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FIGURE LEGENDS

Fig.1 Number of amino acid substitutions per sample in the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) group.

The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the full open reading frame (ORF) (Fig.1, left) and for each HCV protein (Fig.1, right).

Fig.2a Different amino acid usages at each viral amino acid position between the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) patients.

Amino acid variation was determined between SVR and non-SVR patients by Fisher's exact probability test. The longitudinal axis shows the - logP value.

Fig.2b Sequence alignment in the core region.

Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.

Fig.2c Sliding window analysis.

Viral regions affecting treatment outcome are shown as red spots. There are four hot spots: at core amino acid 110, amino acids 400-403 (i.e. the hyper variable region) in Envelope2 (E2) region, amino acids 724-743 in E2 and amino acids 2258-2306 in the nonstructural (NS)5A.

Fig.2d Sequence alignment amino acids in the nonstructural (NS)5A around amino acids 2258 to 2306.

Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.

Fig.3 Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2258 to 2306.

Spearman's correlation coefficient by rank test is demonstrated.

Table

Table 1. Baseline Characteristics of All Patients (Group 1 and 2)

Characteristic	SVR (n = 58)			non-SVR (n = 20)			*P* value△
	Group 1 (n = 36)	Group 2 (n =22)	Combined (n = 58)	Group 1 (n = 7)	Group 2 (n = 13)	Combined (n = 20)	
Gender(Male/Female)	20 / 16	9 / 13	29 / 29	4 / 3	5 / 8	9 /11	0.80†
Age(yrs)	50.0 ± 12.5*	57.3 ± 10.0	52.4 ± 12.1	55.0 ± 9.7	59.8 ± 6.4	58.1 ± 7.8	0.058‡
ALT(IU/l)	86.6 ± 86.6	71.2 ± 50.4	80.5 ± 74.2	52.9 ± 29.3	88.1 ± 90.1	75.8 ± 75.5	0.81‡
Platelet($x10^4/mm^3$)	20.8 ± 6.2	19.0 ± 5.2	20.1 ± 5.8	14.7 ± 7.1	19.1 ± 4.9	17.6 ± 6.0	0.11‡
Fibrosis score(0-2 / ≥3)§	34 / 1	19 / 2	53 / 3	4 / 3	11 / 2	15 / 5	0.049†
HCV RNA(KIU/ml)	760(2-3100)**	340(54-3600)	550(12-3600)	1300 (350-30000)	1400 (180-5000)	1300 (180-30000)	0.002‖
IFN dose(≥80% / 60-80%)¶	28 / 4	21 / 1	49 / 5	4 / 3	11 / 2	15 / 5	0.12†
Ribavirin dose(≥80% / 60-80%)¶	27 / 5	17 / 5	44 / 10	4 / 3	5 / 8	9 / 11	0.003†
RVR rate (%)	87.5	54.5	74.1	33.3	46.1	42.1	0.022†
EVR rate (%)	100	100	100	66.7	100	89.4	0.07†

* : mean ± SD ** : median (range) † : Fisher's exact probability test ‡ : Student t test ‖ : Mann-Whitney's U test △: *P* values between all SVR (n = 58) vs. all non-SVR (n = 20)

Several clinical charcteristics listed below were unavailable in some patients

§ : SVR : n = 56 (35 in group1, 21 in group2), non-SVR : n = 17 (7 in group1, 10 in group2) ¶ : SVR : n = 54 (32 in group1, 22 in group2)

Table 2a. Variations in each Amino Acid Position and SVR rate

Position		Group 1 (n = 43)	*P* value	Group 2 (n = 35)	*P* value	Combined (n = 78)	*P* value
Core aa 110	T	100% (19 / 19)	0.01	92.9% (13 / 14)	0.004	97% (32 / 33)	5E - 05
	non T	70.8% (17 / 24)		42.9% (9 / 21)		57.8% (26 / 45)	
p7 aa 773	V	77.4% (24 / 31)	0.16	53.6% (15 / 28)	0.03	66.1% (39 / 59)	0.002
	non V	100% (12 /12)		100% (7 / 7)		100% (19 / 19)	
NS5A aa 2099	R	92.9% (13 / 14)	0.40	91.7% (11 / 12)	0.01	92.3% (24 / 26)	0.01
	non R	79.3% (23 / 29)		47.8% (11 / 23)		65.4% (34 / 52)	
NS5B aa 3013	L	78.9% (26 / 33)	0.17	47.8% (11 / 23)	0.01	66.1% (37 / 56)	0.008
	non L	100% (10 / 10)		91.7% (11 / 12)		95.5% (21 / 22)	

Table 2b. Number of Amino Acid Substitutions in each Region and SVR rate

Region		Group 1 (n = 43)	*P* value	Group 2 (n = 35)	*P* value	Combined (n = 78)	*P* value
E2 aa 400-403	mutation ≥2	89.3% (25 / 28)	0.22	100% (11 / 11)	0.002	92.3% (36 / 39)	0.0005
	mutation 0-1	73.3% (11 / 15)		45.8% (11 / 24)		56.4% (22 / 39)	
E2 aa 724-743	mutation ≥1	100% (28 / 28)	0.0002	72% (18 / 25)	0.12	86.8% (46 / 53)	0.0006
	no mutation	53.3% (8 / 15)		40% (4 / 10)		48% (12 / 25)	
ISDR(aa 2213-2248)	mutation ≥2	100% (15 / 15)	0.08	86.7% (13 / 15)	0.02	93.3% (28 / 30)	0.003
	mutation 0-1	75% (21 / 28)		45% (9 / 20)		62.5% (30 / 48)	
NS5A aa 2258-2306	mutation ≥5	100% (19 / 19)	0.01	84.2% (16 / 19)	0.006	92.1% (35 / 38)	0.0006
	mutation 0-4	70.8% (17 / 24)		37.5% (6 / 16)		57.5% (23 / 40)	

Table 3. Multivariate Logistic Regression Analysis

Factor	Odds (95% CI)	*P* value
Age	1.01 (0.91-1.13)	0.85
HCV RNA	1.00 (1.00-1.00)	0.09
Fibrosis score ≥3/0-2	2.37 (0.21-26.7)	0.48
RVR achievement	3.46 (0.54-22.1)	0.19
Ribavirin dose ≥ 80%	16.0 (1.66-153)	0.02
Core aa 110 T	24.7 (1.72-353)	0.02
NS5A aa 2258-2306 mutations 0-4/≥5	11.5 (1.23-108)	0.03

Table 4a. Baseline Characteristics of Patients with NS5A aa 2258-2306 mutations 0-4 or ≥5 (Group 1 and 2)

Characteristic	Mutation 0-4 (n = 40)	Mutation ≥5 (n = 38)	*P* value
Gender (Male/Female)	22 / 18	16 / 22	NS†
Age (yrs)	54.3 ± 11.4*	53.5 ± 11.5	NS‡
ALT (IU/l)	73.8 ± 70.3	85.3 ± 78.7	NS‡
Platelet ($x10^4/mm^3$)	18.0 ± 5.9	21.0 ± 5.7	0.03‡
Fibrosis score (0-2 / ≥3)§	33 / 5	33 / 2	NS†
HCV RNA (KIU/ml)	1100 (99 - 30000) **	380 (12 - 5000)	0.02‖
IFN dose (≥80% / 60-80%)¶	31 / 8	33 / 2	NS†
Ribavirin dose (≥80% / 60-80%)¶	25 / 14	28 / 7	NS†
RVR rate (%)	65.8	62.9	NS†
EVR rate (%)	94.7	100	NS†
Relapse rate (%)	35.9	7.9	0.002†
SVR rate (%)	57.5	92.1	0.0006†

*: mean ± SD, †: Fisher's exact probability test , ‡: Student t test, §: Mutation 0-4 : n = 38, mutation ≥5 : n = 35,

** : median (range), ‖ : Mann-Whitney's U test, ¶: Mutation 0-4 : n = 39, mutation ≥5 : n = 35

Table 4b. Baseline Characteristics of Patients with Core 110 T or N/S (Group 1 and 2)

Characteristic	Core 110 T (n = 33)	Core 110 N/S (n = 45)	*P* value
Gender (Male/Female)	18 / 15	20 / 25	NS†
Age (yrs)	50.4 ± 13.0*	56.4 ± 9.5	0.032‡
ALT (IU/l)	64.5 ± 48.2	88.8 ± 86.2	NS‡
Platelet ($x10^4/mm^3$)	19.3 ± 4.9	19.5 ± 6.6	NS‡
Fibrosis score (0-2 / ≥3)§	30 / 1	36 / 6	NS†
HCV RNA (KIU/ml)	580 (54 - 3600) **	980 (12 - 30000)	NS‖
IFN dose (≥80% / 60-80%)¶	26 / 3	38 / 7	NS†
Ribavirin dose (≥80% / 60-80%)¶	23 / 6	30 / 15	NS†
RVR rate (%)	72.4	59.1	NS†
EVR rate (%)	100	95.5	NS†
Relapse rate (%)	3.0	38.6	9E-05†
SVR rate (%)	97.0	57.8	5E-05†

*: mean ± SD, †: Fisher's exact probability test , ‡: Student t test, §: Core 110 T : n = 31, Core 110 N/S : n = 42,

**: median (range), ‖ : Mann-Whitney's U test, ¶: Core 110 T : n = 29

Figure 1
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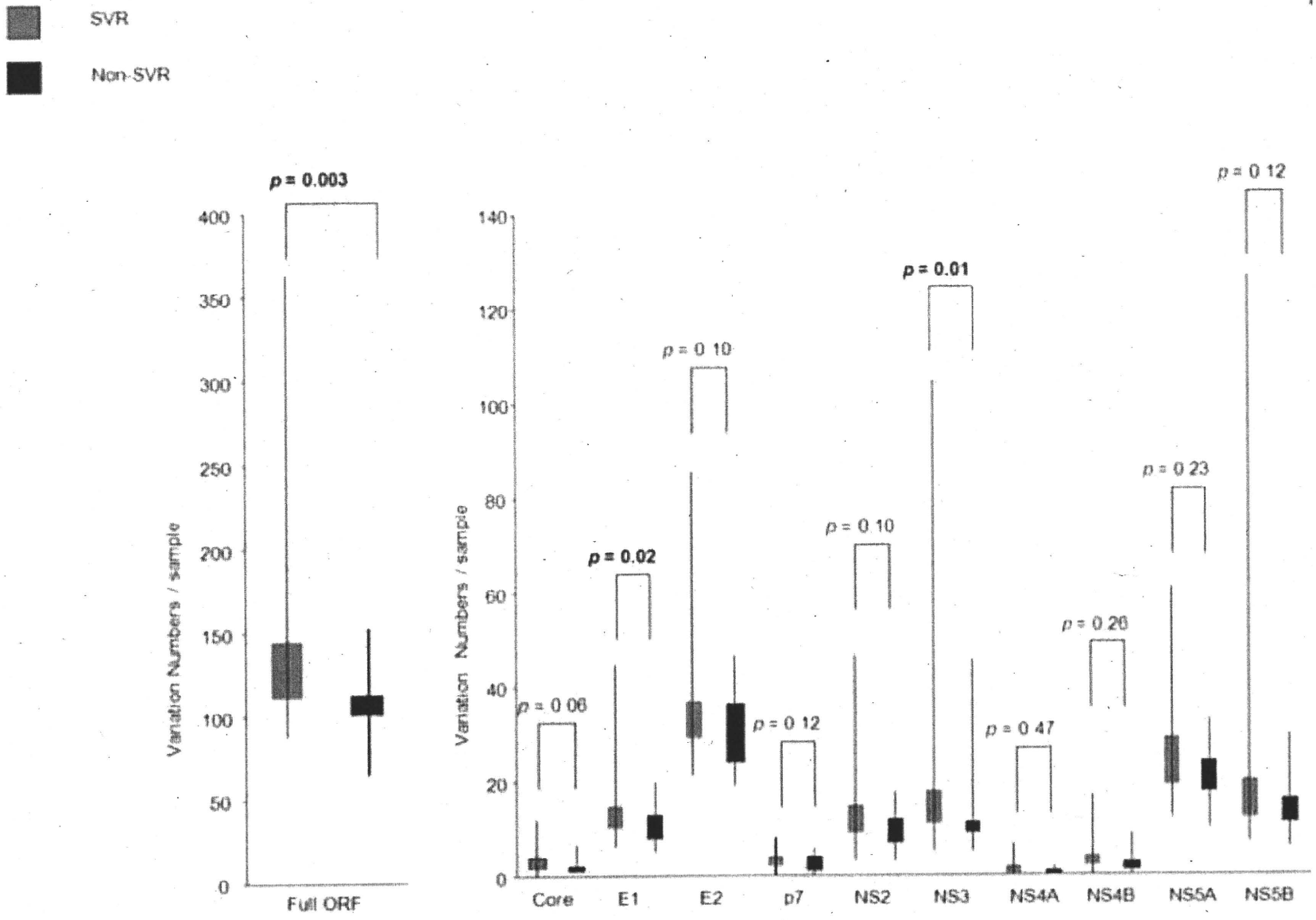

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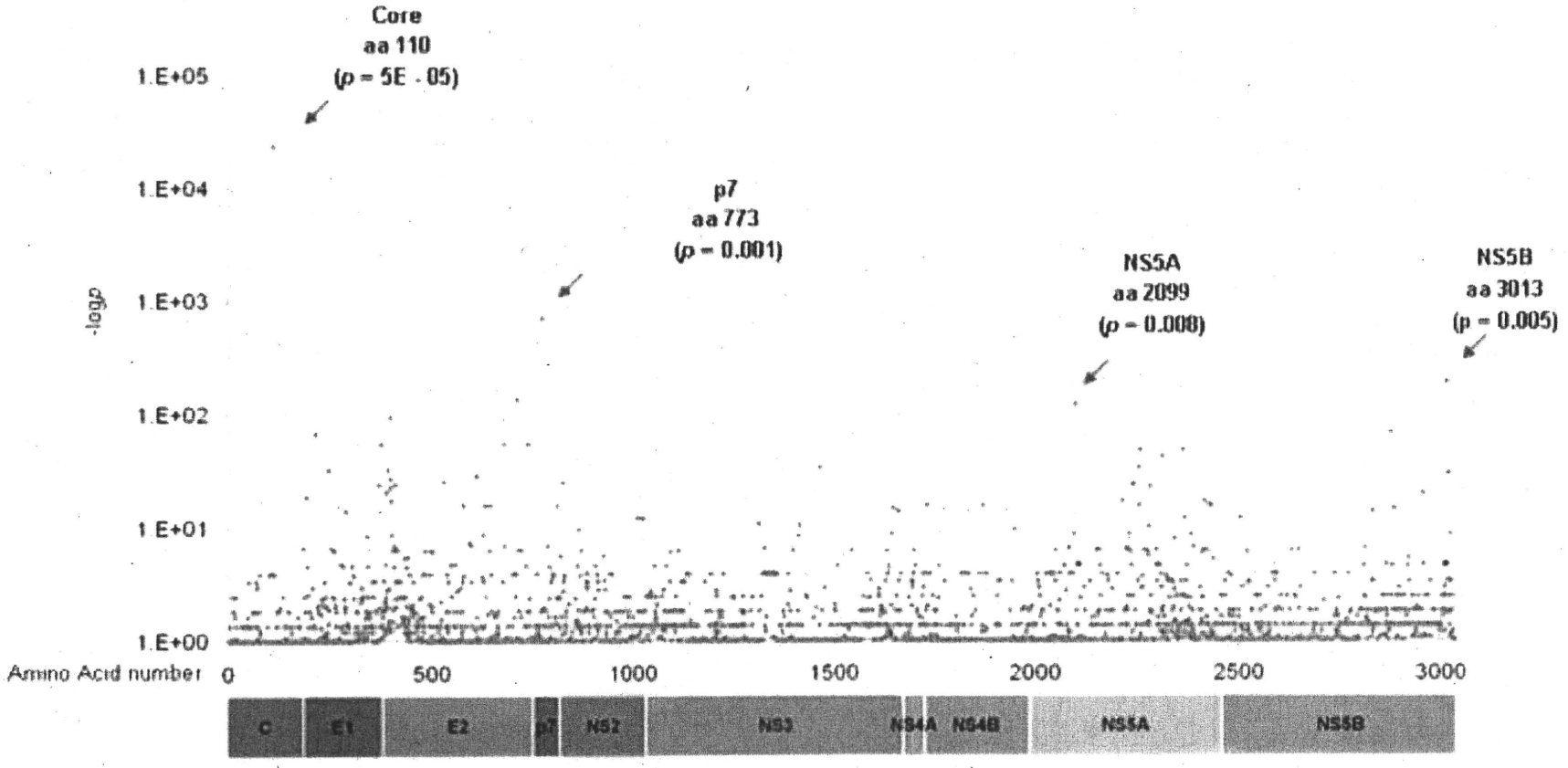

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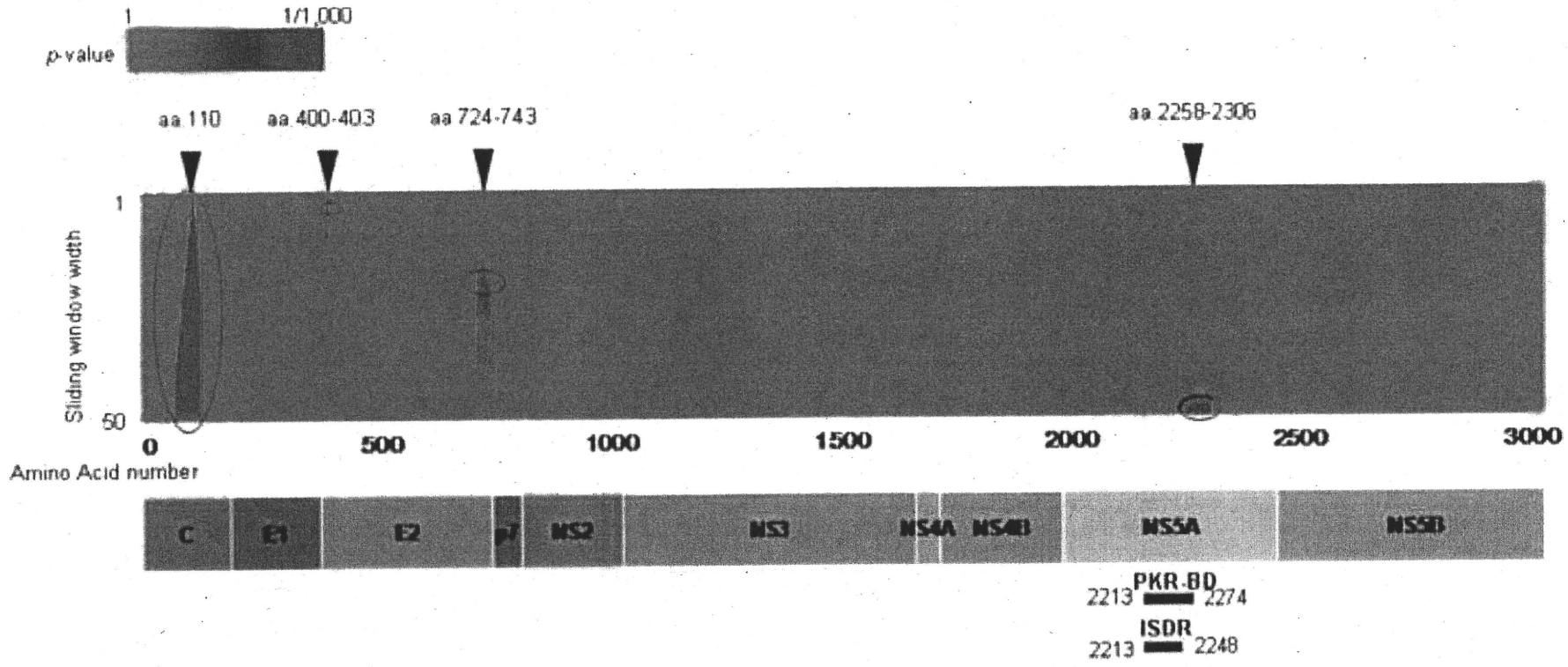

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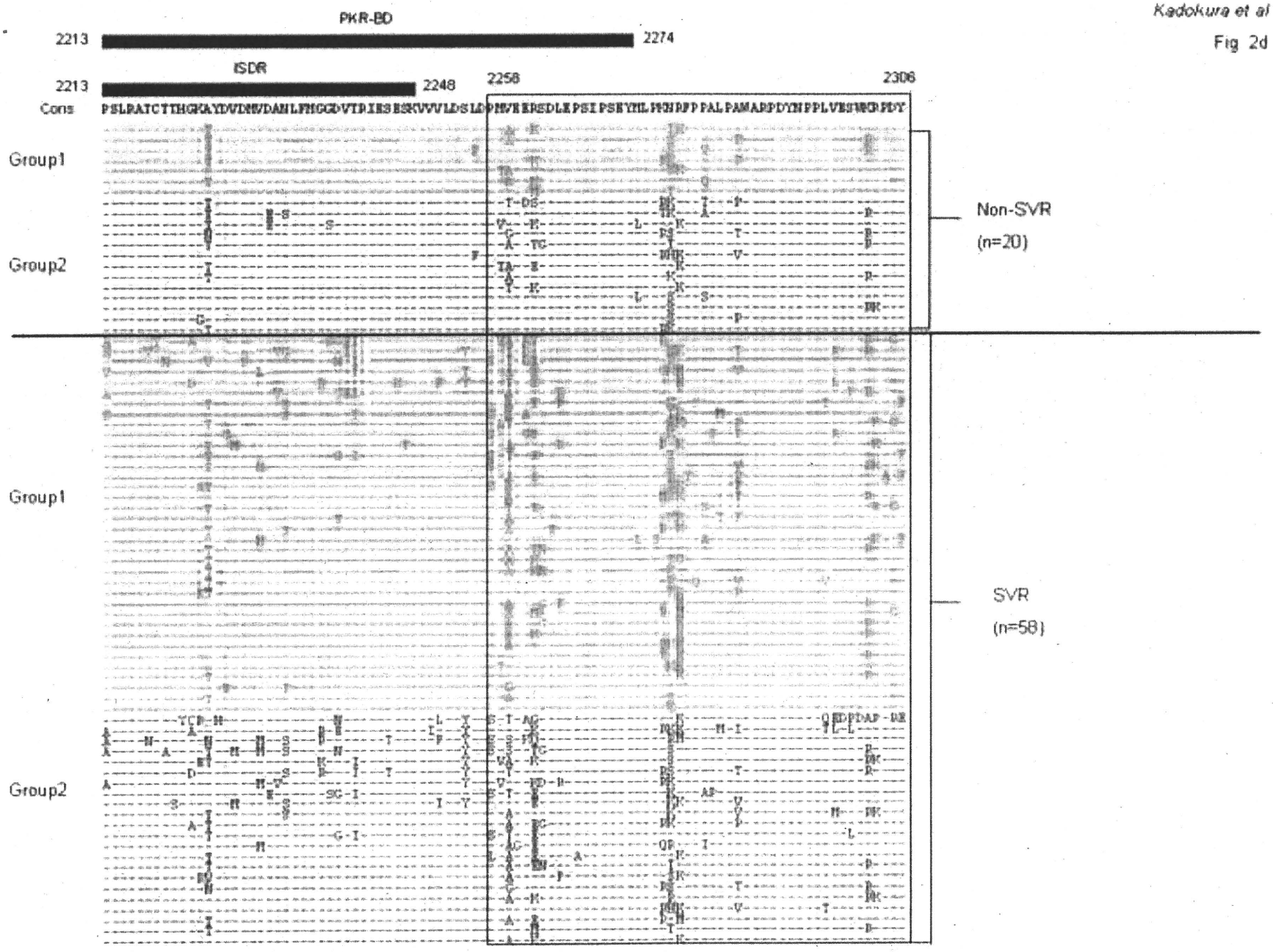

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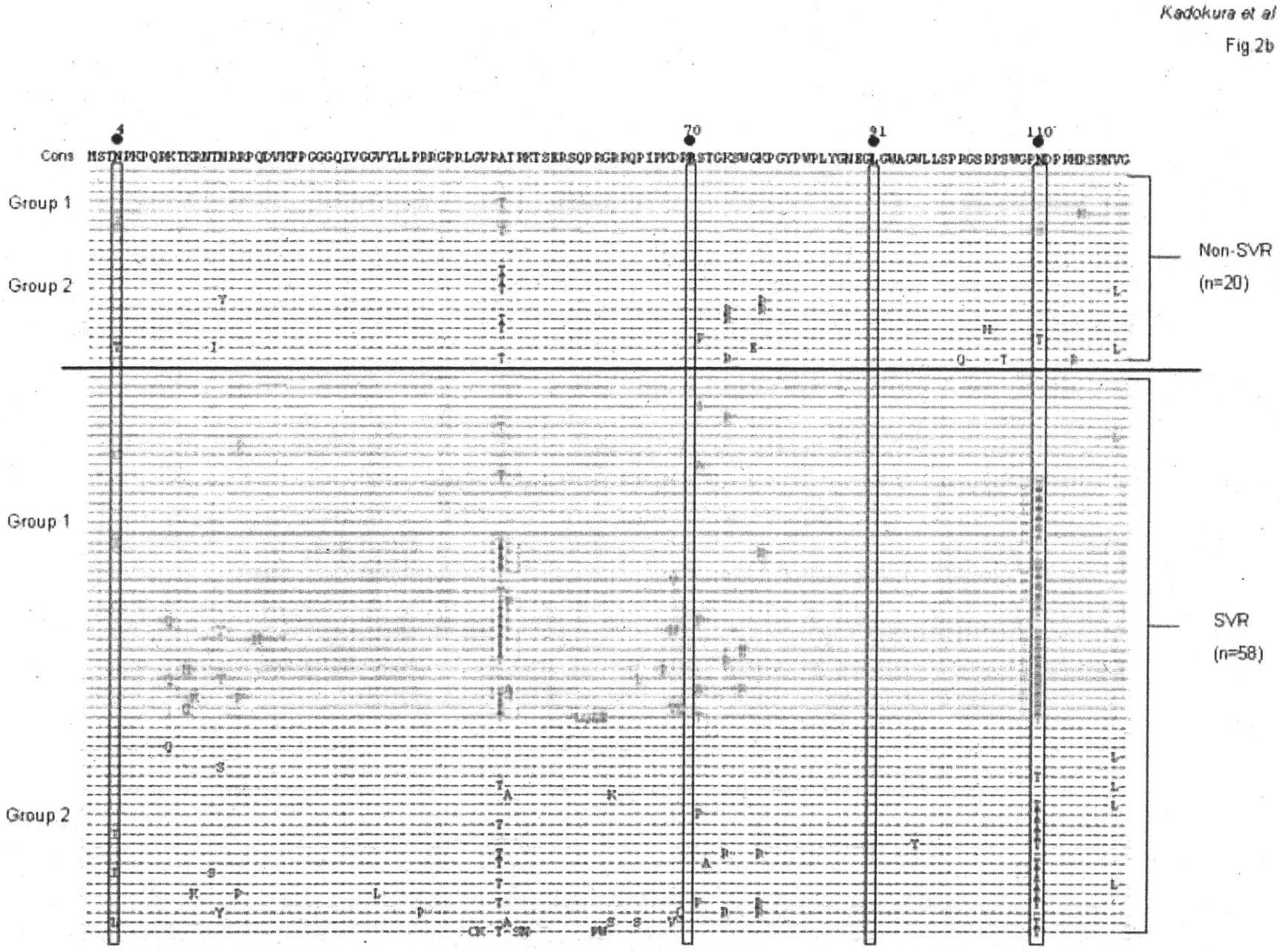

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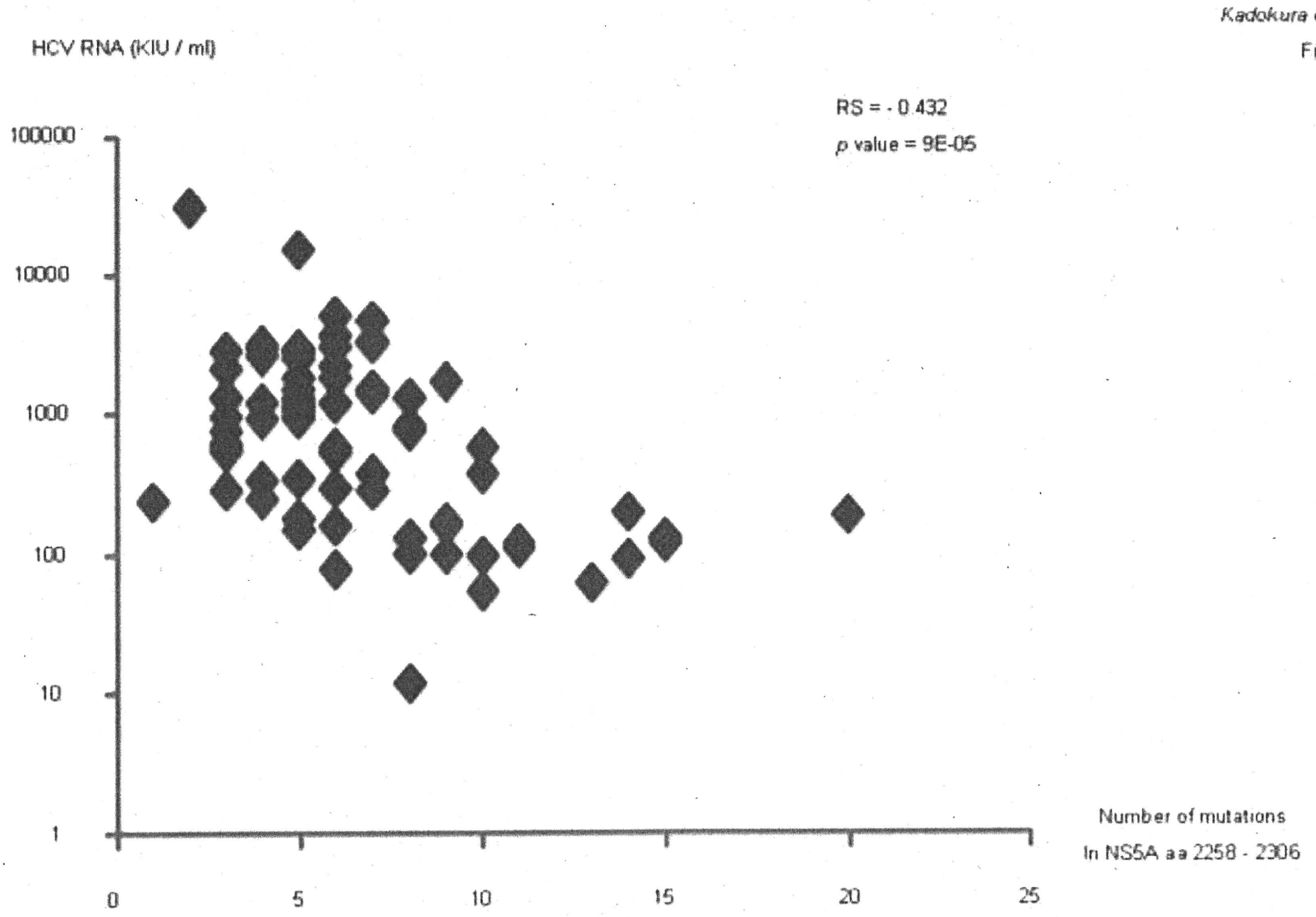

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Sequences in the Interferon Sensitivity-Determining Region and Core Region of Hepatitis C Virus Impact Pretreatment Prediction of Response to PEG-Interferon Plus Ribavirin: Data Mining Analysis

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The aim of the present study was to clarify the significance of viral factors for pretreatment prediction of sustained virological response to pegylated-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C using data mining analysis. Substitutions in the IFN sensitivity-determining region (ISDR) and at position 70 of the HCV core region (Core70) were determined in 505 patients with genotype 1b chronic hepatitis C treated with PEG-IFN plus RBV. Data mining analysis was used to build a predictive model of sustained virological response in patients selected randomly (n = 304). The reproducibility of the model was validated in the remaining 201 patients. Substitutions in ISDR (odds ratio = 9.92, $P < 0.0001$) and Core70 (odds ratio = 1.92, $P = 0.01$) predicted sustained virological response independent of other covariates. The decision-tree model revealed that the rate of sustained virological response was highest (83%) in patients with two or more substitutions in ISDR. The overall rate of sustained virological response was 44% in patients with a low number of substitutions in ISDR (0–1) but was 83% in selected subgroups of younger patients (<60 years), wild-type sequence at Core70, and higher level of low-density lipoprotein cholesterol (LDL-C) (≥120 mg/dl). Reproducibility of the model was validated ($r^2 = 0.94$, $P < 0.001$). In conclusion, substitutions in ISDR and Core70 of HCV are significant predictors of response to PEG-IFN plus RBV therapy. A decision-tree model that includes these viral factors as predictors could identify patients with a high probability of sustained virological response. ***J. Med. Virol. 83:445–452, 2011.***

KEY WORDS: data mining; decision-tree model; ISDR; core region; PEG-interferon

INTRODUCTION

The combination of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) is currently the most effective therapy for chronic hepatitis C, but the rate of sustained virological response after 48 weeks of therapy is about 50% in patients with HCV genotype 1b and a high HCV

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RNA titer [Manns et al., 2001; Fried et al., 2002]. The most reliable means to predict sustained virological response is to monitor the viral response during the early weeks of treatment. The early virological response, defined as undetectable HCV RNA at week 12, is associated with a high rate of sustained virological response [Davis et al., 2003; Lee and Ferenci, 2008]. The rapid virological response, defined as undetectable HCV RNA at week 4 of therapy, is even more predictive of sustained virological response than the early virological response [Jensen et al., 2006; Yu et al., 2008; Izumi et al., 2010]. However, there is no established means that predicts the virological response before commencing treatment. Recent reports have revealed that single nucleotide polymorphisms located near the *IL28B* gene show a strong association with the response to PEG-IFN plus RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Kurosaki et al., 2010c]. These findings indicate that the host factor is an important determinant of the treatment response. On the other hand, the present study's authors have reported that a stretch of 40 amino acids in the NS5A region of HCV, designated as the interferon sensitivity-determining region (ISDR), has a close association with the virological response to interferon mono-therapy [Enomoto et al., 1995, 1996; Kurosaki et al., 1997]. More recently, amino acid substitutions at positions 70 and 91 of the core region have been reported to be associated with response to PEG-IFN plus RBV combination therapy [Akuta et al., 2005, 2007a]. The impact of these HCV substitutions on treatment response is yet to be validated.

Decision-tree analysis is a core component of data mining analysis that can be used to build predictive models [Breiman et al., 1980]. This method has been used to define prognostic factors in various diseases such as prostate cancer [Garzotto et al., 2005], diabetes [Miyaki et al., 2002], melanoma [Averbook et al., 2002; Leiter et al., 2004], colorectal carcinoma [Zlobec et al., 2005; Valera et al., 2007], and liver failure [Baquerizo et al., 2003]. The major advantage of decision-tree analysis over logistic regression analysis is that the results of analysis are easy to understand. The simple allocation of patients into subgroups by following the flowchart form could define the predicted possibility of outcome [LeBlanc and Crowley, 1995].

Decision-tree analysis was used for the prediction of early virological response (undetectable HCV RNA within 12 weeks of therapy) to PEG-IFN and RBV combination therapy in chronic hepatitis C [Kurosaki et al., 2010a], and more recently for the pretreatment prediction of sustained virological response [Kurosaki et al., 2010b]. In the latter model, simple and noninvasive standard tests were used as parameters; specialized tests such as viral mutations and host genetics, or invasive tests such as liver histology, were not included because the aim of that model was for use in general medical practice, especially in some countries or areas where resources are limited. Thus, the impact of viral mutations or liver histology was not considered in that model.

The present study examined whether including viral substitutions in ISDR and the core region of HCV in the decision-tree model could improve its predictive accuracy over the previous model to identify chronic hepatitis C patients who are likely to respond to PEG-IFN plus RBV therapy.

MATERIALS AND METHODS

Patients

This multicenter retrospective cohort study included 505 chronic hepatitis C patients who were treated with PEG-IFN alpha-2b and RBV at Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University Graduate School of Medical Sciences, Yamanashi University, Osaka City University, and their related hospitals. The inclusion criteria were: (1) genotype 1b, (2) HCV RNA titer higher than 100 kIU/ml by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic Systems, Pleasanton, CA), (3) no co-infection with hepatitis B virus or human immunodeficiency virus, (4) no other causes of liver disease, (5) patients having undergone liver biopsy prior to IFN treatment, (6) number of substitutions in ISDR having been determined, (7) substitutions in the amino acid positions 70 and 91 of the core region having been determined, and (8) completion of at least 12 weeks of therapy. Patients were treated with PEG-IFN alpha-2b (1.5 μg/kg) weekly plus RBV. The daily dose of RBV was adjusted by weight: 600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg. For the analysis, patients were assigned randomly to either the model building (304 patients) or validation (201 patients) groups. There were no significant differences in the clinical backgrounds between these two groups (Table I). 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

Hematological tests, blood chemistry, and HCV RNA titer were analyzed before therapy and at least once every month during therapy. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse transcription and polymerase chain reaction as reported previously. At position 70 of the core region (Core70), arginine was defined as the wild type, and glutamine or histidine was defined as the mutant type. At position 91 of the core region, leucine was defined as the wild type and methionine was defined as the mutant type, as described previously [Akuta et al., 2005]. Fibrosis and activity were scored according to the METAVIR scoring system [Bedossa and Poynard, 1996]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of