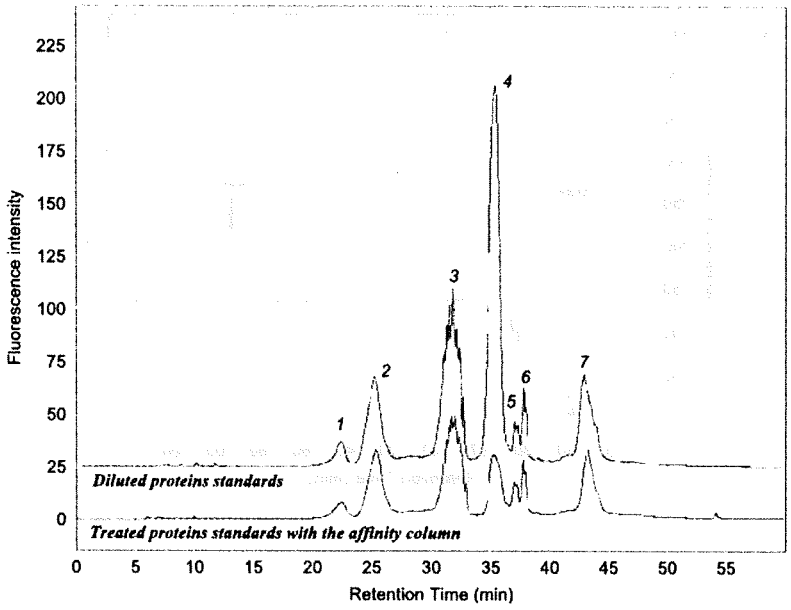

Figure 2. Chromatograms of the protein standards which were treated with Seppro®-IgY12 column and diluted to the same protein amount (4.8 μg/HPLC injection) as the amount for the column treatment. The peak numbers correspond to Table 2.

Table 2. Adsorption ratio to the untreated Seppro®-IgY12 column and protein names obtained by FD-LC-MS/MS method

Peak no.	Adsorption ratio to the untreated Seppro®-IgY12	Protein name
1	38.5%	Peptide
2	30.6%	Peptide
[a]3	45.9%	Lysozyme
4	83.9%	Bovine serum albumin (BSA)
[b]5	18.3%	Trypsin inhibitor
6	28.5%	Trypsin inhibitor
7	30.4%	Phosphorylase B

[a]3, [b]5: Most highest peak

albumin (BSA; 83.9%) as compared with the other proteins in the standards, non-specific binding to the column materials or to carrier proteins such as albumin itself was observed in the 18.3–45.9% range and could result in the loss of presumed biomarkers.

Next, the time series changes of the specific and non-specific adsorption of proteins to the column were investigated. The protein standards mixture was treated with the column periodically after 10 and 20 cycles of treatment of the control plasma sample. The relation of the changes of the protein standards adsorption to the number of uses of the affinity column is shown in Fig. 3(A). The specific adsorption of BSA decreased with an increase in the number of times the column was used. However, the non-specific adsorption for lysozyme, trypsin inhibitor and phosphorylase B reached a maximum at 11 cycles and decreased at 21 cycles. Since the affinity column was optimized for human plasma, the absorption of BSA for the column might be weaker than for plasma albumin. However, the adsorption of albumin in control plasma also decreased with an increase in the number of times the column was used (data not shown). Moreover, since the slopes of decrease differed among the protein standards, the correlation of each adsorption with molecular weight of each protein was calculated [Fig. 3(B)]. The open dots show the value of BSA in Fig. 3(B). The correlation coefficient value was the closest to 1.00 ($R^2 = 0.813$) for 21 cycles [Fig. 3(B-3)], demonstrating that the adsorption ability of the column does not depend any longer on the affinity of the antibody but on the hydrophobicity of the protein. In contrast, the correlation of the values obtained from the first cycle [Fig. 3(B-1); $R^2 = 0.077$] was not fairly observed between the adsorption ability and the hydrophobicity, and the value for BSA was apart from those for other proteins. Therefore, BSA was specifically removed as compared with the other protein standards by the immunoaffinity adsorption. Also, as shown in Fig. 3(B-2), the result obtained from 11 cycles ($R^2 = 0.012$) demonstrated that all proteins bound to the surface of the affinity column materials equally. Therefore, the present data demonstrates that the quantitative changes of the adsorption for the affinity column appear before the limited use of the column (30 times in the manufacturer's instructions).

Understanding the State of the Plasma-treated Column Materials

To understand the state of the column materials of the Seppro®-IgY12 column, the untreated and 11- and 21-times-treated column materials were subjected to electron microscopy and MALDI-TOF-MS analysis.

As shown in Fig. 4, the SEM images show an obvious difference between the untreated and treated materials. The attachment of the unknown bio-molecules to the materials surface appeared and increased with an increase in the number of treatments.

Next, in order to characterize the attached compounds, the same materials were subjected to MALDI-TOF-MS analysis. Since

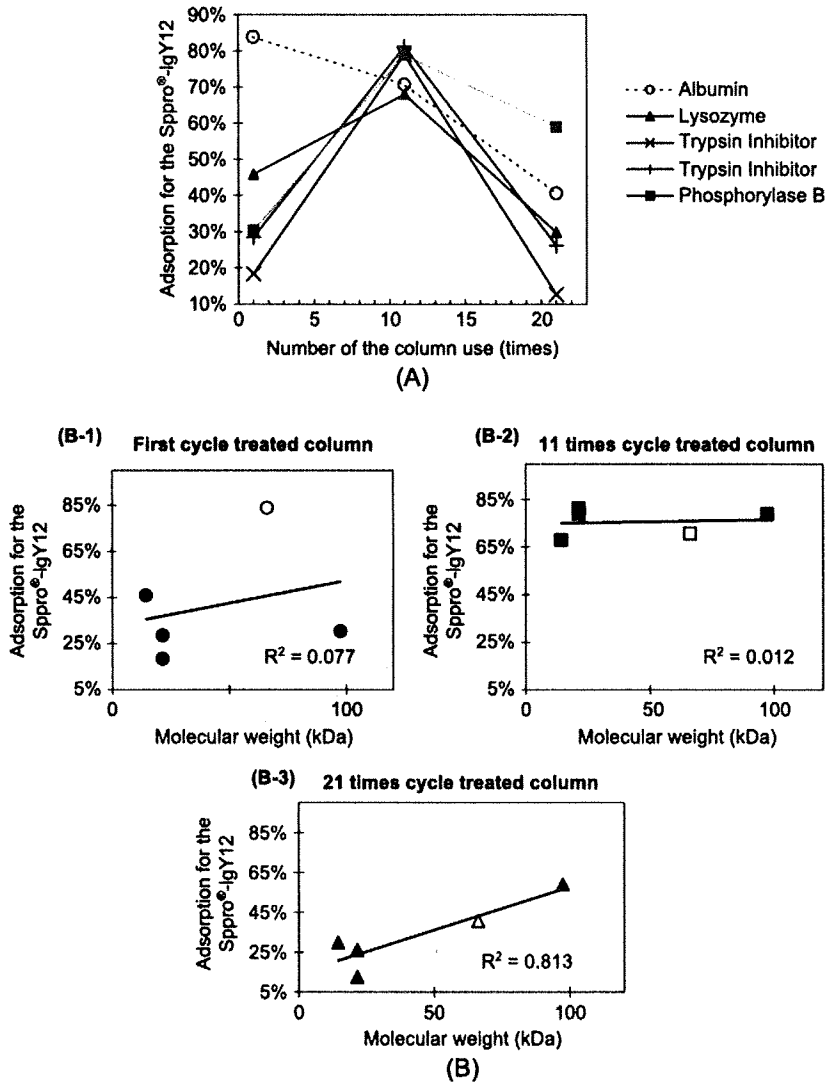

Figure 3. Changes of the adsorption ratio of protein standards for Seppro®-IgY12 column. (A) Relation of the adsorption ratio to the number of times the column was used. (B) Correlation of the adsorption ratio for the column with molecular weight of each protein standard using the column treater one (B-1), 11 (B-2) and 21 (B-3). The open dots show the value for BSA.

direct laser irradiation of the materials could affect the instrument, the positions to be irradiated were the points of the existence of many matrices on a few column materials. Although several peaks existed of less than 70,000 *m/z* in each mass spectrum, the higher molecular weight peaks (7266, 9689, 14,532 and 29,041 *m/z*) appeared in the treated but not in the untreated materials (Fig. 5). After the materials were washed with acetonitrile, the higher molecular weight peaks in the treated materials disappeared (data not shown). Therefore, the compounds attached to the material surface should be hydrophobic high-molecular-weight compounds existing in human plasma.

Two analyses of the column materials surface demonstrated that the hydrophobic high-molecular-weight compounds in plasma adsorbed onto the surface of the affinity column materials and contributed to the changes in the adsorption ability of plasma protein from immunoaffinity into hydrophobic interactions. Howevere, further studies are needed to characterize the exact details of the compounds.

Conclusions

To investigate the ability to remove abundant proteins from plasma by immnoaffinity using the IgY column, FD-LC-MS/MS method was applied to the long-term test of the reproducibility of the column. It was demonstrated that the immunoaffinity column was effective in removing BSA from the protein standards mixture, but, in addition, removing other proteins in the 18.3–45.0% range. The results suggested that the proteins of possible biomarkers could be lost and their quantification made difficult. Moreover, the specific adsorption of BSA in the protein standards mixture and of albumin in the control human plasma samples decreased with an increase in the number of times the column was used with both samples before its use expired. To examine the cause of the functional changes of the immunoaffinity, the correlations between the adsorption ratio for the affinity column and molecular weight of the adsorbed proteins were calculated, and the column materials surface was also investigated by SEM and

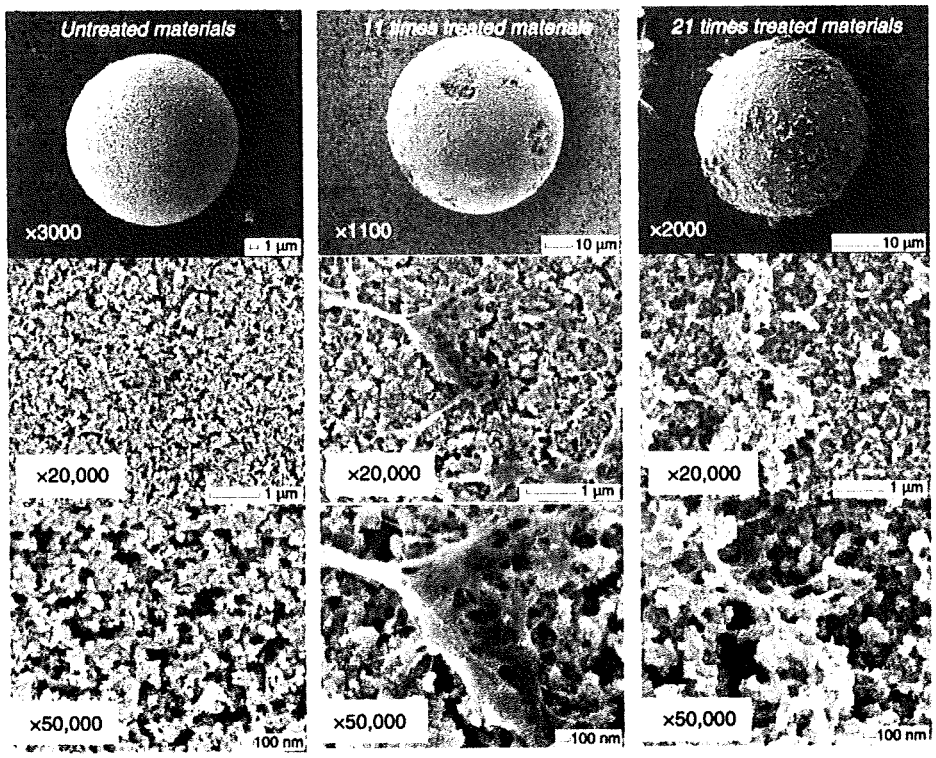

Figure 4. SEM images of the untreated and 11- and 21-times-treated column material surfaces. Magnification in SEM was controlled in a range of × 1100–3000 to show the whole picture of the material.

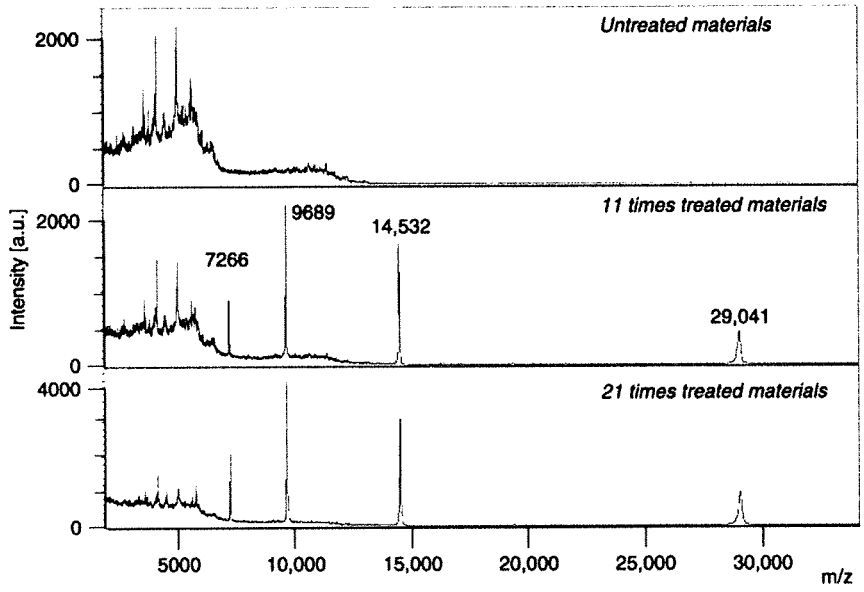

Figure 5. MALDI-TOF-mass spectrum of the untreated and 11- and 21-times-treated column material surface.

MALDI MS analysis. These data demonstrated the attachment of the hydrophobic high-molecular-weight compounds in plasma to the surface, suggesting that on every sample treatment with the affinity column, the adsorption ability of plasma protein changed into hydrophobic interactions. Further studies to characterize the attached compounds are required, and the elucidation of the compounds might lead to the improvement of the affinity column technique and contribute to progress in quantitative plasma proteomics.

Reproducibility is prerequisite for accurate quantitative proteome analysis of clinical samples for biomarker identification and quantification. For this purpose, it is generally essential to

prepare protein samples without high-abundance proteins via specific pre-fractionation techniques to enhance the detection of low-abundance proteins in plasma, and thus, immunoaffinity separation is now chosen as a reliable pre-fractionation method. However, this study indicated that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only nonselective loss but also functional changes of the adsorption ability for the immunoaffinity column.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Abstract
Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.
Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.
Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.
Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/mm^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load ($\geq$100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for $\leq$60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for > 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years ≥ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%≤ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	

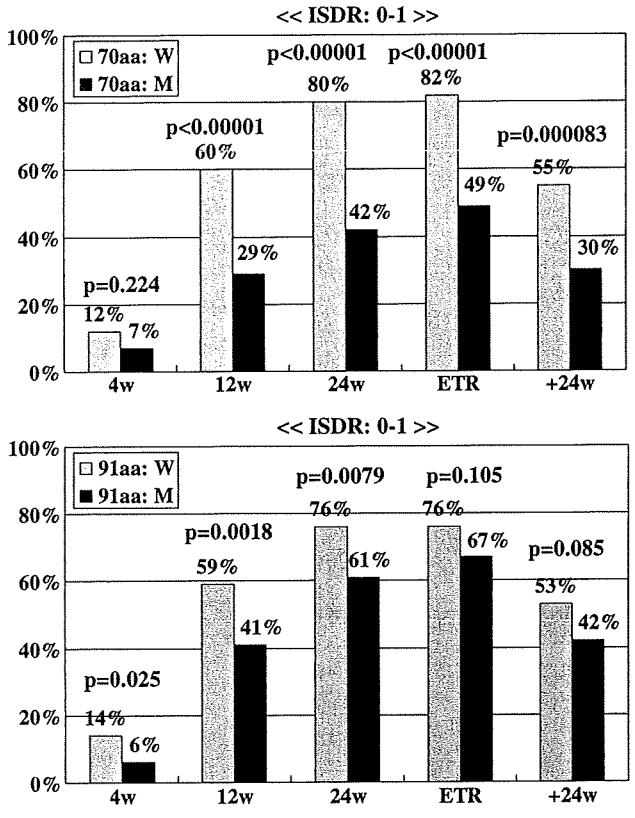

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/mm^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	*p* value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4$/mm^3)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4$/mm^3)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, *M* Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4$/mm^3 of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4$/mm^3. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

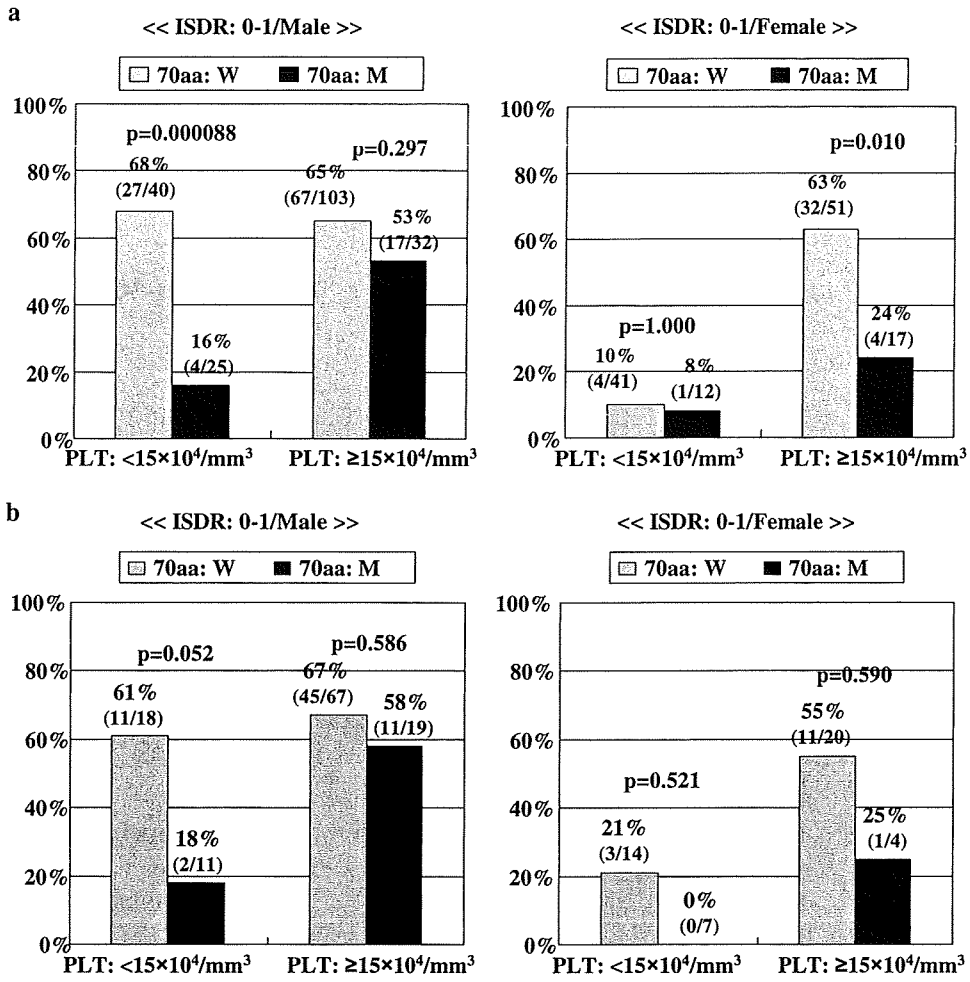

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/mm^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT count of less than $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**a**). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/mm^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/mm^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (**b**)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, p = 0.0037) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

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A novel amplification target, *ARHGAP5*, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells

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ABSTRACT

RhoA, a member of the Rho family of small GTPases, directs the organization of the actin cytoskeleton and is involved in regulating cell shape and movement. Its activity is negatively regulated by p190-B RhoGAP (GTPase-activating protein). We investigated DNA copy number aberrations in human hepatocellular carcinoma and esophageal squamous cell carcinoma cell lines using a high-density oligonucleotide microarray and found a novel amplification at chromosomal region 14q12. We identified *ARHGAP5* (the gene encoding p190-B RhoGAP) as a probable target for the amplification at 14q12, and our results showed that p190-B RhoGAP promotes cells spreading and migration by negatively regulating RhoA activity in Huh-7 hepatocellular carcinoma cells.

1. Introduction

Members of the Rho family of small GTPases act as molecular switches. In response to extracellular signals, they direct the organization of the actin cytoskeleton and alter gene expression [1]. Rho proteins, which include the much-studied Cdc42, Rac1 and RhoA, are involved in regulating cell shape, polarity and movement and establishing cell-cell junctional complexes. Accordingly, their activity is tightly controlled by regulatory proteins that determine whether GTP or GDP is bound. Rho proteins are activated by guanine nucleotide exchange factors, which catalyze the release of GDP and thus allow GTP to bind the proteins. Rho proteins in turn are inactivated by Rho GTPase-activating proteins (GAPs), which bind to the Rho proteins and induce them to hydrolyze their bound GTP to GDP. p190-B RhoGAP, a member of the RhoGAP family, negatively regulates RhoA activity [2,3].

Amplification of DNA in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies, specifically when proto-oncogenic target genes within those amplicons are overexpressed. Oncogenes that are often amplified in cancers include *MYC*, *ERBB2* and *CCND1*.

In the present study, we investigated DNA copy number aberrations in human hepatocellular carcinoma (HCC) and

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esophageal squamous cell carcinoma (ESCC) cell lines and found a novel amplification at chromosomal region 14q12. Because the region may harbor one or more proto-oncogenes whose overexpression following amplification contributes to the initiation or progression of HCC and ESCC, we carried out molecular definition of the amplicon. We show here that the p190-B RhoGAP gene (*ARHGAP5*) within the 14q12 amplicon is amplified and overexpressed, and that p190-B RhoGAP promotes cell spreading and migration in Huh-7 hepatocellular carcinoma cells.

2. Materials and methods

2.1. Cell lines

A total of 10 HCC cell lines (JHH-6, JHH-7, SNU354, SNU398, SNU423, SNU475, Huh-1, Huh-7, HLE and PLC/PRF/5) and 10 ESCC cell lines (T.T, EC-GI-10, KYSE140, KYSE220, TE-4, TE-5, TE-6, TE-10, TE-14 and TE-15) were examined. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Genomic DNA was isolated from each cell line using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA).

2.2. Array analysis

Array analyses were performed using the GeneChip Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. In brief, 250 ng of genomic DNA was digested with a restriction enzyme (StyI), ligated to an adaptor and amplified by PCR. Amplified products were fragmented, labeled by biotinylation and hybridized to the microarrays. Hybridization was detected by incubation with streptavidin–phycoerythrin conjugate, and the array was scanned. Analysis was performed as previously described [4]. Copy number changes were calculated using the Copy Number Analyzer for Affymetrix GeneChip Mapping Arrays (CNAG; http://www.genome.umin.jp) [5].

2.3. Fluorescence in situ hybridization (FISH)

We performed FISH using three bacterial artificial chromosomes (BACs), RP11-113E19, RP11-431H16 and RP11-54H22 as probes (Invitrogen, Carlsbad, CA, USA), as described previously [6]. The BACs were selected based on homology with locations in the human genome according to the database provided by the UCSC (http://genome.ucsc.edu/).

2.4. Real-time quantitative PCR

We quantified genomic DNA and mRNA using a real-time fluorescence detection method, as described previously [6]. The primers used were as follows: *ARHGAP5* mRNA (forward, 5′-CATCTGTTTTTGGCCAACCT-3′; reverse, 5′-gtggaggagccacaatgttt-3′); *HEATR5A* mRNA (forward, 5′-TGTGCCTCCTACTCATGCTG-3′; reverse, 5′-gagatggcctgagct tgaac-3′); *c14orf126* mRNA (forward, 5′-gtgctttttcaaggga gctg-3′; reverse, 5′-ttcctccaagggtagcttga-3′); *NUBPL* mRNA (forward, 5′-cttggccttgtccaaaacat-3′; reverse, 5′-acaattggc tggcctgtatc-3′). These primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *GAPDH* and long interspersed nuclear element 1 (LINE-1) were used as endogenous controls for mRNA and genomic DNA levels, respectively.

2.5. RNA interference (RNAi)

For RNAi, small interfering RNA (siRNA) duplex oligoribonucleotides targeting *ARHGAP5* (5′-CAAGATCATAATATCAATCTA-3′) and control (non-silencing) siRNA duplexes were synthesized by QIAGEN (Valencia, CA, USA). The siRNAs were delivered into Huh-7 cells using HiPerfect Transfection Reagent (QIAGEN), according to the manufacturer's protocol.

2.6. Immunoblotting

Immunoblots were prepared according to previously reported methods [7]. Cell lysates (20 μg protein per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels. Anti-p190-B RhoGAP monoclonal antibody was obtained from BD Transduction Laboratories (Lexington, KY, USA); anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-β-actin monoclonal antibody was from Sigma–Aldrich (Tokyo, Japan). For immunoblotting, we used anti-p190-B RhoGAP, anti-RhoA and anti-β-actin at dilutions of 1:250, 1:100 and 1:5000, respectively. For secondary immunodetection, we used anti-mouse IgG (Amersham, Tokyo, Japan) diluted 1:5000. Protein binding was detected using the ECL system (Amersham).

2.7. RhoA activity assay

Active RhoA levels were measured using the enzyme-linked immunosorbent assay (ELISA)-based G-LISA RhoA activation assay Biochem Kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. In brief, Huh-7 cells were transfected with siRNA targeting *ARHGAP5* or negative control siRNA, or were left untreated. Cells were then cultured under the standard conditions in DMEM containing 10% FCS. After 48 h, cells were harvested for the RhoA activity assay or trypsinized and held in suspension for 1 h in DMEM containing 1% FCS. The suspended cells were then plated on 6-well plates coated with 5 μg/ml fibronectin (BD Transduction Laboratories) and harvested for the RhoA activity assay at the indicated time points. For the RhoA activity assay, cells were lysed in 70 μl of G-LISA lysis buffer, scraped into tubes and snap frozen in liquid nitrogen. Cell lysates were subsequently thawed, clarified for 2 min at 10,000g, and protein concentrations were normalized between the various time points. Equal amounts of total protein were added to a 96-well plate coated with the Rho-binding domain of Rho effector pro-

teins (which bind active GTP-bound Rho) in triplicate and incubated at 4 °C for 30 min with vigorous shaking. Active Rho levels were determined by subsequent incubations with anti-Rho antibody and secondary horseradish peroxidase-conjugated antibody for 45 min each at room temperature. After adding developing solution, the level of active Rho was determined by measuring absorbance at 490 nm using an ELISA plate reader. Equal loading of total RhoA protein at each time point was determined via immunoblotting using anti-RhoA antibody as described above. Experiments were repeated at least three times.

2.8. Immunofluorescence

Huh-7 cells were transfected with siRNA targeting *ARHGAP5* or negative control siRNA or were left untreated. Cells were harvested 48 h after transfection, suspended for 1 h in DMEM containing 1% FCS and then plated on glass slides coated with fibronectin for 10, 20, 40, 60 or 180 min. Cells were fixed for 10 min in 3.7% formaldehyde, permeabilized for 2 min in 1% Triton X-100 and incubated for 1 h with a blocking buffer (phosphate-buffered saline containing 3% bovine serum albumin). The cells were then incubated for 1 h at room temperature with anti-p190-B RhoGAP monoclonal antibody diluted 1:200 in blocking buffer. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Cappel, Aurora, OH, USA) was used to detect the primary antibody. Actin filaments and nuclei were counterstained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) and 4',6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich), respectively.

2.9. Monolayer wound healing assay

Huh-7 cells were transfected with siRNA targeting *ARHGAP5* or negative control siRNA or left untreated. After 24 h, cells in DMEM with 1% FCS were seeded on glass slides coated with fibronectin and allowed to adhere overnight.

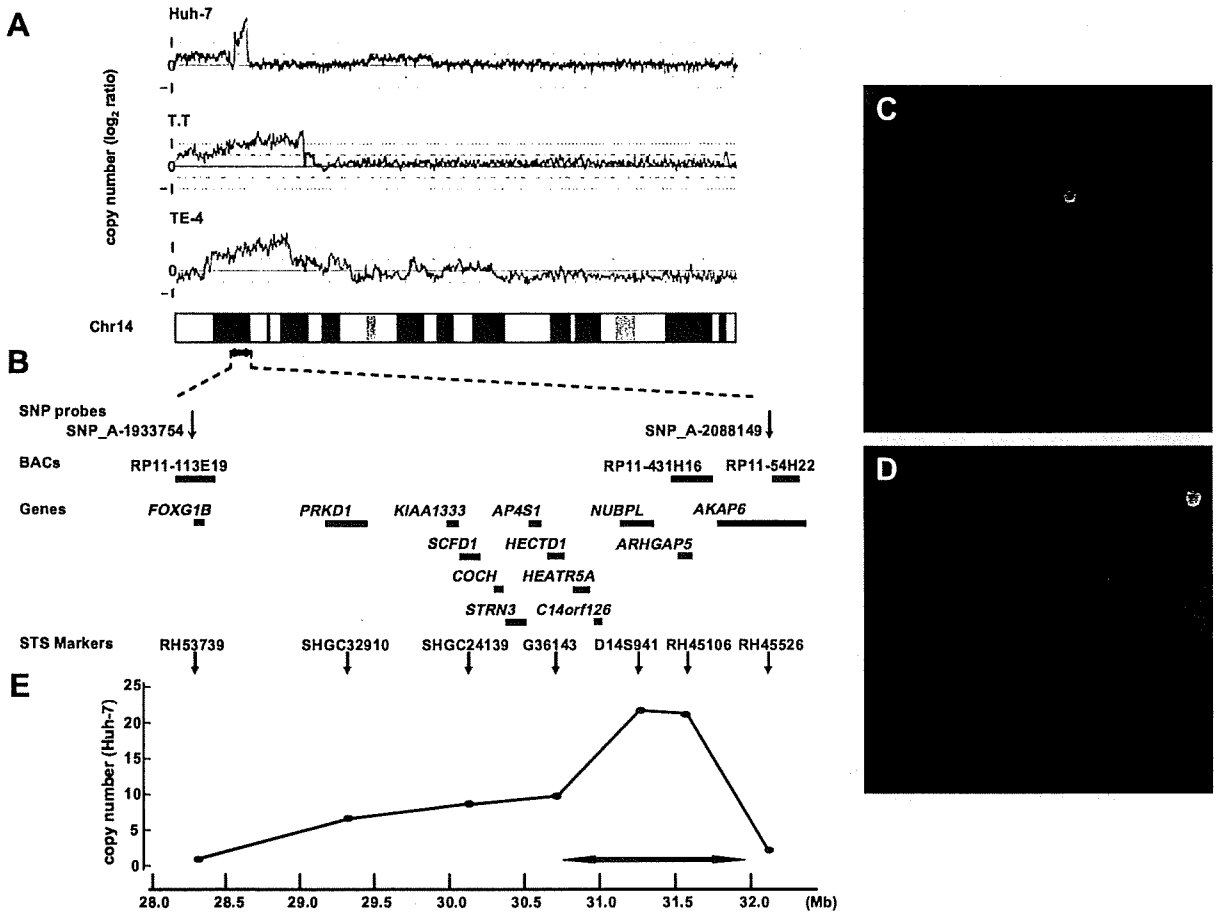

Fig. 1. Map of the amplicon at 14q12. (A) Copy number profiles for chromosome 14 in Huh-7, T.T and TE-4 cells. Copy number values were determined by GeneChip Mapping 250 K array analyses. (B) The positions of the Affymetrix SNP probes, three BACs used as probes for FISH experiments, the 13 genes within the 14q12 amplicon, and the seven STS markers used for real-time quantitative PCR on genomic DNA are shown according to the UCSC genome database (http://genome.ucsc.edu/). (C and D) Representative images of two-color FISH on metaphase chromosomes from Huh-7 cells using BACs: paired RP11-431H16 (green; C) and RP11-113E19 (red; C), or paired RP11-431H16 (green; D) and RP11-54H22 (red; D). (E) Copy numbers at the seven STS marker loci in Huh-7 cells as measured by real-time quantitative PCR with reference to LINE-1 controls. Values are normalized such that the copy number in genomic DNA derived from normal lymphocytes has a value of 2. The smallest region of amplification is indicated (arrow).

We scratched wounds in the cell monolayer using a sterile 200-μl pipet tip, rinsed the cells with phosphate-buffered saline and added DMEM containing 10% FCS with or without mitomycin C (10 μg/ml, Nacalai Tesque, Kyoto, Japan). Cells were allowed to migrate into the wound for 0, 12, or 24 h before fixation. Cells were stained with Giemsa stain (Nacalai Tesque) or were triple-labeled with anti-p190-B RhoGAP, rhodamine-phalloidin and DAPI as described above. Wound widths were measured in three randomly chosen regions. Experiments were repeated at least three times.

2.10. Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). *P* values of <0.05 were considered significant.

3. Results

3.1. Detection of 14q12 amplicon in HCC and ESCC cell lines by array analyses

We screened for DNA copy number aberrations in 10 HCC cell lines and 10 ESCC cell lines using GeneChip Mapping 250 K array analysis. Of the 20 cell lines, one HCC cell line, Huh-7, and two ESCC cell lines, T.T and TE-4, commonly exhibited copy number gains at chromosomal region 14q12 (Fig. 1A). In particular, Huh-7 cells showed a high-level gain indicative of amplification in a narrow region on 14q12 between the positions recognized by the Affymetrix SNP_A-1933754 and SNP_A-2088149 probes. To confirm amplification in Huh-7 cells, we performed FISH analyses using BACs RP11-113E19, RP11-431H16 and RP11-54H22 as probes (Fig. 1B–D). BAC RP11-431H16 generated strong signals as a small homogeneously staining region (HSR), indicating amplification (Figs. 1C, D). In contrast, BACs RP11-113E19 or RP11-54H22 did not show a HSR pattern, indicating their positions outside the amplicon (Fig. 1C and D). Furthermore, we determined gene dosages in Huh-7 cells at the STS markers RH53739, SHGC32910, SHGC24139, G36143, D14S941, RH45106, and RH45526 loci by real time quantitative PCR. (Fig.1B and E). The highest copy number was observed at the D14S941 and RH45106 loci. Taken together, we defined the smallest region of amplification between markers G36143 and RH45526. The extent of the amplicon was estimated to be 1.2 Mb. This region includes four known or predicted protein-coding genes, *HEATR5A*, *c14orf126*, *NUBPL*, and *ARHGAP5*.

3.2. Identification of candidate target genes in the 14q12 amplicon

The 14q12 region may harbor one or more genes (henceforth called 'target genes') that, when activated by amplification, play a role in carcinogenesis. A common criterion for designating a gene as a putative target is that amplification leads to its overexpression [8]. Using real-time quantitative PCR, we determined mRNA levels of all four genes within the amplicon in the 10 HCC cell lines and 10 ESCC cell lines. Among the four genes, *HEATR5A* and *ARHGAP5* were commonly overexpressed in Huh-7, T.T and TE-4 cells, the cell lines that were found to have copy number gains at 14q12 (Fig. 2A). These findings identified *ARHGAP5*, which encodes p190-B RhoGAP, as one of candidate target genes for the 14q12 amplicon.

We determined copy numbers of *ARHGAP5* in the 10 HCC and 10 ESCC cell lines by real-time quantitative PCR (Fig. 2B). Copy number changes were counted as gains if the results of the analysis for a given tumor cell type exceeded the twofold levels of the gene in normal cells. A copy number gain of *ARHGAP5* was observed in six (30%) of the 20 cell lines: Huh-7, T.T, KYSE140, TE-4, TE-6 and TE-10.

We examined the expression of p190-B RhoGAP protein in 4 HCC and 4 ESCC cell lines by immunoblot analysis. As shown in Fig. 2C, expression levels of p190-B RhoGAP were higher in cell lines exhibiting copy number gains of *ARHGAP5* (Huh-7, T.T, KYSE140, TE-4 and TE-10) than other cell lines that did not show gains (SNU354, Huh-1 and PLC/PRF/5).

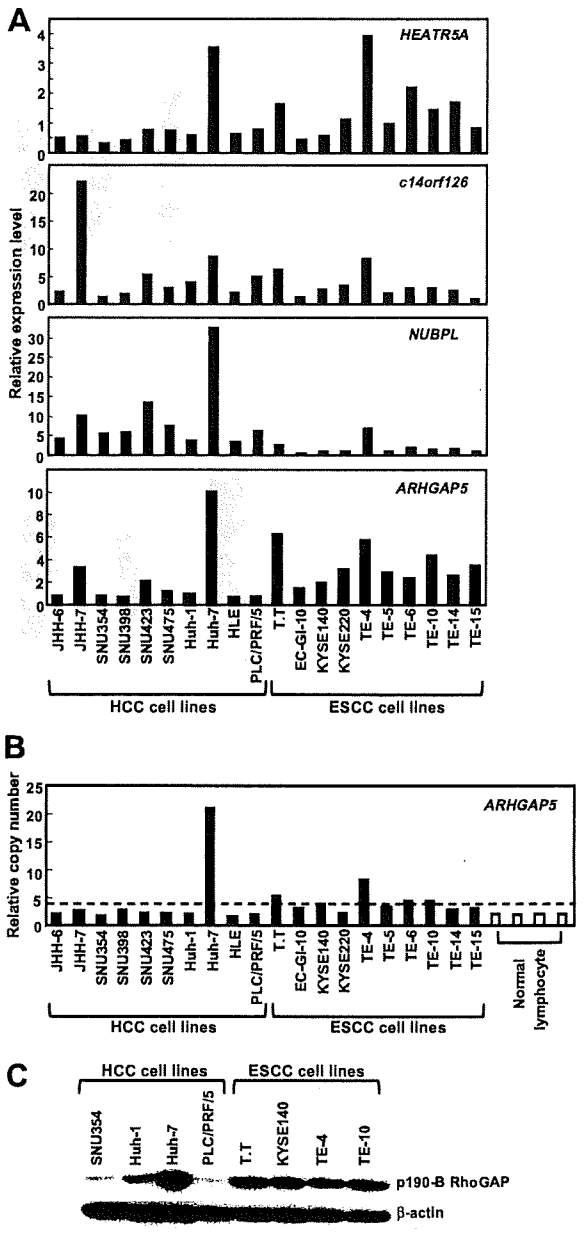

Fig. 2. Amplification and overexpression of *ARHGAP5* in Huh-7, T.T and TE-4 cell lines. (A) Relative expression levels of four genes (*HEATR5A*, *c14orf126*, *NUBPL* and *ARHGAP5*) within the 14q12 amplicon in 10 HCC and 10 ESCC cell lines as evaluated by real-time quantitative PCR. Results are presented as expression levels of each gene relative to a reference gene (*GAPDH*) to correct for variations in the amount of RNA. (B) Copy numbers at the *ARHGAP5* locus (the STS marker RH45106) in 10 HCC cell lines, 10 ESCC cell lines and four normal peripheral blood lymphocytes as measured by real-time quantitative PCR with reference to LINE-1 controls. Values are normalized such that the average copy number in genomic DNA derived from four normal lymphocytes has a value of 2. A value of 4, which is a twofold increase in copy number of normal lymphocytes, was used to determine the cut-off value for copy number gain, shown as a dotted line. (C) Levels of p190-B RhoGAP and β-actin, an internal control, determined by immunoblotting in 4 HCC and 4 ESCC cell lines.