and SVR to PEG-IFN plus RBV combination therapy in chronic hepatitis C [17,27,28]. The results of the analysis are presented as a tree structure, which is easy to understand and use in clinical practice. Patients can be allocated into subgroups by simply following the flowchart form of the decision tree [29].

In the present study, we used decision tree analysis to identify predictors of relapse among patients who achieved EVR to PEG-IFN plus RBV therapy, and to define a more individualized therapeutic strategy beyond response-guided therapy.

Table 1. Background of study population

Characteristic	Value
Age, years	54.9 (10.8)
Gender	-
Male, *n* (%)	557 (59)
Female, *n* (%)	394 (41)
Body mass index, kg/m^2	23.2 (3.3)
Albumin, g/dl	4.1 (1.8)
Creatinine, mg/dl	0.7 (0.2)
AST, IU/l	60.6 (46.2)
ALT, IU/l	80.7 (77.2)
GGT, IU/l	52.0 (60.0)
White blood cell count, cells/μl	4,993 (1,363)
Haemoglobin, g/dl	15.9 (52.6)
Platelets, 10^3/l	174.4 (6.1)
HCV RNA, KIU/ml	1,655 (1,455)
Fibrosis stage	-
F1-2, *n* (%)	626 (66)
F3-4, *n* (%)	98 (10)
NA, *n* (%)	227 (24)
Time to HCV RNA negativity 4/8/12 weeks	-
4 Weeks, *n* (%)	233 (24)
8 Weeks, *n* (%)	386 (41)
12 Weeks, *n* (%)	332 (35)
Treatment duration, weeks	42 (13)
Total RBV dose, g/kg body weight	3.1 (1.3)
Total PEG-IFN dose, μg/kg body weight	62.5 (38.6)
Outcome	-
Relapse, *n* (%)	238 (25)
SVR, *n* (%)	713 (75)

Total *n*=951. Data are expressed as mean (sd) unless otherwise indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; NA, not available; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR: sustained virological response.

Methods

Patients

This is a multicentre retrospective cohort study involving Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University, Yamanashi University, Osaka City University, and their related hospitals. The inclusion criteria were chronic hepatitis C patients treated with PEG-IFN-α2b plus RBV, genotype 1b, pretreatment HCV RNA titre >100 KIU/ml as confirmed by quantitative PCR; Cobas Amplicor HCV Monitor version 2.0; Roche Diagnostic Systems, Pleasanton, CA, USA), an undetectable HCV RNA level within week 12 after the start of therapy, no coinfection with HBV or HIV, and no other causes of liver disease. Patients were treated with PEG-IFN-α2b (1.5 μg/kg) subcutaneously every week plus a daily weight-adjusted RBV dose (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg and 1,000 mg for patients weighing >80 kg). Dose reduction or discontinuation of PEG-IFN and RBV was considered based on the recommendations of the package inserts and the discretion of physicians at each university and hospital. The standard duration of therapy was set at 48 weeks, but extension of duration was allowed and implemented at the discretion of each physician. The duration of therapy was extended beyond 48 weeks in 118 patients (mean duration was 56.3 weeks, ranging from 49 to 72 weeks). Although the exact reason for the prolonged treatment in each case was not available, one reason may be that each physician tried to achieve high adherence of RBV by extending the duration of therapy. Another reason may be the late time point of HCV RNA negativity even within early virological response. Among 118 patients, time to HCV RNA negativity was between 9 to 12 weeks in 56% of patients.

A total of 951 patients fulfilled the study criteria. The baseline characteristics and representative laboratory test results are listed in Table 1. For analysis, patients were randomly assigned to either the model derivation (636 patients) or internal validation (315 patients) groups. There were no significant differences in the clinical backgrounds between these two groups. For external validation of the model, we collaborated with another multicentre study group consisting of 29 medical centres and hospitals belonging to the National

Hospital Organization (Japan). A dataset collected from 598 patients who were treated with PEG-IFN-α2b plus RBV and had undetectable HCV RNA within week 12 were used for external validation. Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committees of all concerned hospitals.

Laboratory tests

Haematological tests, blood chemistry and HCV RNA titre were analysed before therapy and at least once every month during therapy. Rapid virological response (RVR) was defined as an undetectable HCV RNA level at week 4, and complete early virological response (cEVR) was defined as an undetectable HCV RNA level at week 5 through week 12 after the start of therapy. SVR was defined as an undetectable HCV RNA level 24 weeks after the completion of therapy. Detection of HCV RNA level was based on qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor; Roche Diagnostic Systems). A database of pretreatment variables included haematological tests (haemoglobin level, white blood cell count and platelet count), blood chemistry tests (serum levels of creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, total cholesterol, triglycerides and HCV RNA titre), stage of histological fibrosis and patient characteristics (age, sex and body mass index). Post-treatment variables included time to HCV RNA negativity, calculated total RBV dose (g/kg of body weight), and calculated total PEG-IFN dose (μg/kg of body weight).

Statistical analysis

The Student's *t*-test was used for the univariable comparison of quantitative variables and Fisher's exact test was used for the comparison of qualitative variables. Logistic regression models with backward selection procedures were used for multivariable analysis of factors associated with relapse. IBM SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. For the decision tree analysis [30], the data mining software IBM SPSS Modeler 14 (SPSS Inc.) was used, as reported previously [17,26–28]. The decision tree analysis, the core component of the data mining, belongs to a family of non-parametric regression methods based on binary recursive partitioning of data. In this analysis, the software automatically explored the database to determine optimal split variables to build a decision tree structure. A statistical search algorithm evaluate the model derivation group to determine the optimum variables and cutoff values and to yield the most significant division of patients into two subgroups that were as homogeneous as possible for the probability of relapse. Once patients were divided into 2 subgroups, the analysis was automatically repeated on each subgroup in the same way until either no additional significant variable was detected or the number of patients was <20. Finally all patients were classified into particular subgroups that are homogeneous with respect to the probabilities of relapse.

Results

The decision tree model for the prediction of relapse

The overall rate of relapse was 26% in the model derivation group. The decision tree analysis selected three variables that are associated with relapse: time to HCV RNA negativity, age and total RBV dose (Figure 1). Time to HCV RNA negativity was selected as the best predictor of relapse. The rate of relapse was 13% for patients with RVR compared to 30% for patients with cEVR. Among patients with cEVR, age was selected as the variable of second split. Patients <60 years had a lower probability of relapse (22%) compared with those ≥60 years (41%). The total RBV dose was selected as the third variable of split with an optimal cutoff of 3.0 g/kg of body weight. The rate of relapse was lower in patients who received ≥3.0 g/kg of body weight of RBV compared to patients who received <3.0 g/kg of body weight (among patients <60 years rates were 16% versus 32% and among patients ≥60 years rates were 26% versus 52%, respectively).

According to this decision tree, the patients were divided into five groups with different rates of relapse ranging from 13% to 52%. Patients with RVR had the lowest risk of relapse. Among patients with cEVR, patients <60 years who received ≥3.0 g/kg of body weight of RBV also had a low risk of relapse (16%). By contrast, patients who received <3.0 g/kg of body weight of RBV had higher than the average risk of relapse, especially in patients ≥60 years (52%).

Validation of the decision tree model

The decision tree model was validated using an internal validation group that was not included in the model derivation. The rates of relapse for each subgroup of patients were correlated closely between the model derivation and the internal validation group (r^2=0.79; Figure 2A). When validated using an external validation group, the rates of relapse for each subgroup of patients were again correlated closely between the model derivation and the external validation group. (r^2=0.83; Figure 2B).

Multivariable logistic regression analysis for factors associated with relapse

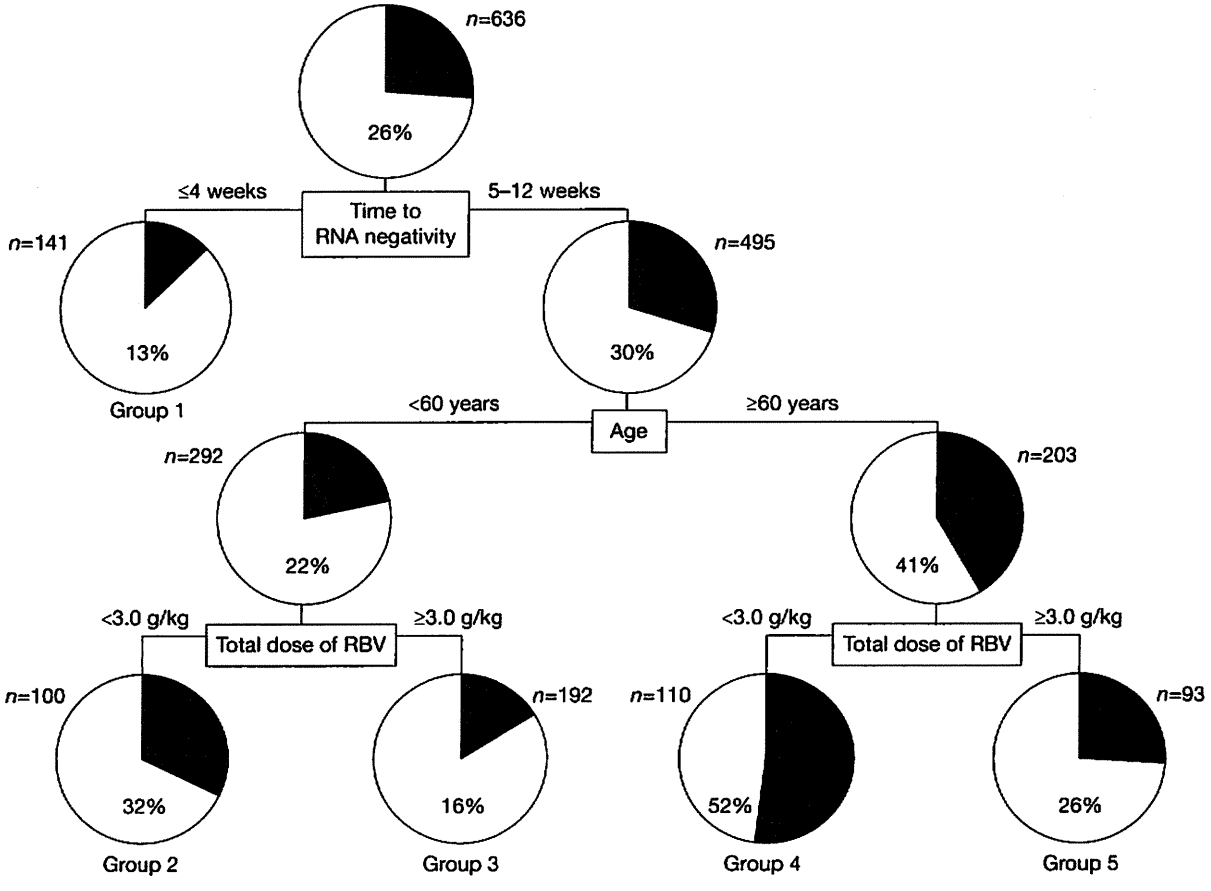
Univariable and multivariable analysis was performed using the combined population of model derivation and internal validation group. Univariable analysis found

that age, sex, serum levels of creatinine, haemoglobin, platelet count, HCV RNA titre, time to HCV RNA negativity, total PEG-IFN dose and total RBV dose were associated with relapse. Duration of therapy was not associated with reduction in relapse rate. Multivariable analysis including these factors showed that age, total RBV dose, serum level of creatinine, and time to HCV RNA negativity were independent predictors of relapse (Table 2). Creatinine was not selected as a splitting variable in data mining analysis probably due to the limitation to stop the analysis when the number of patients was <20. Using the combined population of model derivation and internal validation group, patients in each subgroup of decision tree model were further stratified by creatinine levels and the effect of creatinine level on relapse was analysed. Among patients with RVR, the rate of relapse did not differ between patients with creatinine levels of <0.7 g/dl and ≥0.7 g/dl and were 12% and 12%, respectively. Among patients with cEVR, the rate of relapse was higher in patients with creatinine levels of <0.7 g/dl compared to those with creatinine levels of ≥0.7 g/dl and were 39% versus 23%, respectively, for patients <60 years who received <3.0 g/kg of body weight of RBV, 19% versus 14% for patients <60 years who received ≥3.0 g/kg of body weight of RBV, 58% versus 41% for patients ≥60 years who received <3.0 g/kg of body weight of RBV, and 42% versus 26% for patients ≥60 years who received ≥3.0 g/kg of body weight of RBV.

Effect of age and total RBV dose on relapse among patients with cEVR

The effect of total RBV dose on relapse was analysed among patients with cEVR in a combined group of

Figure 1. The decision-tree model of relapse among patients with rapid virological response or complete early virological response

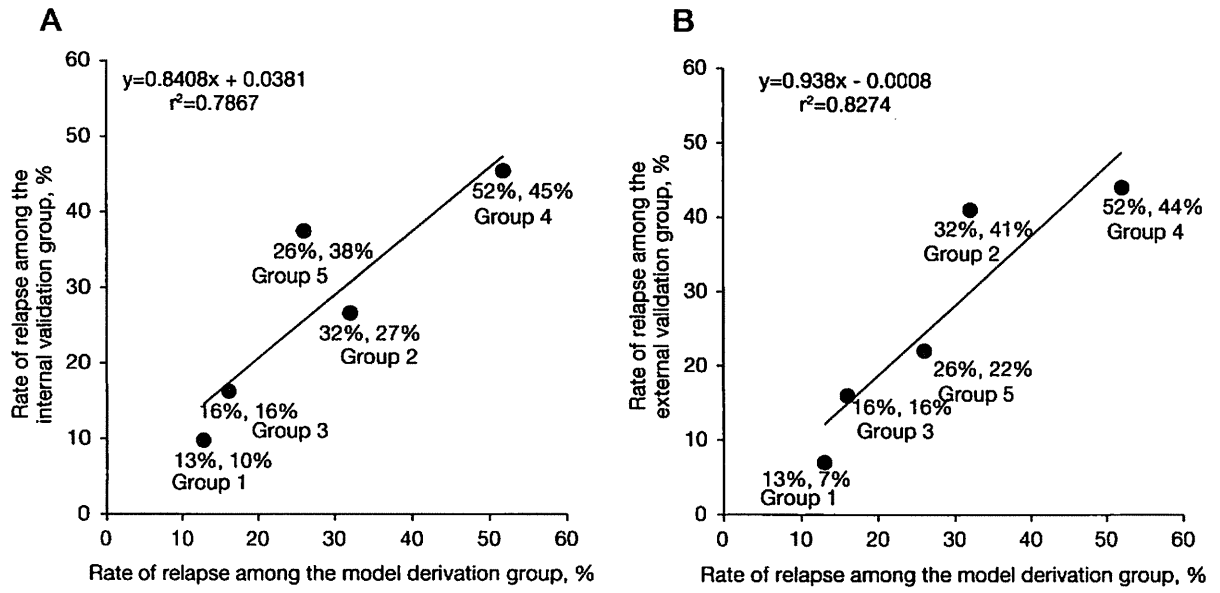


Boxes indicate the factors used for splitting and the cutoff values for the split. Pie charts indicate the rate of relapse for each group of patients after splitting. Terminal groups of patients discriminated by the analysis are numbered from 1 to 5. The rate of relapse was higher than average (>26%) in subgroups 2 and 4, where total ribavirin (RBV) dose was <3 g/kg of body weight.

model derivation and internal validation (n=718). The relapse rate decreased with an increase in RBV dose (Figure 3A). When patients were stratified into two groups according to age, the relapse rate decreased with an increase in RBV dose in patients <60 years. The relapse rate was lowest (11%) in patients <60 years who received ≥4.0 g/kg of body weight of RBV. By contrast, among patients ≥60 years, the relapse rate decreased with an increase in RBV dose up to 3.0 g/kg of body weight, but remained relatively stable despite a further increase in the RBV dose beyond 3.0 g/kg of body weight. The rate of relapse was 31% to 33% in patients who received ≥3.0 g/kg of body weight. Patients ≥60 years had higher relapse rate compared with patients <60 years after stratification by RBV dose (P=0.044 for RBV <2.5 g/kg, P=0.009 for RBV 2.5–2.9 g/kg, P=0.150 for RBV 3.0–3.4 g/kg, P=0.036 for RBV 3.5–3.9 g/kg and P=0.006 for RBV ≥4.0 g/kg).

To exclude the effect of the duration of therapy, patients who received 42–54 weeks of therapy were selected (n=544). Again, the relapse rate decreased with an increase in RBV dose in patients <60 years but remained stable despite a further increase in the RBV dose beyond 3.0 g/kg of body weight in patients ≥60 years (Figure 3B); in addition, patients ≥60 years had a higher relapse rate compared with younger patients after stratification by

Figure 2. Internal and external validation of the decision-tree model: subgroup-stratified comparison of the rate of relapse between the model derivation and validation groups



Each patient in the internal and external validation population was allocated to groups 1 to 5 following the flowchart of the decision tree. The rates of relapse were then calculated for each group and a graph was plotted. The rate of relapse in the (A) internal and (B) external validation groups are shown. The rates of relapse are shown as percentages below data points: the value on the left is from the model derivation group and on the right is from the validation group. The rates of relapse in each group of patients correlated closely between the model derivation group and the validation group (correlation coefficient: r^2=0.79 and 0.83, respectively).

Table 2. Multivariable analysis of factors associated with relapse among patients with RVR/cEVR

Factor	OR	95% CI	P-value
No-RVR	4.07	2.57–6.43	<0.0001
Total RBV dose <3.0 g/kg body weight	2.19	1.58 –3.03	<0.0001
Creatinine <0.7 g/dl	1.67	1.22–2.29	0.001
Age ≥60 years	2.37	1.73–3.24	<0.0001

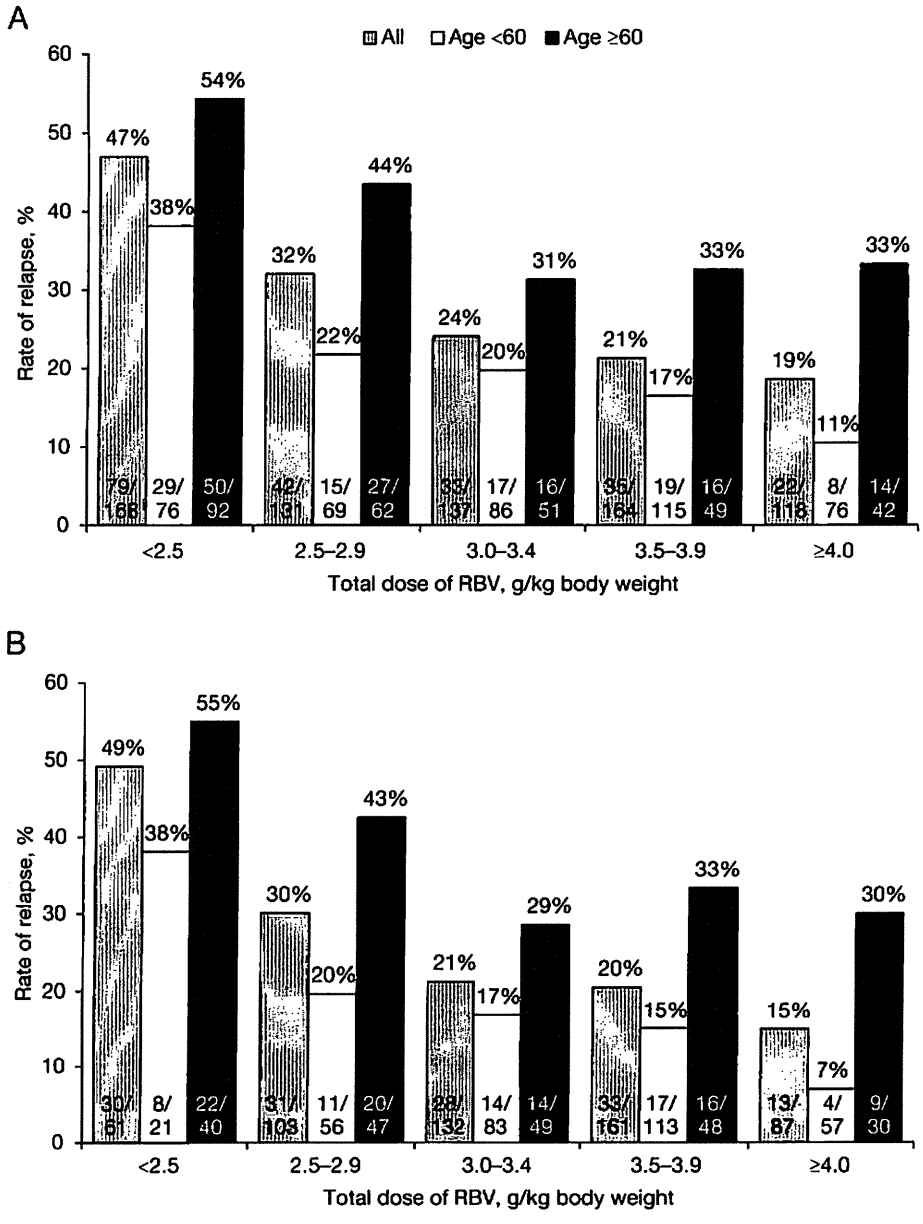
cEVR, complete early virological response (HCV-RNA-positive at week 4, but negative at week 12); RBV, ribavirin; RVR, rapid virological response (HCV-RNA-negative at week 4).

RBV dose (P=0.283 for RBV <2.5 g/kg, P=0.017 for RBV 2.5–2.9 g/kg, P=0.127 for RBV 3.0–3.4 g/kg, P=0.011 for RBV 3.5–3.9 g/kg and P=0.009 for RBV ≥4.0 g/kg).

Total dose of RBV was associated with relapse independently of PEG-IFN dose. The cutoff value of 58 zμg/kg of PEG-IFN was selected, which corresponds to the 80% of 1.5 μg/kg dose for 48 weeks. In patients who received <58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 24% and 42%, respectively. In patients who received ≥58 μg/kg of body weight of PEG-IFN, the rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 21% and 38%, respectively.

The data mining analysis procedure did not select further split variables among RVR patients. However,

Figure 3. Correlation between the rate of relapse and total RBV dose among patients with cEVR after stratification by age



Association between the total ribavirin (RBV) dose and the rate of relapse among patients with complete early virological response (cEVR) is shown. (A) Higher dose of RBV was associated with reduced rate of relapse. (B) These associations were also confirmed in selected patients who received 42–54 weeks of therapy.

when analysed separately, the rate of relapse was also associated with age and total RBV dose among patients with RVR. The rate of relapse for patients who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 5% and 14%, respectively. The rate of relapse for patients <60 and ≥60 years was 9% and 18%, respectively. Collectively, the rate of relapse for patients <60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 2% and 11%, respectively, whereas the rate of relapse for patients ≥60 years who received ≥3.0 g/kg or <3.0 g/kg of body weight of RBV was 12% and 20%, respectively.

Discussion

The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse among patients with cEVR to PEG-IFN plus RBV. Older patients (≥60 years) who received a total RBV dose <3.0 g/kg of body weight had the highest risk of relapse (52%), whereas younger patients who received a total RBV dose ≥3.0 g/kg of body weight had the lowest risk of relapse (16%). The rate of relapse decreased depending on the total RBV dose in younger patients, but remained stable in older patients despite a further increase in the RBV dose beyond 3.0 g/kg of body weight. These findings imply that the target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR, and further increase in RBV dose up to 4.0 g/kg of body weight or greater may be recommended in patients <60 years.

The associations between the drug adherence and virological response had been reported with inconsistent results. In an earlier study, patients who received >80% of the planned dose of PEG-IFN plus RBV for >80% of the planned duration of therapy had a higher rate of SVR compared to those who received a lesser dose (51% versus 34%) [31]. Consistent results were obtained in a study reporting that patients who received >80% of the planned dose of PEG-IFN and RBV within the first 12 weeks of therapy had a higher rate of EVR compared with those who received a lesser dose of both drugs (80% versus 33%) [4]. By contrast, a large-scale multicentre study showed that reducing the PEG-IFN dose during the first 20 weeks reduced SVR; however, reducing RBV did not affect SVR as long as RBV was not prematurely discontinued [32]. The reason for these inconsistencies is unclear. One reason may be the differences in the backgrounds of patients enrolled in the study, and hence the last study was limited to patients with advanced fibrosis and prior non-responders to PEG-IFN therapy. Because the probability of SVR is affected by virological response and relapse after response, the effect of drug dosing should be analysed separately with respect to these two factors.

In the present study, we focused on factors predictive of relapse after early virological response. According to the decision tree model, relapse was less likely in patients with RVR compared with cEVR. Among patients with cEVR, older patients (≥60 years) had a higher risk of relapse compared to younger patients (41% versus 22%). In addition, our results emphasized the effect of RBV dose for the prevention of relapse. In our study, a total RBV dose of ≥3.0 g/kg of body weight was repeatedly associated with a suppressed rate of relapse in the model derivation and validation groups. The rate of relapse in patients <60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 32% versus 16%, 27% versus 16%, and 41% versus 16%, respectively. The rate of relapse in patients ≥60 years who received an RBV dose of <3.0 versus ≥3.0 g/kg of body weight in the model derivation, internal validation and external validation groups were 52% versus 26%, 45% versus 38%, and 44% versus 22%, respectively. It has been reported that the rate of relapse is suppressed in 48 weeks of IFN plus RBV combination therapy compared to IFN monotherapy, indicating that RBV contributes to the increase in SVR by reducing relapse [2,3]. Another study, focused on the associations between the drug dose reduction and relapse in patients with virological response, found that maintaining RBV dose ≥12 mg/kg/day during 48 weeks of treatment, which can be translated into a total dose of 4.0 g/kg of body weight, suppressed relapse [33]. Results of the present study are in accordance with this report.

The importance of drug dosing on reduction in relapse is also supported by the findings that extending therapy from 48 to 72 weeks in patients with delayed virological response improved SVR rates by reducing relapse [9–13]. Apart from these clinical studies, in the real world of clinical practice, duration of therapy is extended – even in patients with cEVR – at the physician's discretion. The relationship between duration of therapy or RBV dose, and relapse among patients with cEVR and treated with various lengths of therapy has not been examined. In the combined group of our study, extending the duration of therapy was not associated with a reduction in relapse rate. Rather, the rate of relapse decreased depending on the total RBV dose. These findings suggest that acquiring a sufficient total RBV dose, either within 48 weeks or by extending the duration of therapy, is essential to prevent relapse among patients with cEVR. The limitation of the present study was that the mean duration of therapy was only 56.3 weeks in patients whose duration of therapy was extended beyond 48 weeks. It is probable that extended duration of therapy was not long enough for the prevention of relapse. Further studies with

longer durations of therapy are necessary to confirm the effect of extended duration of therapy on reduction of relapse among patients with cEVR.

Previous reports did not consider the effects of age in setting the optimal dose of RBV. In the present study, the relapse rate decreased with an increase in RBV dose from <2.5 to 3.0–3.5 g/kg of body weight, but remained relatively stable despite a further increase in the RBV dose in older patients. Thus, a total RBV dose ≥3.0 g/kg of body weight should be the target dose for patients ≥60 years with cEVR. By contrast, ≥3.0 g/kg of body weight of RBV was associated with lower risk of relapse in patients <60 with cEVR (16% versus 32%), and a further increase in RBV dose led to a more profound reduction in relapse rates, as low as 11% in patients who received ≥4.0 g/kg of body weight. Thus, a total dose of ≥4.0 g/kg of body weight or even greater should be the target dose in patients <60 years.

In the near future, more potent therapies, such as direct antiviral agents [34,35], may become available. These drugs require RBV and PEG-IFN in combination. However, not all patients may be able to tolerate this triple combination therapy due to adverse drug reactions, such as severe anaemia or skin eruption. In particular, it may be difficult to administer a full dose of triple drugs to older patients. Thus, personalizing the PEG-IFN and RBV combination therapy based on this model may be beneficial to patients who were intolerant to triple combination therapy.

In the present study creatinine was an independent predictor of relapse by multivariable logistic regression analysis. However creatinine was not selected as a splitting variable in decision tree, which may be due to the unique property of data mining analysis. In data mining analysis, limitation is imposed to stop the analysis when the number of patients is <20. This limitation is used to avoid dividing patients into too small subgroups which lead to the generation of rules that only apply to the model derivation population and not reproduced when applied to other populations. This phenomenon is called the over-fitting of the model. Due to this limitation, the variables selected in the data mining analysis are not necessarily identical to the variables that are significant by ordinary multivariable analysis. In a separate analysis, lower level of creatinine was associated with higher rate of relapse in each subgroup of patients with cEVR. The reason for this association is not clear, but lower creatinine level may be related to more efficient clearance of RBV leading to lower serum level of RBV. Further research is needed to confirm this speculation.

A potential limitation of the present study is that data mining analysis has an intrinsic risk of showing relationships that fit to the original dataset, but are not reproducible in different groups. Although internal and external validations showed that our model had high reproducibility, we recognized that further validation on a larger external validation cohort, especially in groups other than Japanese, may be necessary to further verify the reliability of our model.

In conclusion, we built a decision tree model for the prediction of relapse among patients with EVR to PEG-IFN plus RBV. The result of the present study shows that older age and insufficient dose of RBV are significant and independent risk factors for relapse. The target dose of total RBV can be set at 3.0 g/kg of body weight in patients who achieved cEVR. A further increase in RBV dose up to 4.0 g/kg of body weight may be warranted in patients <60 years.

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Disclosure statement

The authors declare no competing interests.

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Identification of Novel *N*-(Morpholine-4-Carbonyloxy) Amidine Compounds as Potent Inhibitors against Hepatitis C Virus Replication

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To identify novel compounds that possess antiviral activity against hepatitis C virus (HCV), we screened a library of small molecules with various amounts of structural diversity using an HCV replicon-expressing cell line and performed additional validations using the HCV-JFH1 infectious-virus cell culture. Of 4,004 chemical compounds, we identified 4 novel compounds that suppressed HCV replication with 50% effective concentrations of ranging from 0.36 to 4.81 μM. *N*′-(Morpholine-4-carbonyloxy)-2-(naphthalen-1-yl) acetimidamide (MCNA) was the most potent and also produced a small synergistic effect when used in combination with alpha interferon. Structure-activity relationship (SAR) analyses revealed 4 derivative compounds with antiviral activity. Further SAR analyses revealed that the *N*-(morpholine-4-carbonyloxy) amidine moiety was a key structural element for antiviral activity. Treatment of cells with MCNA activated nuclear factor κB and downstream gene expression. In conclusion, *N*-(morpholine-4-carbonyloxy) amidine and other related morpholine compounds specifically suppressed HCV replication and may have potential as novel chemotherapeutic agents.

Hepatitis C virus (HCV) is a major human pathogen. It is associated with persistent liver infection, which leads to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (13). Treatment with pegylated interferon (IFN) and ribavirin is associated with significant side effects and is effective in only half the patients infected with HCV genotype 1 (6). More effective and more tolerable therapeutics are under development, and direct-acting antiviral agents (DAAs) for HCV infection are currently in advanced clinical trials. In combination with IFN and ribavirin, the HCV protease inhibitors telaprevir and boceprevir have recently been approved for treatment of genotype 1 HCV infection in the United States, Canada, Europe, and Asian countries (11, 12, 22). Although these two drugs can achieve higher sustained virologic response rates than IFN and ribavirin, their effects could be compromised by the emergence of highly prevalent drug-resistant mutants (25). Thus, it is crucial to use several different classes of DAAs in combination to improve efficacy and reduce viral breakthrough.

The HCV subgenomic replicon system has been widely used to screen compound libraries for inhibitors of viral replication, using reporter activity as a surrogate marker for HCV replication. We previously reported the successful adaptation of the Huh7/Rep-Feo replicon cell line to a high-throughput screening assay system (28). This approach contributed to the discovery of antiviral compounds, such as hydroxyl-methyl-glutaryl coenzyme A reductase inhibitors (10) and epoxide compounds (20). In our present study, we used the Huh7/Rep-Feo replicon cell line to screen a library of small molecules with various amounts of structural diversity to identify novel compounds possessing antiviral activity against HCV. We showed that the screening hit compounds inhibited HCV replication in an HCV genotype 2a (JFH-1) infectious-virus cell culture (29). The most potent compound was *N*′-(morpholine-4-carbonyloxy)-2-(naphthalen-1-yl) acetimidamide (MCNA). Structure-activity relationship (SAR) analyses revealed that the *N*-(morpholine-4-carbonyloxy) amidine moiety was a key structural element for antiviral activity. We also investigated the possible mechanisms of action of these compounds and showed that MCNA likely inhibited HCV replication through activation of the nuclear factor κB (NF-κB) pathway.

MATERIALS AND METHODS

Reagents and chemicals. Recombinant human alpha 2b interferon (IFN-α2b) was obtained from Schering-Plough (Kenilworth, NJ), the NS3/4A protease inhibitor BILN 2061 from Boehringer Ingelheim (Ingelheim, Germany), beta-mercaptoethanol from Wako (Osaka, Japan), and recombinant human tumor necrosis factor alpha (TNF-α) from Sigma (St. Louis, MO). The library of chemicals that were screened was provided by the Chemical Biology Screening Center at Tokyo Medical and Dental University. Information about the library is available at http://bsmdb.tmd.ac.jp. The important features of the library were the abundance of pharmacophores and the great diversity. Lipinski's rule of five was used to evaluate drug similarity (15). The purity of each chemical from the library was greater than 90%. For SAR analyses, 27 compounds were purchased from Assinex (Moscow, Russia), ChemBridge (San Diego, CA), ChemDiv (San Diego, CA), Enamine (Kiev, Ukraine), Maybridge (Cambridge, United Kingdom), Ramidus AB (Lund, Sweden), SALOR (St. Louis, MO), Scientific Exchange (Center Ossipee, NH), or Vitas-M (Moscow, Russia). The chemicals were all prepared at concentrations of 10 mM in dimethyl sulfoxide (Sigma) and stored at −20°C until they were used.

Cell lines and cell culture maintenance. Huh7 and Huh7.5.1 cell lines (32) were maintained in Dulbecco's modified Eagle's medium (Sigma)

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supplemented with 10% fetal bovine serum and incubated at 37°C under 5% CO_2. The maintenance medium for the HCV replicon-harboring cell line, Huh7/Rep-Feo, was supplemented with 500 μg/ml of G418 (Nacalai Tesque, Kyoto, Japan).

HCV replicon construction and cell culture. An HCV subgenomic replicon plasmid that contained Rep-Feo, pHC1bneo/delS (Rep-Feo-1b), was derived from the HCV-N strain. RNA was synthesized from pRep-Feo and transfected into Huh7 cells. After culture in the presence of G418, a cell line that stably expressed the replicon was established (28, 31).

Cell-based screening of antiviral activity. Huh7/Rep-Feo cells were seeded at a density of 4,000 cells/well in 100 μl of medium in 96-well plates and incubated for 24 h. Test compound solutions, 10 mM in 100% dimethyl sulfoxide (DMSO), were added to the wells; for primary screening, the final concentration was 5 μM. The assay plates were incubated as described above for another 48 h, and luciferase activity was measured with a luminometer (Perkin-Elmer) using the Bright-Glo Luciferase assay system (Promega) following the manufacturer's instructions. Assays were performed in triplicate, and the results were expressed as means and standard deviations (SD) as percentages of the controls. Compounds were considered hits if they inhibited >50% of the mean control luciferase activities. Compounds were considered cytotoxic if they reduced cell viability below 70% of the control in dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays and were discarded. The hit compounds were then validated by secondary screening, which determined the antiviral activities of each compound serially diluted at concentrations ranging from 0.1 μM to 30 μM under Huh7/Rep-Feo cells cultured in an identical manner to the primary screen. Compounds inhibiting replication with a 50% effective concentration (EC_{50}) of <5 μM and a selectivity index (SI) of >5 were selected for further analysis.

MTS assay. To evaluate cell viability, MTS assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's directions.

Calculation of the EC_{50}, CC_{50}, and SI. The EC_{50} indicates the concentration of test compound that inhibits replicon-based luciferase activity by 50%. The 50% cytotoxic concentration (CC_{50}) indicates the concentration that inhibits cell viability by 50%. The EC_{50} and CC_{50} values were calculated using probit regression analysis (2, 26). The selectivity index was calculated by dividing the CC_{50} by the EC_{50}.

Reporter and expression plasmids. The plasmid pC1neo-Rluc-IRES-Fluc was constructed to analyze the HCV internal ribosome entry site (IRES)-mediated translation efficiency (19). The plasmid expressed a bicistronic mRNA containing the *Renilla* luciferase gene translated in a cap-dependent manner, and firefly luciferase was translated by HCV-IRES-mediated initiation. The plasmid pISRE-TA-Luc (Invitrogen, Carlsbad, CA) expressed the firefly luciferase reporter gene under the control of the interferon stimulation response element (ISRE). The plasmid pNF-κB-TA-Luc (Clontech Laboratories, Franklin Lakes, NJ) expressed the firefly luciferase reporter gene under the control of NF-κB. The plasmid pRL-CMV (Promega, Madison, WI), which expressed the *Renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used as a control for the transfection efficiency of pISRE-TA-Luc and pNF-κB-TA-Luc (8).

Western blot analysis. Fifteen micrograms of total cell lysates was separated using NuPage 4-to-12% Bis-Tris gels (Invitrogen) and blotted onto polyvinylidene difluoride membranes. Each membrane was incubated with primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence reaction using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, United Kingdom). The primary antibodies were anti-NS5A (BioDesign, Saco, ME), anti-HCV core (kindly provided by T. Wakita), anti-phospho-p65 (Ser536) (93H1; Cell Signaling Technology, Beverly, MA), anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma) antibodies.

HCV-JFH1 virus cell culture. HCV-JFH1 RNA transcribed *in vitro* was transfected into Huh7.5.1 cells. The transfected cells were subcultured every 3 to 5 days. The culture supernatant was subsequently transferred onto Huh7.5.1 cells.

TABLE 1 Effects of the leading hit compounds on HCV replication[a]

Compound	EC_{50} (μM)	CC_{50} (μM)	SI
1	0.36 (0.22–0.58)	45.2 (35.9–56.9)	126
2	0.86 (0.73–1.02)	>100	>116
3	0.94 (0.76–1.06)	25.3 (19.8–32.3)	26.9
4	4.81 (3.79–6.12)	27.1 (17.1–58.0)	5.64

[a] The EC_{50} and CC_{50} values are reported, with 95% confidence intervals in parentheses, from a representative experiment performed in triplicate.

Real-time RT-PCR analysis. The protocols and primers for real-time RT-PCR analysis of HCV RNA have been described previously (17). Briefly, total cellular RNA was isolated using an RNeasy Minikit (Qiagen, Valencia, CA), reverse transcribed, and subjected to real-time RT-PCR analysis. Expression of mRNA was quantified using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystems).

Analyses of drug synergism. The effects on HCV replication of antiviral hit compounds plus IFN-α or BILN 2061 were analyzed according to classical isobologram analyses (24, 28). Dose-inhibition curves for IFN or BILN 2061 and the test compounds were drawn, with the 2 drugs (IFN or BILN 2061 and each test compound) used alone or in combination. For each drug combination, the concentrations of IFN or BILN 2061 and test compound that inhibited HCV replication by 50% (EC_{50}s) were plotted against the fractional concentration of IFN or BILN 2061 and the compound on the *x* and *y* axes, respectively. A theoretical line of additivity was drawn between plots of the EC_{50}s obtained for either drug used alone. The combined effects of the 2 drugs were considered to be additive, synergistic, or antagonistic if the plots of the combined drugs were located on, below, or above the line of additivity, respectively.

Statistical analyses. Statistical analyses were performed using Welch's *t* test. *P* values of less than 0.01 were considered statistically significant.

RESULTS

Screening results. To identify novel regulators of HCV replication, 4,004 chemical compounds were screened using the Huh7/Rep-Feo replicon assay system. The primary screens identified 117 compounds that inhibited ≥50% of replicon luciferase activity at 5 μM. Of the 117 compounds, 74 were cytotoxic and could not be further evaluated. In the secondary screen, nontoxic primary hits were evaluated by determining the antiviral activities of serial dilutions at concentrations ranging from 0.1 μM to 30 μM. This screen identified 19 compounds with EC_{50}s of less than 5 μM and CC_{50} values 5-fold greater than the EC_{50} values. The effect of each secondary hit on HCV-NS5A protein expression was examined using Western blot analysis. Of the 19 compounds, 4 compounds, designated 1, 2, 3, and 4, suppressed HCV subgenomic replication, with EC_{50}s ranging from 0.36 to 4.81 μM and SIs ranging from 5.64 to more than 100 (Table 1 and Fig. 1A and B; see Table S1 in the supplemental material). By Western blot analysis, compounds 1, 2, and 3 decreased HCV-NS5A protein levels at concentrations of 5 μM after incubation for 48 h (Fig. 1C). Compared with compounds 1, 2, and 3, the effect of compound 4 on HCV-NS5A protein expression was not remarkable at a concentration of 5 μM, similar to the results from the luciferase assay shown in Fig. 1B. The effects of the compounds on the HCV replicon were further validated in the JFH-1 cell culture. As shown in Fig. 1D, compounds 1, 2, 3, and 4 significantly inhibited intracellular RNA replication of HCV-JFH1. Although compound 4 was negative by Western blot analysis, it decreased HCV replication in the other

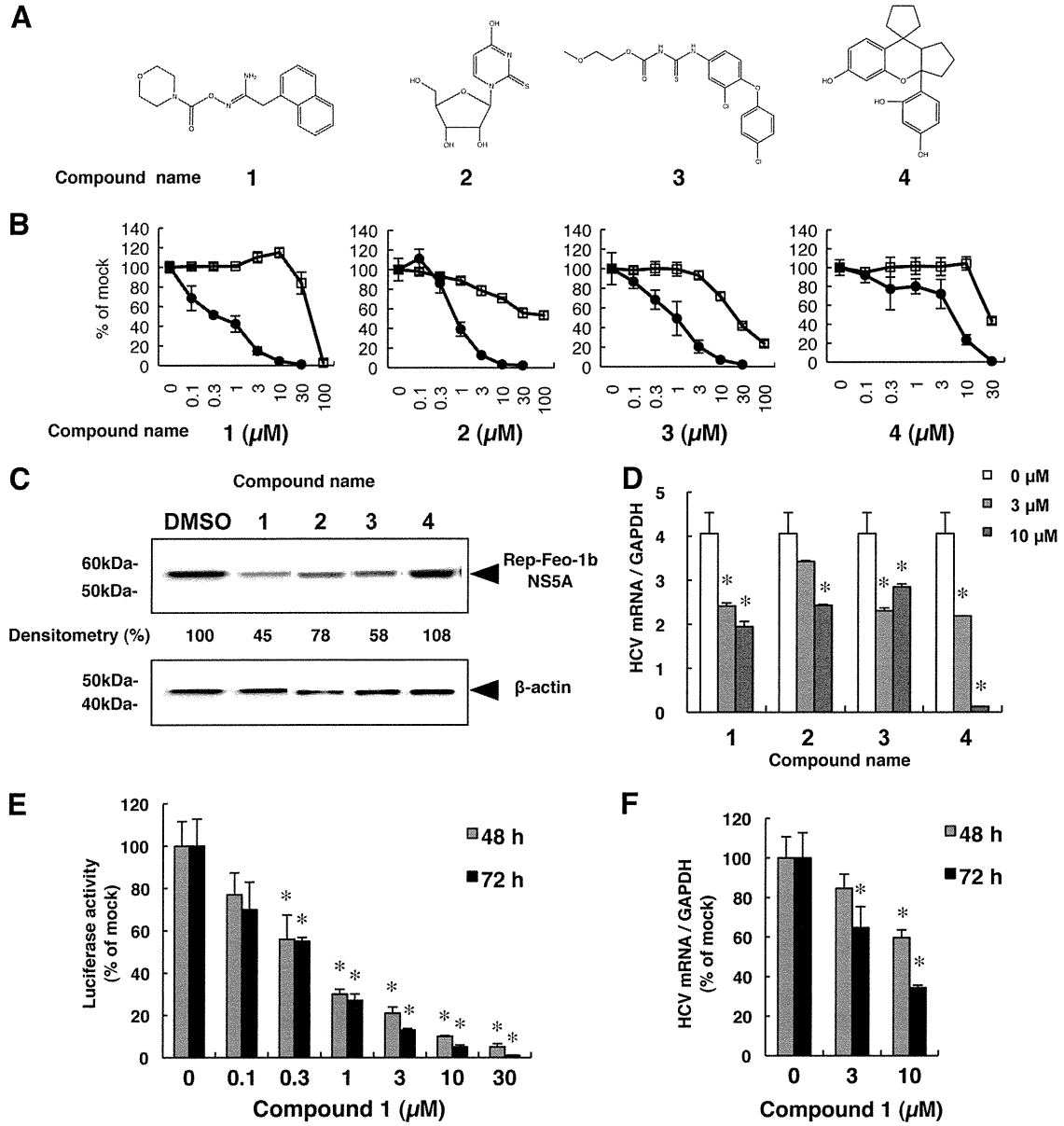

FIG 1 Effects of 4 screening hit compounds on HCV replication. (A) Chemical structures of hit compounds. (B) Huh7/Rep-Feo cells were treated with the indicated concentration of each compound for 48 h. Luciferase activities representing HCV replication are shown as percentages of the DMSO-treated control luciferase activity (solid circles). Cell viability is shown as a percentage of control viability (open squares). Each point represents the mean of triplicate data points, with the standard deviations represented as error bars. (C) Huh7/Rep-Feo cells were treated with DMSO or compounds 1 through 4 at 5 μM for 48 h, and Western blotting was performed using anti-HCV NS5A and anti-β-actin antibodies. Densitometry of NS5A protein was performed, and the results are indicated as percentages of the DMSO-treated control. The assay was repeated three times, and a representative result is shown. (D) Huh7.5.1 cells were transfected with HCV-JFH1 RNA and cultured in the presence of the indicated compounds at 3 μM or 10 μM. At 72 h after transfection, the cellular expression levels of HCV-RNA were quantified by real-time RT-PCR. The bars indicate means and SD. (E) Time-dependent reduction of luciferase activities in Huh7/Rep-Feo cells induced by compound 1. Luciferase activities are shown as percentages of the DMSO-treated control luciferase activity. The bars indicate means and SD. (F) Time-dependent reduction of cellular expression levels of HCV-RNA in HCV-JFH1-transfected cells induced by compound 1. HCV RNA levels are shown as percentages of the DMSO-treated control HCV-RNA level. The bars indicate means and SD. The asterisks indicate P values of less than 0.01.

assays, including the replicon and HCV-JFH1 virus assays. Thus, we concluded that compound 4 was an antiviral hit. These results indicated that the 4 compounds identified by cell-based screening suppressed subgenomic HCV replication and HCV replication in an HCV-based cell culture.

Hit compounds did not suppress HCV IRES-mediated translation. To determine whether the leading antiviral hits suppressed HCV IRES-dependent translation, we used the Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc. Treatment of these cells with the test compounds did not result in significant change in the internal luciferase activities at compound concentrations that suppressed expression of the HCV replicon (Fig. 2), suggesting that the effect of the hit compounds on HCV replication does not involve suppression of IRES-mediated viral-protein synthesis.

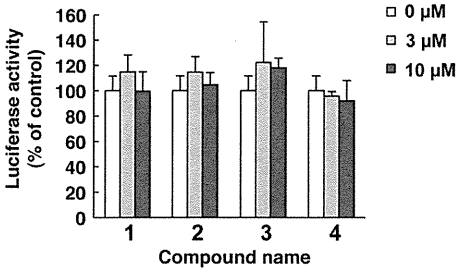

FIG 2 Hit compounds do not affect HCV IRES-mediated translation. The bicistronic reporter plasmid pC1neo-Rluc-IRES-Fluc was transfected into Huh7 cells. The cells were cultured in the presence of the indicated concentrations of compounds 1 through 4. After 6 h of treatment, luciferase activities were measured, and the values were normalized against *Renilla* luciferase activities. The assays were performed in triplicate. The bars indicate means and SD.

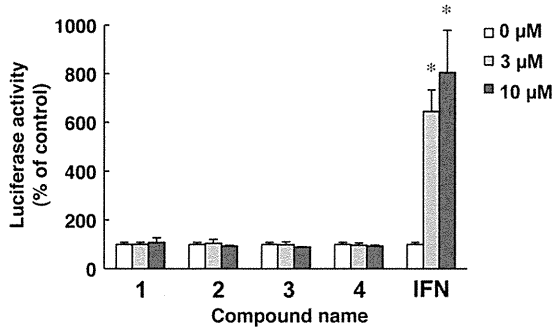

FIG 3 Hit compounds do not activate interferon-stimulated gene responses. Plasmids pISRE-TA-Luc and pRL-CMV were cotransfected into Huh7 cells. The transfected cells were cultured in the presence of the indicated concentrations of the hit compounds. After 6 h of treatment, luciferase activities were measured, and the values were normalized against *Renilla* luciferase activities. As positive controls, cells were treated with IFN-α at a concentration of 0, 3, or 10 U/ml. The bars indicate means and SD. The asterisks indicate *P* values of less than 0.01.

Hit compounds do not activate interferon-stimulated gene responses. To study whether the actions of the hit compounds involved IFN-mediated antiviral signaling that would induce expression of an IFN-stimulated gene, an ISRE-luciferase reporter plasmid, pISRE-TA-Luc, was transfected into Huh7 cells, and the transfected cells were cultured in the presence of the 4 compounds at concentrations of 0, 3, or 10 μM. In contrast to interferon, which elevated ISRE promoter activities significantly, the hit compounds showed no effects on the ISRE-luciferase activities (Fig. 3). These results indicated that the action of the hit compounds is independent of interferon signaling.

Drug synergism with IFN-α or BILN 2061. To investigate whether the hit compounds were synergistic with IFN-α or the protease inhibitor BILN 2061, we used isobologram analyses (24, 28). HCV replicon cells were treated with a combination of IFN-α or BILN 2061 and each hit compound at an EC_{50} ratio of 1:0. 4:1, 3:2, 2:3, 1:4, or 0:1, and the dose-effect results were plotted (Fig. 4A and C). The fractional EC_{50}s for IFN-α or BILN 2061 and each compound were plotted on the *x* and *y* axes, respectively, to generate an isobologram. As shown in Fig. 4B, all plots of the fractional EC_{50}s of compound 1 and IFN-α fell below the line of additivity, while the plots were located closed to the line of additivity for the treatments using IFN-α plus compound 2 or 3 and above the line for the treatment using IFN-α plus compound 4. Those results indicated that the anti-HCV effect of compound 1 was synergistic with IFN-α, the anti-HCV effects of compounds 2 and 3 were additive, and the effect of compound 4 was antagonistic. In the BILN 2061 combination study, the combination with compound 2 was slightly synergistic, while the combination with compound 1 or 3 was additive, and the combination with compound 4 was antagonistic (Fig. 4D).

SARs of compound 1 and similar compounds. We next conducted SAR analyses for hit compound 1 by screening 69 compounds with structures similar to that of compound 1 (see Table S2 in the supplemental material). Out of those compounds, we identified 4 structural analogues that suppressed subgenomic HCV replication with EC_{50}s ranging from 1.82 to 4.03 μM and SIs of 6.01 through >43.7 (Table 2 and Fig. 5A and B). Similarly, the 4 compounds designated 5, 6, 7, and 8 substantially decreased HCV-NS5A protein expression levels following treatment with the compounds (Fig. 5C). Consistent with the replicon results, the compounds significantly suppressed HCV-JFH1 mRNA in cell culture (Fig. 5D). Although the suppressive activities of the 4 compounds were similar, the original compound 1 showed the greatest anti-HCV activity. Therefore, we conducted SAR analyses of the compound 1 *N*-(morpholine-4-carbonyloxy) amidine and *N*-acyloxy-1-naphthalenacetamidine moieties. We screened 13 compounds containing *N*-(morpholine-4-carbonyloxy) amidine and 11 with *N*-acyloxy-1-naphthalenacetamidine (Fig. 6A; see Table S3 in the supplemental material). Intriguingly, 11 out of the 13 *N*-(morpholine-4-carbonyloxy) amidine compounds suppressed the subgenomic HCV replicon without cytotoxicity at a fixed concentration of 5 μM. In contrast, only 2 *N*-acyloxy-1-naphthalenacetamidine compounds decreased HCV replication (Fig. 6B and C). We also conducted dose-dependent suppression assays for HCV replicon. As shown in Table 3, 11 out of 13 *N*-(morpholine-4-carbonyloxy) amidine compounds consistently decreased subgenomic HCV replication, with EC_{50}s ranging from 1.52 through 8.62 μM and SIs of 14.2 to >61.4. Of these 11 compounds, compound 14 was the most potent, with an EC_{50} of 1.63 μM and an SI of >61.4. The antiviral effect of compound 14 against HCV-JFH1 was identical to that of the original compound 1. To identify the moiety conferring anti-HCV activity, we tested the morpholine-4-carboxyl moiety within the *N*-(morpholine-4-carboynloxy) amidine structure (Fig. 6D). Three compounds bearing the morpholine-4-carboxyl moiety were tested, and none showed suppressive activity toward the HCV replicon. These results suggested that the entire *N*-(morpholine-4-carbonyloxy) amidine moiety was important for efficient anti-HCV activity.

Effect of compound 1 on the NF-κB signaling pathway. NF-κB, composed of homo- and heterodimeric complexes of Rel-like domain-containing proteins, including p50 and p65, is a key regulator of innate and adaptive immune responses through transcriptional activation of several antiviral proteins (9, 23). We performed luciferase reporter assays, a p65 phosphorylation assay, and an IκB-α degradation assay to assess the effect of compound 1 on NF-κB signaling in host cells. Intriguingly, treatment of both Huh7 cells and HCV replicon-expressing cells with compound 1 increased NF-κB reporter activity in a dose-dependent manner (Fig. 7A and B). Consistently, treatment with compound 1 increased phosphorylated NF-κB p65 in Huh7 cells (Fig. 7C). Acti-

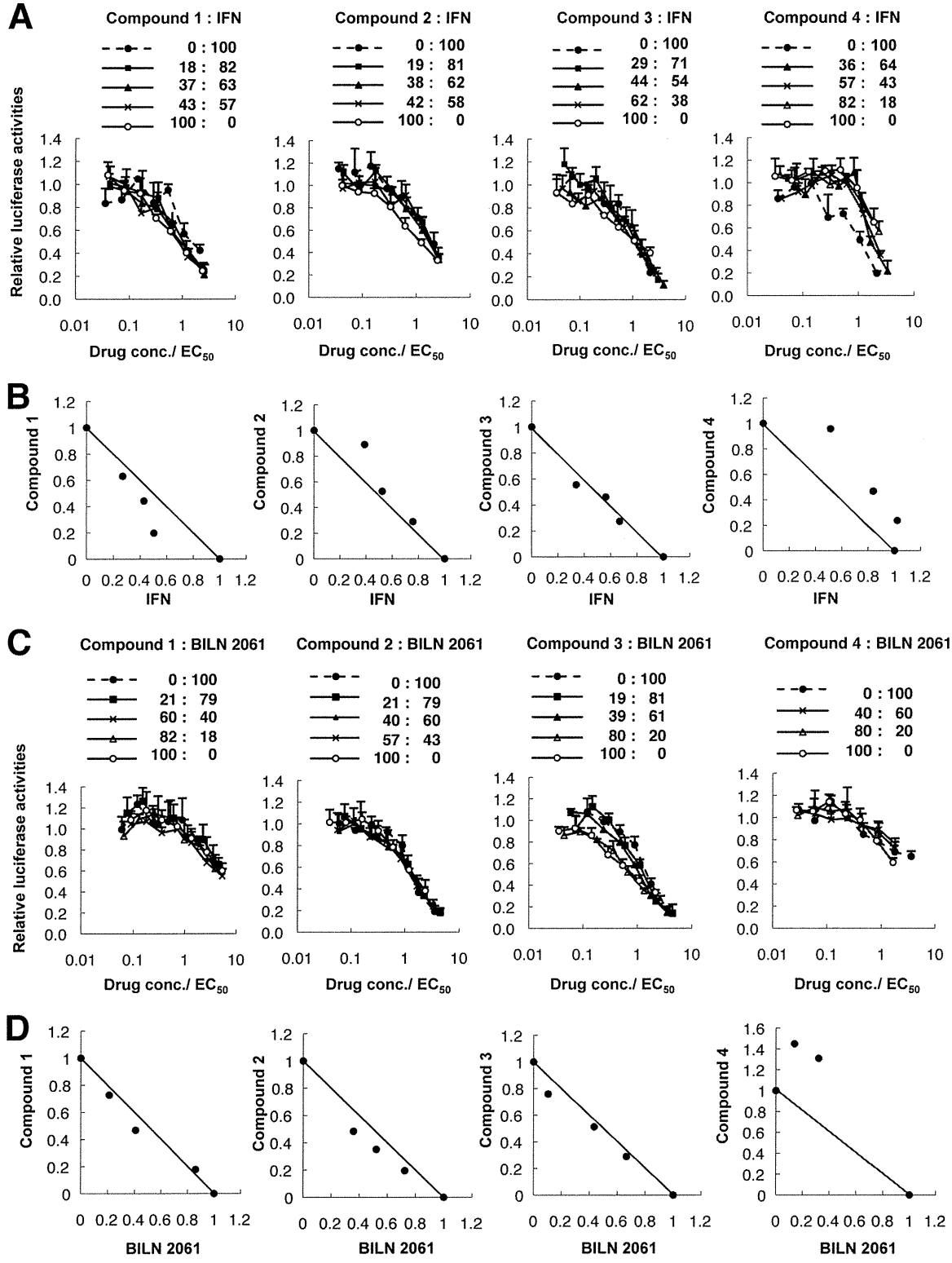

FIG 4 Drug synergism analyses: effects of each of the 4 antiviral hit compounds combined with IFN-α or BILN 2061 on HCV replication. (A and C) Huh7/Rep-Feo cells were cultured with a combination of IFN-α or BILN 2061 and antiviral hit compound 1, 2, 3, or 4 at the indicated ratios, adjusted by the EC_{50} of the individual drug. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Shown are means and SD. (B and D) Graphical presentations of isobologram analyses. For each drug combination in panels A and C, the EC_{50}s of IFN-α or BILN 2061 and compound 1, 2, 3, or 4 for inhibition of HCV replication were plotted against the fractional concentrations of IFN-α or BILN 2061 and each compound, as indicated on the x and y axis, respectively. A theoretical line of additivity is drawn between the EC_{50}s for each drug alone.