total mutations, respectively, in the three patients treated with IFN monotherapy, and 43.2%, 38.3% and 37.8%, respectively, in those treated with combination therapy. These results showed no obvious increase in key mutations of C-to-U and G-to-A associated with the combination therapy (Table 1).

Sequence analyses of NS5b region in 81 patients treated with IFN and RBV therapy

To study the correlation between the genetic structures of NS5B and the outcome of IFN plus RBV combination therapy, amino acid sequences of HCV NS5B (aa. 61–407), including motif A-F, were analyzed in 81 patients treated with IFN plus RBV combination therapy. The clinical characteristics of the patients are shown in Table 2. Nineteen (23.5%) patients were SVR, 40 (49.4%) were ETR, and 22 (27.2%) were NR. Clinical variables were analyzed according to the results of the combination therapy. Univariate analysis identified fibrosis stage as significantly lower in the SVR patients than in the other patients. No other clinical parameters were significantly correlated with the responses.

The amino acid sequences of the essential motif B to E of NS5B in these 81 patients are aligned with consensus sequences in Figure 3. Comparison of the NS5B sequences between patients with SVR and patients with non-SVR (ETR and NR) showed no obvious differences. Instead, when we compared the sequences of a patient group of SVR plus ETR with those of patients with NR, the mutations at position NS5B 300–358, including motif B to E between, were more frequent in the SVR plus ETR group than in the NR group. When we analyzed mutations of individual amino acid positions, the frequencies of mutations at aa 309, 333, 338 and 355 of NS5B (the four sites) were found to be more frequent in patients with SVR or ETR than those with NR (Fig. 4). The total number of amino acid changes at these four sites was significantly higher in patients with SVR or ETR than those with NR (0.93 ± 0.89 *vs* 0.27 ± 0.70, $P = 0.0004$). In 19 SVR patients, five patients had no mutations, 10 patients had one mutation, and four patients had two or more mutations at the four sites. In the 40 ETR patients, 18 patients had no mutations, 13 patients had one mutation, and nine patients had two or more mutations at the four sites. In 22 NR patients, 19 patients had no mutations, two patients had one mutation, and one patient had three mutations at the four sites (Fig. 5a). The SVR rates were 11.9% (5 of 42) and 35.9% (14 of 39) in patients who had none and one or more mutations at the four sites, respectively (Fig. 5b). Patients with increased mutations at the four sites tended to be in the SVR or ETR groups. We subsequently analyzed various clinical factors by multivariate analysis among the three response groups to determine the independent predictors for SVR and NR (Table 3). Among these clinical factors, the NS5B mutation described above was independently associated with NR ($P = 0.0185$).

Mutations of the NS5B region, which codes for the viral RdRp, may alter its enzymatic activities which may influence serum virus load of each patient. In our results, however, there was no obvious correlation between the number of NS5B mutations and serum viral loads in each patient, nor was there a difference in the serum virus loads between the patient groups categorized by the numbers of mutations at aa 309, 333, 338 and 355 of NS5B.

Table 1 Sequence analysis of full genome of hepatitis C virus (HCV) RNA treated with interferon (IFN) plus ribavirin

	G-to-A and C-to-U	Other transition (A-to-G and U-to-C)
IFN plus ribavirin	58.3	72.5
No ribavirin (IFN monotherapy)	60.8	70.4

Mutations per 10 000 nucleotides. A total of 56 538 nucleotides were sequenced.

DISCUSSION

In the present study, we have demonstrated that particular amino acid changes in the NS5B region of HCV

Table 2 Baseline characteristics of the group of 81 patients, segregated according to the clinical outcome of interferon (IFN) plus ribavirin combination therapy

	SVR	ETR	NR	*P*-value
Number of patients	19	40	22	
Age (years)	49.5 ± 12.2	55.9 ± 8.1	57.2 ± 10.6	NS
Sex (male/female)	15/4	27/13	11/11	NS
Baseline ALT (IU/L)	122.2 ± 88.0	80.2 ± 43.2	107.4 ± 73.7	NS
Platelet count (10^3/mm^3)	16.0 ± 5.5	16.3 ± 5.5	14.7 ± 4.5	NS
Fibrosis stage (SD)	1.41 ± 0.71	1.92 ± 0.94	2.10 ± 0.72	0.012†
Serum HCV RNA at baseline (KIU/mL)	480.5 ± 295.7	594.6 ± 239.3	599.9 ± 271.3	NS
Number of ISDR mutations	1.73 ± 2.92	0.80 ± 1.22	1.00 ± 1.80	NS

†Significant differences between SVR and others. Values are expressed as mean ± SD, except where noted. ALT, alanine aminotransferase; ETR, end-of-treatment responder; NR,-non-responder; NS, not significant; SR, sustained responder.

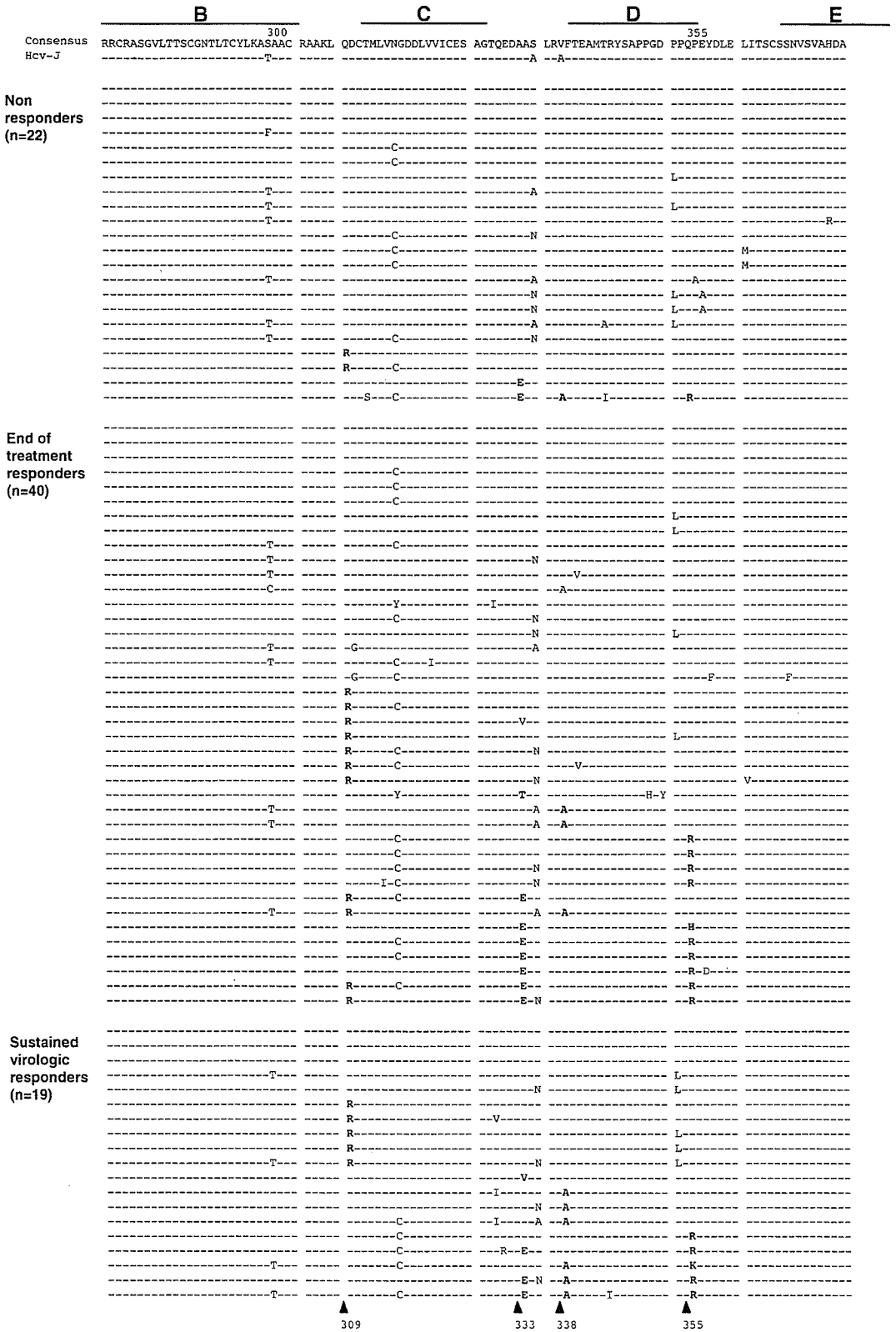

Figure 3 Amino acid sequence alignments of the NS5B motif B-E in 81 patients with chronic hepatitis C virus (HCV)-1b infection and treated with interferon (IFN) plus ribavirin for 24 weeks. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate the identical amino acid residues with consensus sequence and HCV-J shown at the top. At four amino acid positions (NS5B 309, 333, 338 and 355), usages of amino acid residues differ between non-responders (NR) and others. Changes of this part are indicated by bold letter. Outcome of combination therapy is shown on the left side.

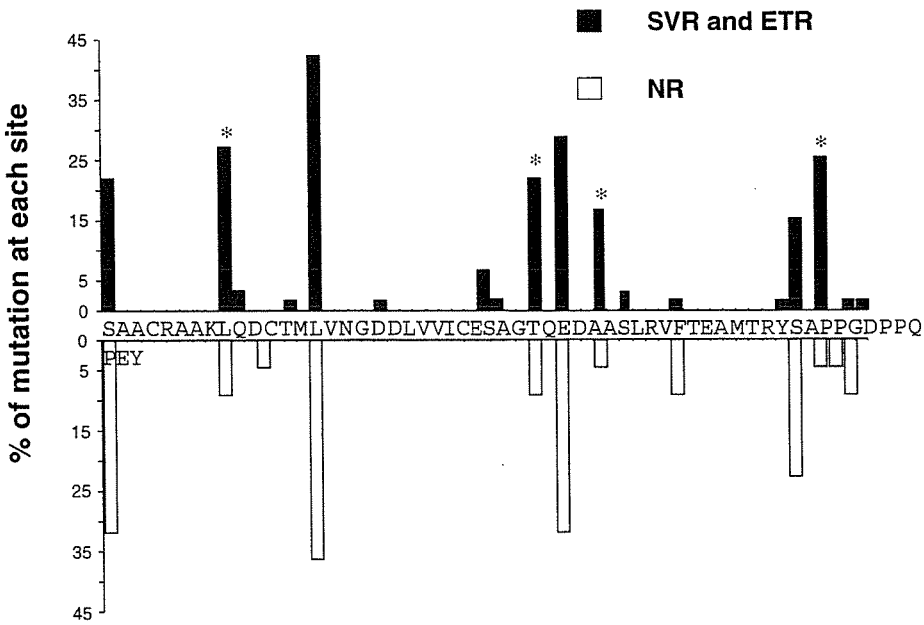

Figure 4 Relationship between frequency of mutations at each site in NS5B 300–358 and the efficacy of interferon (IFN) plus ribavirin treatment. Amino acid residues are indicated by the standard single-letter codes. Among these 59 sites, mutations of aa NS5B 309, 333, 338 and 355 (identified by *) are frequent in sustained virologic response (SVR) and end-of-treatment response (ETR) patients. NR, non-response.

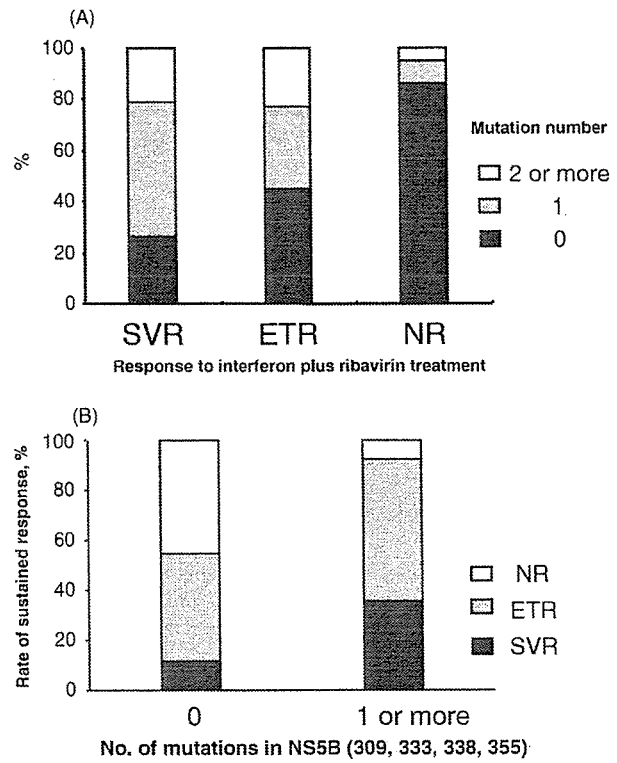

Figure 5 Relationship between number of mutations in NS5B 309, 333, 338, 355 and the outcome of interferon (IFN) plus ribavirin treatment. (a) Distribution of total numbers of mutations at aa. 309, 333, 338 and 355 of NS5B according to sustained virologic response (SVR), end-of-treatment response (ETR) and non-response (NR) patients. (b) Proportion of SVR, ETR and NR patients between groups with or without mutations at aa. 309, 333, 338 and 355 of NS5B.

Table 3 Multivariate analysis for the clinical and virological factors affecting virological responses (SVR and NR) to interferon (IFN) plus ribavirin combination therapy in the group of 81 patients

	Patient with SVR *P*-value	Patient with NR *P*-value
Age (years)	0.572	0.598
Sex (male/female)	0.814	0.158
Baseline ALT (IU/L)	0.022	0.981
Platelet count (10^3/mm^3)	0.749	0.627
Mean fibrosis stage (SD)	0.037	0.330
Serum HCV RNA at baseline	0.227	0.890
No. of ISDR mutations	0.491	0.754
No. of NS5B mutations (309,333,338,355)	0.057	0.019

ALT, alanine aminotransferase; ETR, end-of-treatment response; ISDR, interferon sensitivity determining region; NR, non-response; SR, sustained response.

correlate with the clinical outcome of combination therapy. Pair-wise comparisons of the full-length HCV genome in three patient sera obtained before and 12 weeks after the start of IFN plus RBV therapy did not show consistent amino acid changes. The results suggest negative evidence against the presence of treatment-resistant viral sub-populations. On the contrary, subsequent analyses of mutation patterns in the NS5B region in 81 patients showed a significant correlation between particular amino acid mutations of NS5B and the outcome of the combination therapy. Mutations of aa. 309, 333, 338 and 355 of the NS5B were significantly more frequent in SVR and ETR patients, in which the virus has been persistently or at least tempo-

rarily eliminated. Total numbers of mutations at the four amino acid positions were significantly more in SVR and ETR patients compared to NR patients (0.93 ± 0.89 *vs* 0.27 ± 0.70; $P = 0.0004$). These data suggest that particular amino acid mutations of NS5B-RdRp protein may confer sensitivity to combination therapy.

Recently, several studies on mutational analyses of HCV NS5B have identified several key residues responsible for its RdRp activity. Lohmann *et al.* noted that one single amino acid substitution in NS5B increased the efficacy of colony formation by 500-fold in HCV subgenomic replicon.[39] Cheney *et al.* noted that several amino acid substitutions (K155A, R168A, D225N and R386Q) were detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells.[40] Recently, Young *et al.* suggested that NS5B F415Y mutation in HCV-1a was a key resistant variant for RBV monotherapy.[33] However, Y415 is the consensus residue for all genotypes except for 1a and 6a. In the present study of three non-responders, there was no difference at NS5B Y415 between sera collected before treatment and sera collected 12 weeks after the start of treatment with combination therapy.

The locations of the four mutations within the calculated tertiary structure of NS5B RdRp are illustrated in Figure 6a,b. The mutations in NS5B, which were more frequently found in the SVR and the ETR patients, were clustered in motif B to E of RdRp. The amino acid 309 and 355 are both located on the enzyme surface of the substrate entry site. NS5B 333 and 338 are adjacent to the NTP tunnel (Fig. 6b). Because mutations found in HBV and HIV DNA polymerase/reverse transcriptase are known to be located on the surface of the catalytic domain, the mutations in HCV RdRp that were found in the present study may considerably affect their enzymatic activity. Our preliminary data have shown that the HCV subgenomic replicon carrying point mutations in aa. 141 in NS5B less efficiently than the original sequences. Further studies are needed to clarify the role of these point mutations in NS5B in determining the activity of RdRp.

A recent study by Crotty *et al.* has shown that direct antireplicative effects of RBV on viruses include 'error catastrophe' theory in which misincorporations of RBV triphosphate into the viral genome lead to accumulation of mutations in the viral genome and yield defective virus genome. Characteristic pattern of nucleotide mutations by RBV are an increase of G-to-A and C-to-U transition mutations.[17,18] In our present study, although the majority of the mutations were transitions, there was no significant difference in the ratios of the G-to-A and C-to-U mutations between IFN monotherapy and combination therapy (Table 1). One explanation for the discrepancy is that the concentration of RBV in clinical use is too low to act as a mutagen. The clinically achievable blood concentration of RBV is 10–30 μM.[41] On the contrary, an *in vitro* study of polio virus has shown that RBV concentration of 100 μM is required to increase the mutation frequency by at least 1.2-fold.[17] Highly mutated HCV can be excluded or escape detection by RT-PCR and minor clone of HCV quasi-species are excluded by direct sequence of nested PCR products. Therefore, although it is not clear whether RBV is a mutagen against viral genome, our results suggest other mechanisms of RBV contribute to suppress HCV replication, such as inhibition of enzymatic activities of viral RNA polymerase.

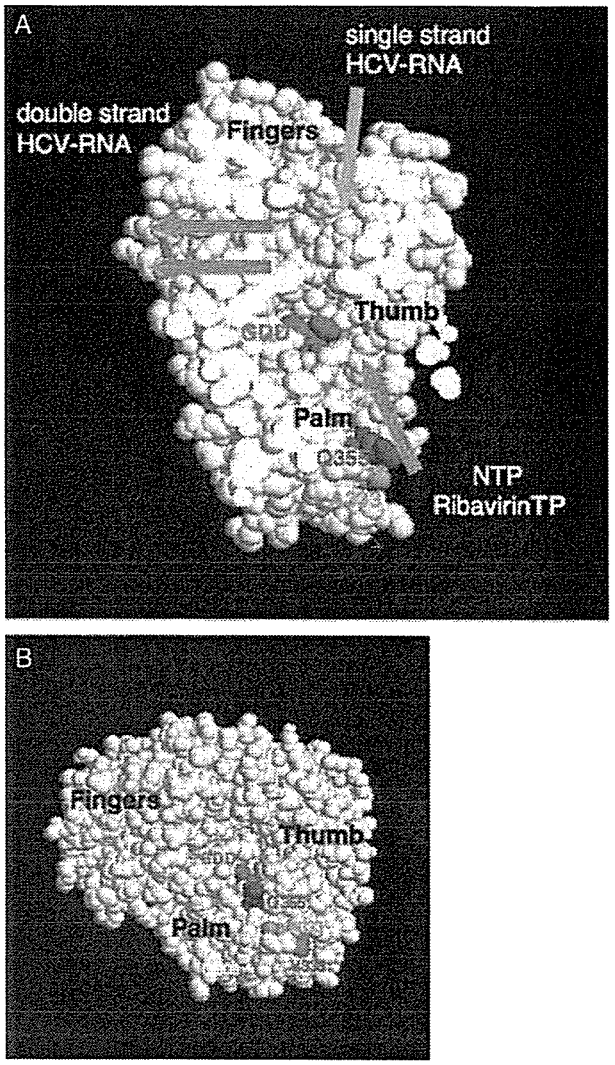

Figure 6 Crystal structure of the hepatitis C virus (HCV) NS5B-RNA dependent RNA polymerase (RdRp). The molecular model of NS5B was constructed using 1QUV from Protein Data Bank (PDB). A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. (a) Cross-section of the RdRp at level of nucleotide tunnels. The single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and the NTP or ribavirin enters the enzyme through the right lower dNTP tunnel (between β fingers and thumb). The essential GDD motif is shown in pink. NS5B 309, 333, 338 and 355 are shown in yellow, orange, green and red, respectively. (b) View from the dNTP entry site.

Many studies have endeavored to identify factors predictive of the outcome of IFN plus RBV combination therapy. Factors that have been examined include pretreatment clinical parameters such as baseline viral load, degree of fibrosis, and gender.[42] One study has

found early viral response (two-log decline of HCV RNA) to be predictive of SVR.[43] Another study showed that ISDR mutations were correlated with the SVR in chronic HCV 1b infection in Taiwan.[44] In the present study, multivariate analysis identified baseline ALT and the degree of fibrosis as independent factors for SVR. Further multivariate analysis showed that the number of mutations at positions NS5B 309, 333, 338 and 355 were independently associated with NR ($P = 0.0185$). The possible implications of our results are that the number of the above-described NS5B mutations is an independent predictive factor and that the parameter predicts NR patients exclusively from SVR or ETR patients. Our results which may enable prediction of NR before initiation of therapy might be of value when we consider indication for IFN plus RBV antiviral therapy or when making a decision about early cessation of the therapy, which may avoid possible side-effects and therapy costs. Although further studies of a larger population of patients are needed, the mutation number might be used to tailor therapy and is a useful factor for clinicians in making a clinical decision to stop treating HCV infection with combination therapy.

Given the absence of proven anti-HCV agents other than IFN and RBV, these combinations will continue to dominate therapy against HCV. Our present results provide evidence of a significant correlation between the response to IFN plus RBV combination therapy in patients with chronic HCV-1b infection and the amino acid changes that were present before therapy in conserved regions of NS5B. Certain amino acid changes in the HCV NS5B-RdRp domain may correlate with the clinical outcome of combination therapy and could thus be an initial predictor for response to IFN plus RBV combination therapy.

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Viral load change and sequential evolution of entire hepatitis C virus genome in Irish recipients of single source-contaminated anti-D immunoglobulin*

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SUMMARY. In hepatitis C virus (HCV) infection, serum viral load is important in the prediction of therapeutic efficacy. However, factors that affect the viral load remain poorly understood. To identify viral genomic elements responsible for the viral load, we investigated samples from a population of Irish women who were iatrogenically infected from a single HCV source by administration of HCV 1b-contaminated anti-D immune globulin between 1977 and 1978 (Kenny-Walsh, *N Engl J Med* 1999; 340: 1228). About 15 patients were divided into two groups, viral load increasing group (11 patients) and decreasing group (4 patients). Pairs of sera were collected from each patient at interval between 1.1 and 5.8 years. Full-length sequences of HCV genome were determined, and analyzed for changes in each patient. Sliding window analysis showed that the decreasing group had significantly higher mutation rates in a short segment of NS5B region that may affect the activity of RNA-dependent RNA polymerase. By comparing each coding regions, significantly higher mutation numbers were accumulated in NS5A region in the increasing group than the decreasing group (0.92 *vs* 0.16 nucleotides/site/year, $P = 0.021$). The mutation in certain positions of the HCV genome may be determinant factors of the viral load in a relatively homogeneous patient population.

Keywords: anti-D immunoglobulin, full genome sequence, hepatitis C virus.

INTRODUCTION

Hepatitis C virus (HCV) is globally distributed virus that causes chronic inflammation in liver, and may leads to liver cirrhosis and hepatocellular carcinoma over the course of 20–30 years [1–3]. Efficacy of anti-viral therapy based on pegylated interferon and ribavirin for chronic hepatitis C ranges between 40 and 80% [4–6]. Viral load is an important predictive factor for therapeutic outcome. We have previously demonstrated a close relationship between HCV genomic structures with patients' viral load [7], hepatitis activities [8,9], and ALT levels [10]. However, all of these observations were on the basis of heterogeneous hosts infected with different viral strains, making the role of the viral genetic structures ambiguous. A study using homogeneous host populations infected with a homogeneous viral strain would be ideal to clarify the role of viral gene in determination to the viral load.

There were two outbreaks of HCV infection in the world through usage of the virus-contaminated blood products from a single donor. One was in Ireland and one in Germany [11,12]. In Ireland, from May 1977 to November 1978, 704 women were iatrogenically infected with HCV through administration of a virus-contaminated anti-D immune globulin to prevent rhesus isoimmunization [12,13]. All recipients were female, had same ethnic origin, similar

*Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. There is no conflict of interests for present study.

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; LC, liver cirrhosis; HCC hepatocellular carcinoma; ALT, alanine aminotransferase; E, envelope; NS, nonstructural; PKR, protein kinase R; HLA, human histocompatibility leukocyte antigen; RT, reverse transcription; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction.

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duration of disease, infected with HCV genotype 1b from a single donor. In this homogeneous clinical setting, the amount of serum HCV has been shown to fluctuated [14]. HLA DR locus and DQ locus were shown to be associated with viral load in this patients group [15]. The HCV genomic determinants of the viral load set point are currently unknown.

The purpose of this study is to clarify the relationships between viral genetic structures and the serum viral load in this Irish patient population. We analyzed the full viral genomic sequences in each patient within the study population, and compared those patients whose had increased viral load with those who had decreased viral load within the time frame under retrospective investigation of approximately 20 years.

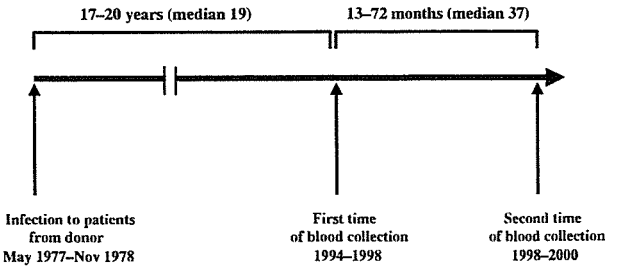

Fig. 1 Study design. A total of 30 sera were acquired from 15 patients. Each patient's sera were collected as part of the routine clinical management of chronic hepatitis C at Cork University Hospital, Ireland. Initial viral load assessment was approximately 19 years post-infection with HCV 1b contaminated anti-D immunoglobulin. The second samples selected for analysis in this study were dated to a further 6 years post-infection. Sequences were analysed by direct sequence from PCR generated amplicons.

PATIENTS AND METHODS

Patients

We determined the HCV sequence of donor's preserved serum (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916). In total, 15 Irish female patients infected with HCV-contaminated anti-D immunogobulin were used in this study, all of them have never been treated before enrolled into this study. About 25 patients were randomly chosen from the anti-D patients, DNA sequence analysis was attempted on all specimens, and 15 pairs of sera yielded the full length viral genomic sequence information. Each patient in the study population had two sera samples retrospectively analyzed from a biobank of specimens prospectively collected as a part of the routine clinical management and viral load quantification in this patient population at Cork University Hospital. The median temporal separation between samples was 39.2 months (13–72 months). All patients were infected by HCV genotype 1b from virus-contaminated anti-D immunoglobulin injections during the period from May 1977 to November 1978. All patients attended the hepatitis C clinic at Cork University Hospital, Cork, Ireland. Serum HCV-RNA levels were determined by a polymerase chain reaction assay (PCR assay; Roche HCV Monitor kit, F. Hoffmann-La Roche Ltd., Basel, Switzerland). The study design is shown in Fig. 1. Standard deviations have previously been reported [16], the 95% confidence interval of the viral load was ±0.032 viral copies/mL. A viral load increase or decrease over the range 0.062log10 viral copies/mL, was used as the criteria for change in viral load. The patients were classified into two groups. The characteristics of group one were as follows: the viral load was increased over time, $n = 11$, hence, increasing group (group-I). The second group of patients had a decreased or remained stable in viral load over the time investigated, decreasing group (group-D, $n = 4$). All patients were seronegative for HBV markers, had no autoimmune liver disease or competing risk factors such as excessive alcohol intake or hepatotoxic drugs. All patients had liver biopsy performed as part of their routine clinical assessment. Informed consent was obtained from each patient for liver biopsy, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Sera were stored at −80 °C storage facility.

RNA extraction and RT-PCR analysis

RNA extraction, cDNA synthesis and PCR analysis were performed as reported by us previously [17]. Briefly, serum RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). The extracted RNA was reverse-transcribed by moloney murine leukemia virus reverse transcriptase (MMLV-RT, GIBCO BRL) using random hexamers (Takara, Kyoto, Japan). The full-length HCV genome was amplified by the nested PCR with 21 partially overlapping sets of primers using Advantage cDNA Polymerase Mix (Clontech, California, USA) according to the manufacturer's instructions. 3′UTR and X tail were not uniformly amplified probably because of the condition of storage. Therefore, these regions were not analyzed in this study.

Sequence determination

Each PCR product was purified and residual primers were removed with the column (Suprec 02, Takara, Kyoto, Japan). Thereafter, both strands of the PCR products were cycle sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Tokyo, Japan) using forward and reverse sequencing primers, respectively. The products were purified by the column (Quickspin column, Boehringer Mannheim, Indiana, USA) and sequenced using an automated DNA sequencer (model 373S; Applied Biosystems, Tokyo, Japan). Each sequence

Table 1 Patients' basic characteristics

No.	Duration from infection (year)		Interval time (month)	Viral load (log_{10}kcopy/mL)		Rate change of viral load (log_{10}kcopy/mL/year)
	1st	2nd		1st	2nd	
1	18	22	29	5.62	7.41	0.741
2	19	22	33	5.48	7.28	0.655
3	17	22	31	4.64	6.27	0.633
4	20	23	37	6.18	7.59	0.459
5	17	20	45	4.38	5.94	0.416
6	17	23	56	5.23	6.88	0.354
7	20	22	69	5.46	7.15	0.293
8	17	23	37	4.71	5.15	0.203
9	20	22	39	4.82	5.38	0.173
10	17	19	72	5.38	5.91	0.089
11	19	22	26	4.96	5.04	0.052
12	19	23	18	5.36	5.36	0.000
13	21	22	24	5.00	4.89	−0.054
14	19	22	13	5.86	5.75	−0.099
15	19	22	59	5.46	4.55	−0.184
Group-I*	18.7 ± 0.3	22.2 ± 0.2	41.0 ± 4.8	5.19 ± 0.15	6.28 ± 0.27	0.37 ± 0.07
Group-D*	19.0 ± 1.1	21.0 ± 1.0	32.0 ± 13.9	5.44 ± 0.25	5.07 ± 0.36	−0.08 ± 0.04

*no. 1–11: group-I, no. 12–15: group-D.

Table 2 Patients' basic characteristics (continued)

No.	ALT value (microkat/L)		HLA DQB1 locus	HLA DRB1 locus	DR 51–53
	1st	2nd			
1	0.50	0.44	0201, −02/0501	01/03	53
2	0.98	0.91	0201, −02/0602, −11	03/15	51/52
3	0.65	0.70	Not typed	Not typed	Not typed
4	1.43	1.56	Not typed	Not typed	Not typed
5	0.72	0.51	0201, −02/0602, −11	0701/15	51
6	0.63	0.59	05031/05031	13/14	52
7	1.26	2.29	0201, −02/0602, −11	0701/15	51/53
8	0.72	0.75	0201, −02/03032, −06	03/0701	53
9	0.56	0.57	0201, −02/03032, −06	0701/0701	52
10	1.70	0.98	0201, −02/03011	03/04	Not typed
11	0.54	0.98	0301/0501	01/13	52
12	0.71	0.59	Not typed	Not typed	Not typed
13	0.32	0.43	Not typed	Not typed	Not typed
14	0.79	0.67	0301/0301	0701/11	52/53
15	0.69	0.64	0402/0604	08/13	52
Group-I†	0.91 ± 0.12	0.97 ± 0.16			
Group-D†	0.63 ± 0.11	0.58 ± 0.06			

Normal range of ALT value was 0.12–0.60 microkat/L.
†no. 1–11: group-I, no. 12–15: group-D.

was confirmed twice with direct sequencing method for sense and anti-sense strands'. Subcloning study was not done, therefore HVR were not mentioned in the present study. HLA profile was available for 11 individuals study population and was performed as outlined previously reported [16].

Statistical analyses and phylogenetic analysis

For phylogenetic analysis, the nucleotide and deduced amino acid sequences of the patients were compared with a sequence of HCV-J strain [18] as a reference. The nucleotide and amino acid sequences were compared longitudinally in each patient. Amino acid changes were picked up between the paired samples from each patient. Serum viral load changes were defined as increasing status when they were above zero, and defined as decreasing status when equal to or under than zero. Calculation of amino acid mutation rates and the phylogenetic analyses were performed by the Mega software version 2.1 with neighbour-joining method (NJ) [19], and PHYLIP version 3.62 with maximum likelihood method (ML) [20]. NJ tree and NG distances were evaluated using 1000 bootstrap samples, *P* values for the branches of the ML tree were also calculated. Nucleotide mutation rates were calculated by the Mega ver.2.1. Statistical analyses of the two groups were done by Mann–Whitney's *U* test using the program Stat View 5.0 (SAS institute inc.). All tests of significance were two-tailed, with *P* values of <0.05 considered to indicate statistical significance.

RESULTS

Characteristics of patients

The clinical characteristics were compared between the two groups, 11 patients with increasing viral load status (group-I) and 4 patients with decreasing status (group-D), as shown

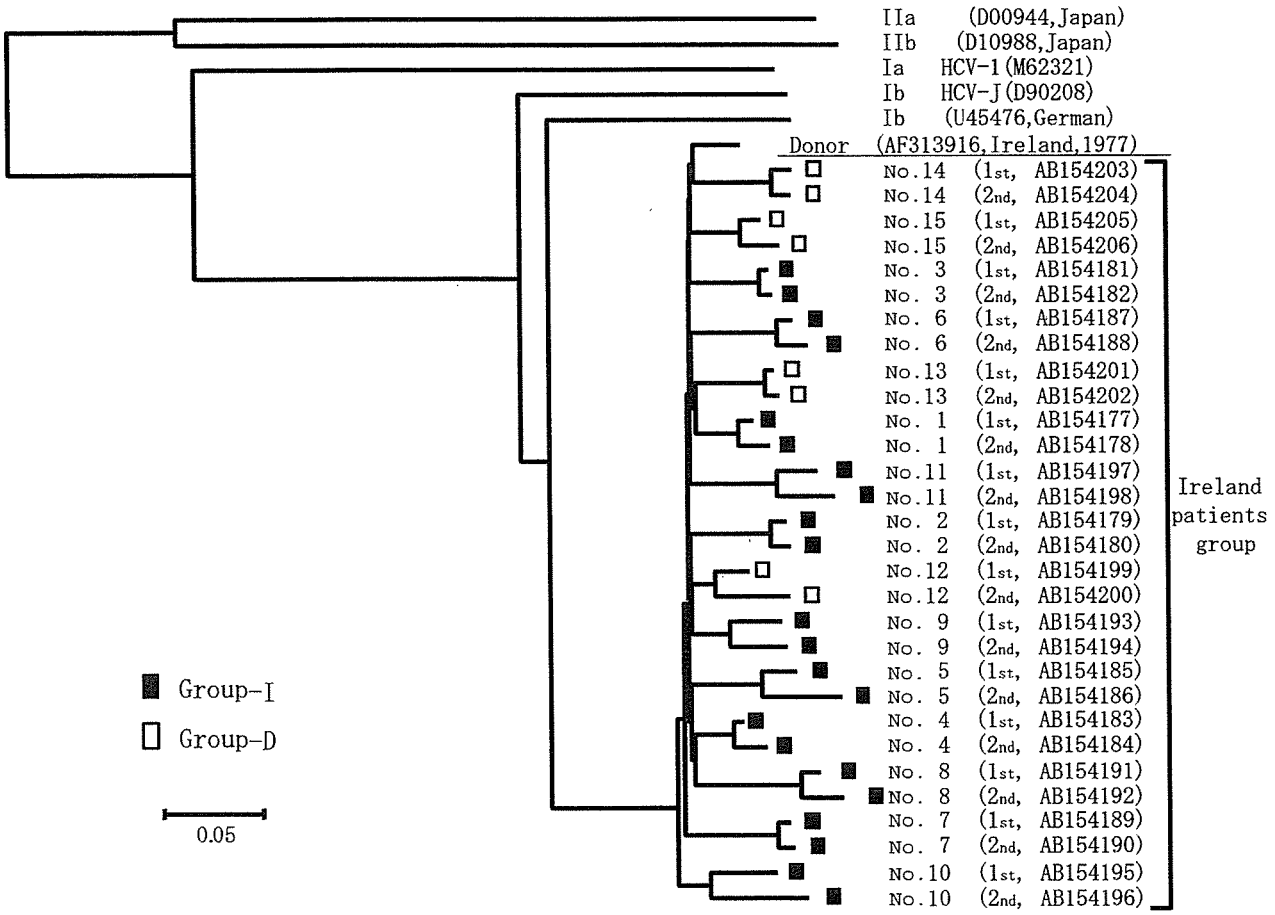

Fig. 2 Phylogenetic analysis with entire amino acid sequences of anti-D recipients, the donor, and representative sequences of genotype 1b, 2a and 2b. A phylogenetic tree was constructed by the neighbour-joining tree method with 10 000 bootstraps supports. The donor-sequence is shown in the 6th from the top. Closed squares show the recipients sequences belonging to the viral load increasing group (group-I), and open squares show them belonging to the decreasing group (group-D), and the bar at the left below is the reference for the distance which length is 0.05. Patients' numbers are matched with Table 1, and ordinal numbers after patients' number indicates the order of collection of serum from each patient. The mutations cluster in one group involving donor's sequence, but are individual to each patient. The distances from donor's sequence to patients' sequences did not segregate with change in viral load. The average distance from donor's sequence are as follows: to 1st acquired serum, 0.058 ± 0.0028 in group-I and 0.051 ± 0.0034 in group-D, and to 2nd, 0.065 ± 0.0030 in group-I and 0.060 ± 0.0021 in group-D, and within each pairs, 0.028 ± 0.0060 in group-I and 0.024 ± 0.0080 in group-D.

in Table 1. No significant difference could be found in age, time intervals between the two-blood sampling of each patient, and the intervals between the time on infection and the first blood sampling. The amounts of serum HCV-RNA were not significantly different between the two groups at first time of blood collection (5.17 ± 0.16 $\log_{10}$kcopy/mL in group-I, 5.42 ± 0.18 $\log_{10}$kcopy/mL in group-D, respectively). The viral load was significantly higher in the group-I at the second serum samples (6.37 ± 0.29 $\log_{10}$kcopy/mL in group-I, 5.14 ± 0.26 $\log_{10}$kcopy/mL in group-D, $P = 0.027$). The mean rate of change was significantly different between two groups (0.37 ± 0.07 Log_{10}kcopy/mL/year to group-I, −0.08 ± 0.04 $\log_{10}$kcopy/mL/year to group-D, $P = 0.004$). Average ALT values were not different between both the groups (Table 2).

Phylogenetic analysis

To clarify the tendency of genomic changes of HCV in each patient (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916 for donor sequence, AB154177 to AB154206 for recipients), phylogenetic analyses were done based on the entire amino acid sequence by the neighbour-joining tree method (Fig. 2). The phylogenetic tree analysis showed that all sequences studied in the present study belonged to genotype 1b cluster (100% bootstrap support, $P < 0.01$ in the maximum likelihood tree), and they were more closely related to the donor's sequence than any other known 1b sequences (100% bootstrap support, $P < 0.01$). The two sets of sequence data derived from the each patient segregated together on the phylogenetic tree (100% bootstrap support, $P < 0.01$). The genetic distance between each specimen of each pair was, as anticipated, less than the distance from donor's sequence (100% bootstrap support, $P < 0.01$). There was no relationship between the genetic distance calculated with amino acid sequence and time intervals of recipient's serum samplings or viral load fluctuations. This suggests that the genetic evolution speed was different among patients independent of viral loads.

Mutations from donor to recipients

We initially analyzed the average rate of HCV amino acid sequence mutation between the donor and the pair of samples from each recipient (Fig. 3). Comparison of the number of mutations between group-I and group-D in the open reading frame revealed no significant differences. Further analyses restricted to small functional regions including PKR eukaryotic initiation factor-2α phosphorylation homology domain, ALT response related element, PKR-binding domain, interferon sensitivity determining region (ISDR), nuclear localization signal and variable region 3 region showed no difference in mutations number in both groups (raw sample data not shown).

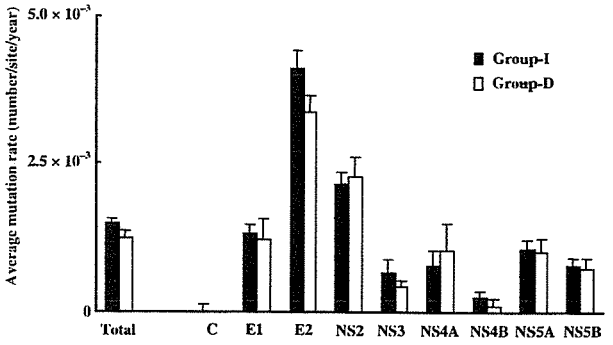

Fig. 3 Average rates of amino acid mutations from donor's sequence to recipients'. The mutation rates are those calculated from the first acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations in both groups indicated that there are differences between regions, but the analysis did not achieve significance.

Genomic analysis of paired sera

The average rates of mutations in amino acids for the entire HCV genome in group-I were similar to that in group-D ($3.4 \times 10^{-3} \pm 0.6 \times 10^{-3}$ numbers/site/year in group-I and $3.3 \times 10^{-3} \pm 1.2 \times 10^{-3}$ numbers/site/year in group-D). A comparison of each coding region revealed that the average number of mutations was significantly higher in group-I than group-D in NS5A ($2.07 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year *vs* $0.36 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year in average, $P = 0.02$) (Fig. 4). Individual points of mutations in each patient were aligned as outlined in Fig. 5. The majority of the mutations clustered at E2, NS5A and NS5B in each patient. However, no specific position was found to be unique to group-I or group-D.

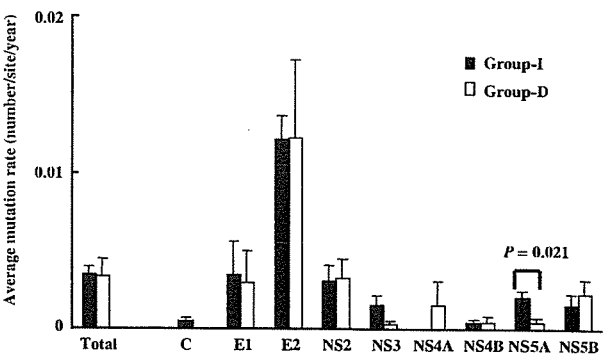

Fig. 4 Average rates of viral sequential mutations within each recipients belonging to two groups. The mutation rates are those calculated from the first to second acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations indicated that there are defined differences in the observed mutation frequency between the group-I and group-D for NS5A. This difference was significantly different ($P = 0.021$).

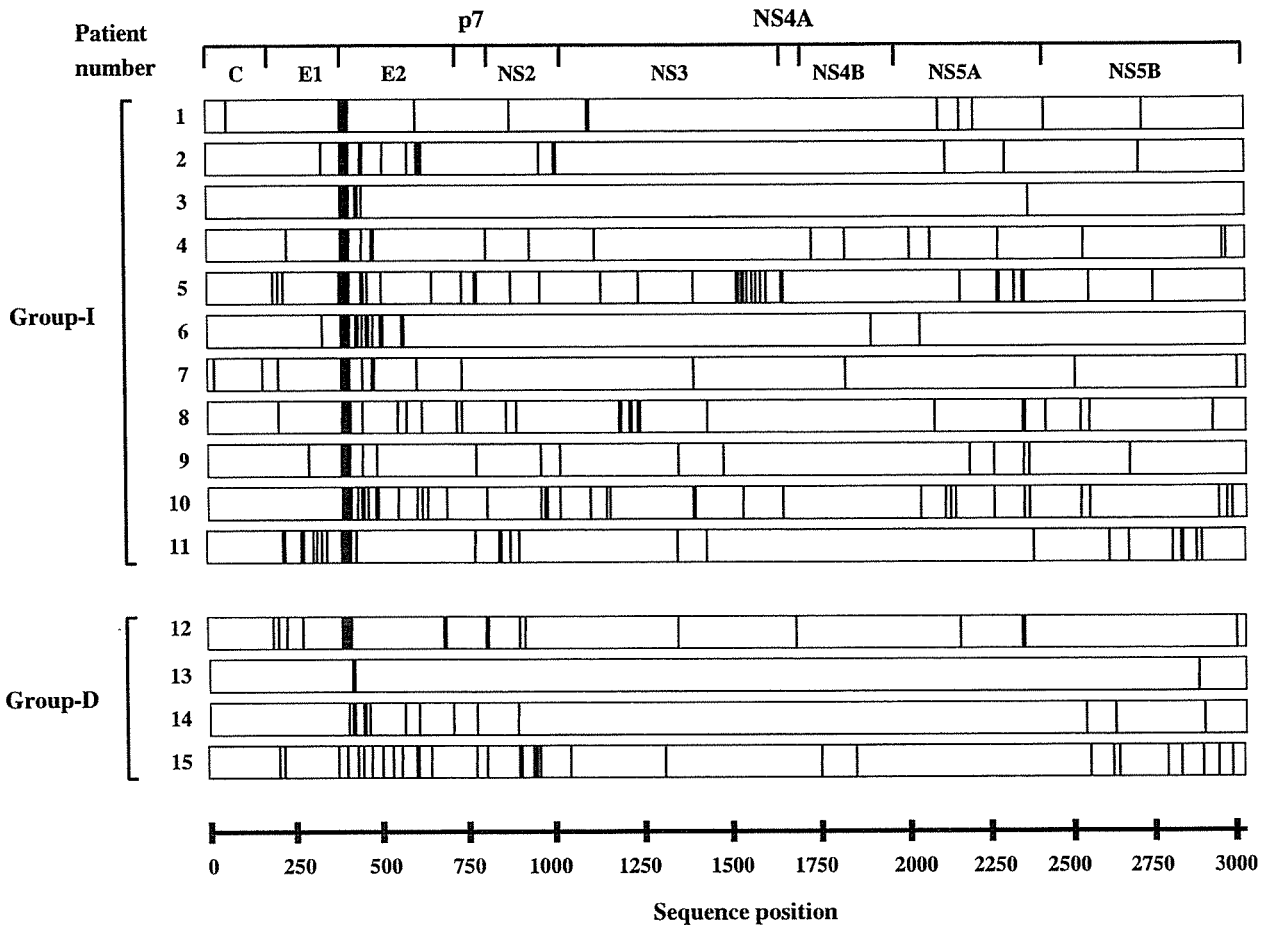

Fig. 5 Plots of mutations in full sequence in each recipients. Every mutation of amino acids has plotted on the bar for each patient. No specific point mutation was detected that was associated with change in viral load.

Sliding-window analysis

We analyzed the sequence data using a method that we previously reported [21], to find any accumulation of mutations related to viral load change across the entire sequences. We summated the total number of mutation recognized in each window constructed by 10 amino acids among all the sequence-pairs after corrected by the observation period (Fig. 6a), and compared by Mann–Whitney's *U* test (Fig. 6b). A correlation between viral load change and NS5B revealed, by collapsing the window to 2 amino acids, at position 2508–2509. These results are in agreement with those published by Qin *et al.* [22]. Qin suggested that mutations within this region have been associated with reduced NS5B activity [22].

Synonymous and nonsynonymous mutations and the molecular clock analysis

To analyze the profile of the nucleotide mutations underling the amino acid changes, we examined the synonymous and the nonsynonymous mutations of each sequence generated from this population with Nei–Gojobori model and Jukus–Cantor method. Ratios of the genetic distance of the nonsynonymous changes (d*N*) per distance of the synonymous changes (d*S*) were calculated for the respective regions as well as the entire HCV genome (Fig. 7a). High *dN*/*dS* ratio reflects the immune selective pressure, and the low *dN*/*dS* reflects the speed of genomic evolution which is dependent on the fidelity of viral genomic replication [23]. The comparison of the *dN*/*dS* ratio showed that there was no overall difference between group-I and group-D.

In addition, we investigated the viral mutational rate in each patient. Averaged mutation rates of each genome were calculated by dividing distances of synonymous changes (d*S*) by the time interval between paired specimens. The viral mutational rates of both groups were almost same across the HCV genome. The rate of mutation between the donor sequence and (1) the first sera of each pair were $2.81 \times 10^{-3} \pm 0.51 \times 10^{-3}$ nucleotides/site/year in the group-I, $2.75 \times 10^{-3} \pm 0.34 \times 10^{-3}$ nucleotides/site/year in the group-D, and (2) the second sera of each pair were $2.77 \times 10^{-3} \pm 0.49 \times 10^{-3}$ in the group-I, $2.72 \times 10^{-3} \pm 0.36 \times 10^{-3}$ nucleotides/site/year in the group-D. The average mutational rate for the entire HCV genome during

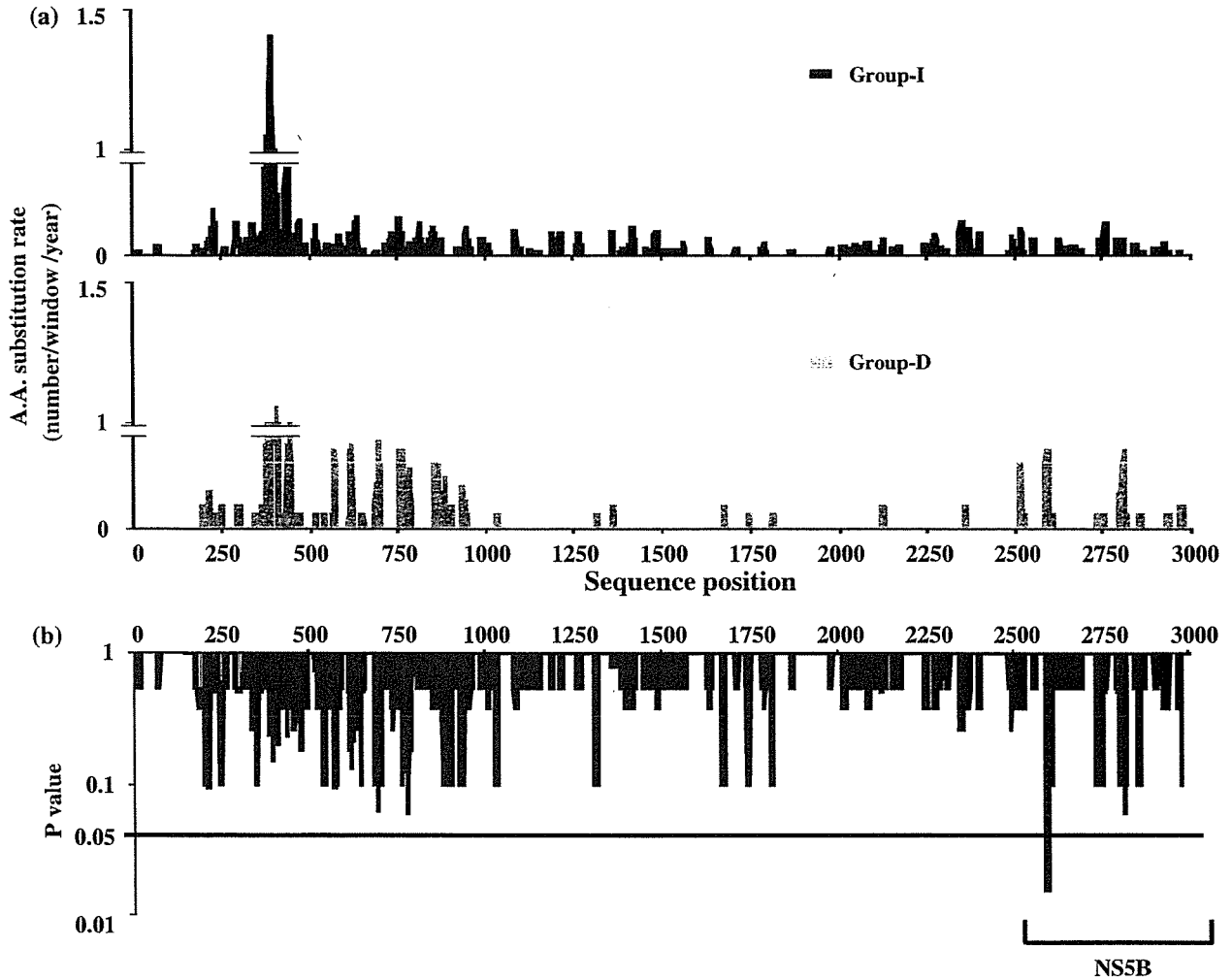

Fig. 6 Sliding-window analysis in full sequences. (a) Average mutation rates in every window are plotted according to the groups. Each window constructed with 10 amino acids. In NS5B region, calculated annual mutation numbers were high in group-D than group-I. (b) The results of statistical analysis are plotted. Longitudinal axis is appeared in logarithmic scale, areas under the bar of 5×10^{-2} indicate statistical significance. Mutation rates of two groups in E2 and NS5B region, showed difference in (a), have significance when tested by Mann–Whitney's U test ($P = 0.015$).

the two sampling points was as follows: $8.20 \times 10^{-3} \pm 2.0 \times 10^{-3}$ nucleotides/site/year for group-I and $10.0 \times 10^{-3} \pm 3.4 \times 10^{-3}$ nucleotides/site/year for group-D, respectively (Fig. 7b). The mutational rate of the viral genome did not differ between the two patient groups examined.

DISCUSSION

In the present study, we aimed to clarify the relationship between amino acid substitutions of HCV and serum viral load in Irish women, who were infected by an HCV-contaminated serum from a single donor during a period between 1977 and 1978. We analyzed full sequences of HCV derived from the patients whose viral load set point have increased (group-I), and those with patients whose viral load set point has decreased (group-D). Serum ALT values and nucleotide mutation rates were not significantly different between the two groups. These results suggested that the mutation pressure is almost equal in across the two groups. Specific points of substitution directly related to the viral load were not found. However, significantly more substitution changes of amino acid were observed in NS5A in group-I when analyzed at the level of the polyprotein. Analysis using a sliding windows method revealed that the numbers of mutations in a short segment in the NS5B region was significantly higher in group-D than in group-I. These regions of NS5B are closely related to the position that is reported to be important to RNA-dependent RNA polymerase (RdRP) activity [22]. These results suggest that the determinants of hepatitis C viral load include, at least in part, virological factors.

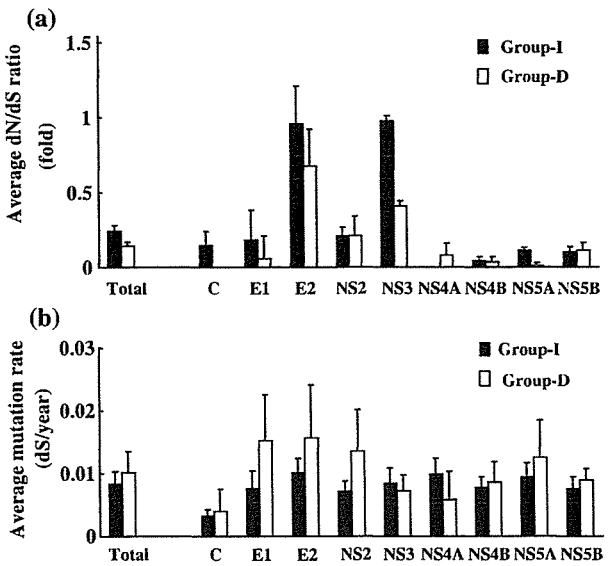

Fig. 7 The analysis of selection bias and mutation rates in RNA level. (a) Distance of nonsynonymous changes (d*N*) per distance of synonymous changes (d*S*) ratio of each coding region in comparison with two groups. Only in NS5A is the ratio significantly different between the two groups. (b) Mutation rate calculated by d*S*/interval time. There were no significant rate differences between two groups.

Clusters of mutations within the NS5B region of the genome were evident in this study group. The RdRP coded by NS5B contains conserved amino acid sequence motifs which essential for RdRP activity [24–26]. The mutation profiles correlated to viral load in the present study were not within these conserved motifs. However, Qin *et al.* and Labonte *et al.* reported that point mutations outside of this latter NS5B motif caused a change in the RdRP activity [22,27]. Qin *et al.* showed that the mutation of amino acid no. 191 of NS5B, co-incident with the region identified in the study reported here, lowered the RdRP activity from 100 to 3%. Furthermore, all mutations identified in our present study altered the polarity of amino acids, and the mutations in this site were only found in group-D. The mutations in the region may affect the replication capacity of the virus resulting in changes in the serum viral load. Further study will be needed which assess the relationship between the RdRP activity of the wild type HCV and those HCV with mutations observed in this study.

We found a significantly different pattern of the mutations in NS5A regions between group-I and group-D. NS5A possess transcriptional activator properties [28,29], and also considered to affect to virus–host interaction [30]. NS5A protein has been reported to bind to cellular RNA-dependent protein kinase R (PKR), a protein activated by double stranded viral RNA. The NS5A inhibition PKR prevents the down regulation of protein translation [31,32], mutations in ISDR, which within PKR-binding domain, have been shown to effect the efficacy of interferon based anti-viral therapy [17,33]. Several researchers have previously identified a correlation between ISDR mutation and viral load [7,17,34–36]. However sequence of ISDR were highly conserved in the present study. In addition, no such relationship was found to exist in a longitudinal study of patients which were followed 2 years [37,38], or in patients with normal ALT levels [39]. These three studies suggest that ISDR is not mutation-prone region. The role of transcriptional activator properties or affection to interaction may explain the different mutation trends found between two groups in this study.

A unique feature of the anti-D patients analyzed in this study is that they are infected with a single strain genotype, and that they are young and may have an immune system that possess different potency to influence the interaction between host and virus than other less homogeneous cohorts. Seven hundred and four individuals were identified as having being iatrogenically infected through HCV 1b contaminated anti-D immunoglobulin. Three hundred and ninety of these women were viremic [12]. Three hundred and seventy six patients had been evaluated for the clinical outcome in 1997; 55% of them had evidence of elevated alanine aminotransferase. The disease progression in this group is slower than the dogma relating to the natural progression of chronic hepatitis C. Only 7 patients had evidence of cirrhosis on the liver 17 years post-infection. Fanning *et al.* have previously reported an association between HCV RNA titers and the degree of inflammation, and between the degree of inflammation and serum ALT levels [40], but Creedon *et al.* reported no association between viral load and the progression of disease [41]. HLA class II data previously generated on this group was analyzed in an attempt to identify any association between host HLA and modulations in viral load [16,42]. HLA class II, has previously been reported to be associated with changes in viral load. The number of individuals examined does not give this study the power to fully address this question, however all of the patients with the HLA DRB1 15/DQB1 0602 phenotype belonged to the increasing group.

Two previously published studies which investigated this cohort of Irish women infected by contaminated anti-D immune globulin, estimated the mutational rate to be 2×10^{-3} synonymous substitutions per site per year by using Core E1/E2 and NS5 region sequences [43,44]. Other researcher reported that the molecular clock of HCV in Japan and USA are ranged in $1–2 \times 10^{-3}$ synonymous substitutions per site per year [45]. In the previous Japanese investigations of the entire HCV genome in all but one individual, the mutational rates were calculated about 2×10^{-3} base substitutions per site per year in human and chimpanzee over a 10 year period [46,47]. The mutation rates calculated with the first and the second samples in the present study are likely to be over estimated in comparison with those described in the previous investigations. The mutational rates between the donor sequence and the patients' samples used in this study, approximate that of these other studies [43–47]. In fact,

Allain *et al.* reported the evolutionary rate of HCV genome between blood donor and recipient, the range were 3.4×10^{-4} to 4.51×10^{-3} nucleotide substitutions per site per year, which were close to the data presented here [48]. The temporal difference between the first and second samples is too small to determine the mutation rate of the viral genome accurately.

In conclusion, we have shown that the hepatitis C viral genomic mutation patterns are associated with changes in viral load in this patient group infected from a single source. Interestingly, specific small regions within NS5B were identified as associated with changes in serum viral load. Mutations in NS5A regions were correlated with the viral load, when analyzed from the viewpoint of each polyprotein as a unit. These results suggest that the viral genome composition is a determinant of the set point of viral load for the hepatitis C virus.

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Journal of Gastroenterology

Editorial

Genetic changes in the interferon sensitivity-determining region of hepatitis C virus (HCV) during the natural course of infection: an implication for the gene function in the role of chronic infection

Article on page 43
Nonstructural 5A gene variability of hepatitis C virus (HCV) during a 10-year follow up
FAN W, ZHU W, WEI L, et al.

The interferon sensitivity-determining region (ISDR; aa2209–2248 of HCV-J) in the nonstructural 5A region (NS5A) of hepatitis C virus (HCV) was originally identified as the genomic functional element wherein missense mutations were closely related to the clinical efficacy of interferon treatment, as well as to serum viral loads, in genotype-1b HCV infection.[1,2] After the first reports of the ISDR, controversy arose as to its predictive value for the outcome of interferon therapy, because clinical studies in Europe and North America did not always support the relevance of ISDR,[3] although most studies in Japan, Spain, and Italy supported it.[4,5] However, recent meta-analyses have clearly supported the universal correlation between ISDR sequence and interferon resistance.[6] It is speculated that the initial discrepancy of the results might have been caused by differences in interferon regimens and patient sources.

After identification of ISDR as the key genomic element for interferon efficacy and viral replication, the molecular function of NS5A protein and its relevance to ISDR structures has been vigorously and intensively studied using NS5A protein expression in vitro or in transgenic mice. A variety of putative NS5A functions were postulated, such as binding to cellular protein kinase R (PKR),[7] TRADD,[8] Grb-2,[9] p21,[10] hVAP-33,[11] or other proteins that may influence the pathogenesis of hepatitis C by antiviral effects, apoptosis, signal transduction, cell cycle regulation, or formation of viral replication complex. Much attention has been paid to PKR, because NS5A protein was found to block the antiviral effect of PKR in an ISDR sequence-dependent manner by directly binding to PKR through the so-called PKR-binding domain, which includes the ISDR plus an additional 26 aa stretch located at the C-terminal portion (aa2209–2274). The recently developed HCV subgenomic replicon system also disclosed the importance of NS5A proteins in intracellular viral replication, because specific mutations called "cell culture-adaptive mutations" needed in its genome for efficient replication in cultured cells clustered in the central region of NS5A, particularly in the serine cluster region immediately upstream to the ISDR or the ISDR itself.[12,13] Because these mutations possibly affect phosphorylation of NS5A proteins, the role of phosphorylated NS5A protein in viral replication and interferon sensitivity has become the recent target of molecular research.

How does the HCV–ISDR structure change in a host during the natural course of disease? The answer to this question should give us important clinical information about when to start interferon therapy, whether earlier or later in HCV infection. In the current issue of the *Journal of Gastroenterology*, Fan et al.[14] report the genetic evolution of the *NS5A* gene during a 10-year follow-up of natural HCV infection in 7 patients, focusing on ISDR, PKR-binding domain, serine cluster region, and other functional domains in *NS5A* gene. To investigate changes of the genetic variability during the natural course, they performed subcloning analysis. As a result, serine residues at positions 2194, 2197, 2201, and 2204 in the serine cluster region, suggested to be important for hyperphosphorylation of NS5A protein, were highly conserved in all patients and within the quasipecies of each patient, suggesting that phosphorylation plays the crucial role in NS5A protein function. Meanwhile, subcloning analysis of the ISDR quasispecies disclosed that the wild-type ISDR (no aa substitution relative to HCV-J), or the intermediate-type ISDR (one to three substitutions) was dominant and stable throughout the observation periods in all patients. However, the ISDR quasispecies decreased over time, and the quasispecies had a tendency gradually to converge to the wild-type ISDR.

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Before the report by Fan et al., several studies already had been conducted for analyzing the natural genetic change of the ISDR.[15–19] Although these previous studies also demonstrated that the wild-type ISDR or the intermediate-type ISDR was generally stable, the observation periods were rather short, and the quasispecies complexity was not investigated in most of the studies. Importantly, Fan et al. demonstrate that the ISDR quasispecies finally tended to converge to the wild-type ISDR with a decrease of the quasispecies complexity, indicating that sequence motif of the wild-type ISDR had a crucial role functionally in establishing chronic HCV infection. Although the mutant-type ISDR (four or more aa substitutions) was not included in the study, the mutant-type ISDR was reported to be rather unstable, because nonsynonymous mutations (63%) were higher than synonymous mutations (37%), indicating that strong selective pressure of the host was exerted on the mutant-type ISDR.[16] This finding coincides with the results by Fan et al. that the HCV of wild-type ISDR ultimately survives in the course of chronic infection.

If the quasispecies complexity of the ISDR subtypes finally converges to the wild-type ISDR, it might be better to start interferon therapy early for chronic HCV infection. It is not clear why the mutant-type ISDR is unstable in a host. Part of the mechanism, however, might be explained by different interaction with cellular proteins, such as PKR induced by endogenous interferon. On the other hand, if the mutant-type ISDR was unstable and easily defeated by the wild-type ISDR in chronic infection, this weak subtype might have disappeared in the world of HCV infection. However, although the distribution is rather small, the mutant-type ISDR is still frequently found in clinical samples. Is it on the way to disappearing? Or does it have an advantage over the other types in a certain phase of infection other than the chronic phase? Recent advances in understanding of the innate immune system have disclosed that mammalian cells have two distinct innate immune pathways protecting cells from the virus: the interferon regulatory factor (IRF) system, and the interferon (IFN)—signal transduction and activator of transcription (STAT) system. In the phase of chronic infection, the IFN–STAT system might have a dominant role in viral suppression, and the wild-type ISDR is supposed to inhibit this pathway, giving a survival advantage to HCV with the wild-type ISDR. In contrast, the IRF system might be dominant in the phase of acute infection. Does the mutant-type ISDR inhibit this IRF pathway, and give a survival advantage to HCV with the mutant-type ISDR? The answer to this question requires further study, but these analyses might go far toward helping understand the mysterious function of the ISDR.

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SPECIAL ARTICLE

Consensus Proposals for a Unified System of Nomenclature of Hepatitis C Virus Genotypes

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International standardization and coordination of the nomenclature of variants of hepatitis C virus (HCV) is increasingly needed as more is discovered about the scale of HCV-related liver disease and important biological and antigenic differences that exist between variants. A group of scientists expert in the field of HCV genetic variability, and those involved in development of HCV sequence databases, the Hepatitis Virus Database (Japan), euHCVdb (France), and Los Alamos (United States), met to re-examine the status of HCV genotype nomenclature, resolve conflicting genotype or subtype names among described variants of HCV, and draw up revised criteria for the assignment of new genotypes as they are discovered in the future. A comprehensive listing of all currently classified variants of HCV incorporates a number of agreed genotype and subtype name reassignments to create consistency in nomenclature. The paper also contains consensus proposals for the classification of new variants into genotypes and subtypes, which recognizes and incorporates new knowledge of HCV genetic diversity and epidemiology. A proposal was made that HCV variants be classified into 6 genotypes (representing the 6 genetic groups defined by phylogenetic analysis). Subtype name assignment will be either confirmed or provisional, depending on the availability of complete or partial nucleotide sequence data, or remain unassigned where fewer than 3 examples of a new subtype have been described. In conclusion, these proposals provide the framework by which the HCV databases store and provide access to data on HCV, which will internationally coordinate the assignment of new genotypes and subtypes in the future. (HEPATOLOGY 2005;42:962-973.)

Abbreviations: HCV, hepatitis C virus; IDU, injection drug user; RF, recombinant form; ICTV, International Committee for the Taxonomy of Viruses.

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