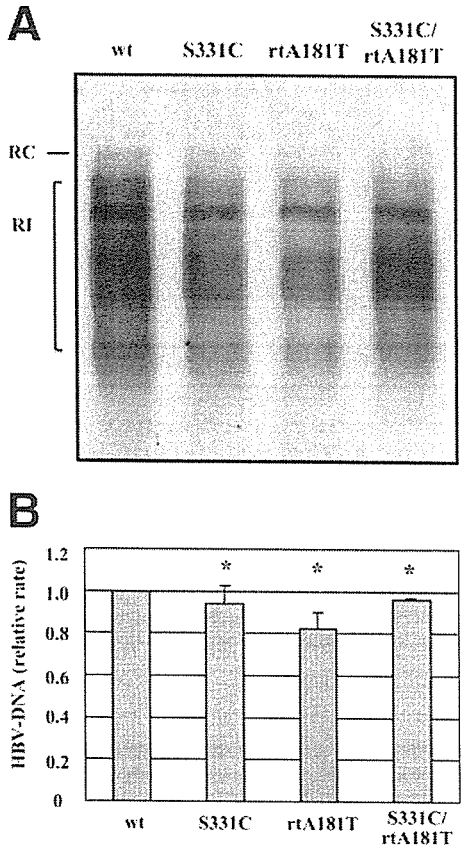

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means ± SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase (polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

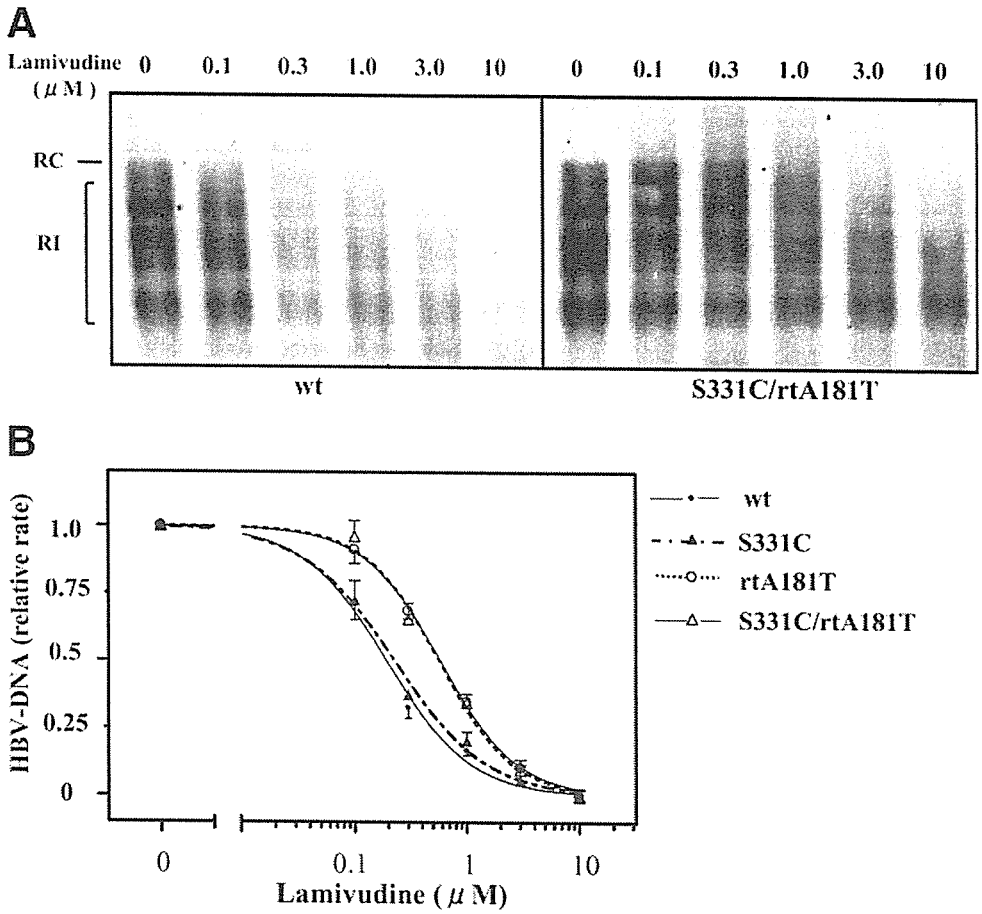

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50}s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means ± SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

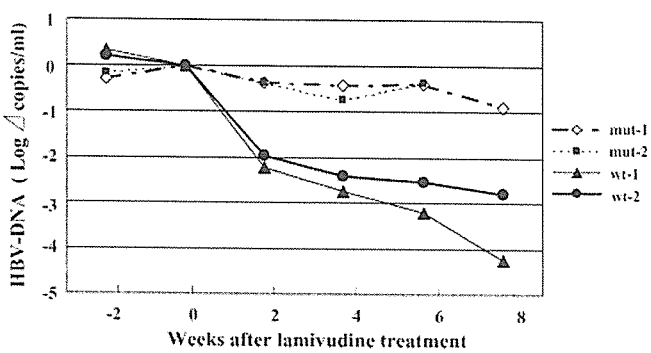

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50}s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir[a]

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC_{50} (μM)	Resistance (fold)	IC_{50} (μM)	Resistance (fold)	IC_{50} (nM)	Resistance (fold)
WT	−/−	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

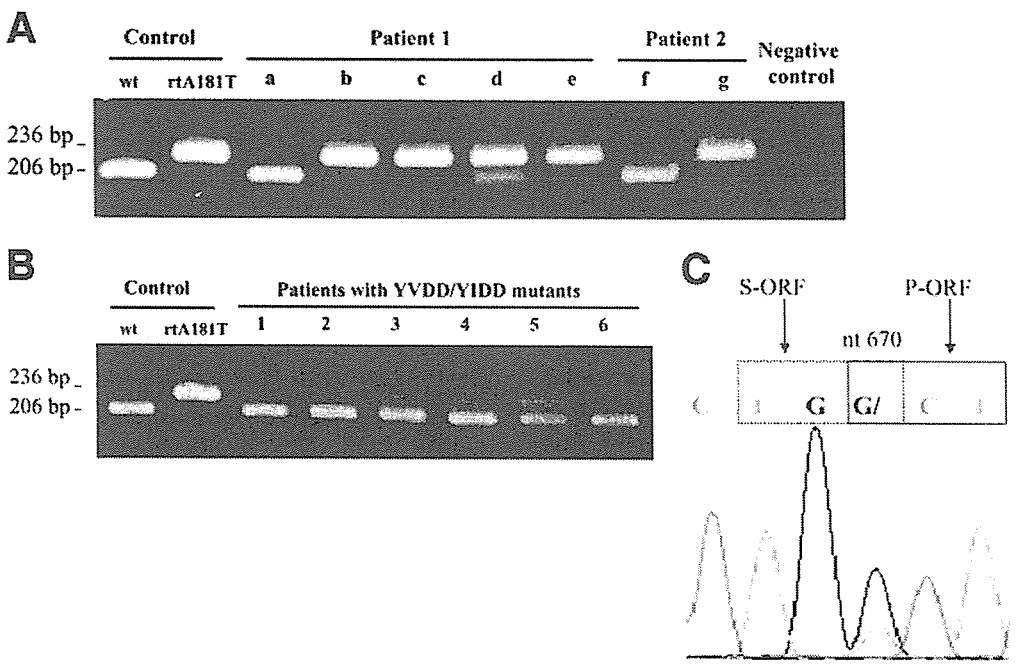

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses. f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type–mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

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Evolution of Hepatitis C Virus Quasispecies during Ribavirin and Interferon-Alpha-2b Combination Therapy and Interferon-Alpha-2b Monotherapy

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Key Words
Hepatitis C virus quasispecies · Viral resistance · Error catastrophe · Chronic hepatitis C virus infection · Ribavirin

Abstract
Objective: Ribavirin and interferon combination therapy is more effective than interferon monotherapy in patients with chronic hepatitis C virus (HCV) infection. To test the hypothesis that ribavirin induces nucleotide substitutions in the viral genome and reduces viral load by forcing it into error catastrophe in the combination therapy, we investigated the molecular evolution of HCV quasispecies in 3 patients who received combination therapy and 2 patients who received interferon monotherapy. ***Methods:*** The quasispecies were analyzed before and after therapy by sequencing at least 8 clones in five regions of the HCV genome; 5′ untranslated region, E1, E2, NS5A and NS5B. ***Results:*** Marked genetic drift was observed in the NS5A and NS5B regions in patients treated with combination therapy. However, genetic distances between clones obtained after therapy were closer than those obtained before therapy. ***Conclusion:*** Our results suggest that the combination therapy modified HCV quasispecies, but that this did not reflect the induction of error catastrophe by ribavirin. Modification of quasispecies by this therapy requires further investigation in a larger number of patients to elucidate the possible mechanism of viral resistance against the combination therapy.

Introduction

Hepatitis C virus (HCV) infection is a serious health problem worldwide [1–4]. Ribavirin and interferon (IFN) combination therapy induces a significantly higher response rate than IFN monotherapy as shown in recent randomized studies [5–7]. McHutchison et al. [5] and Poynard et al. [6] studied patients with chronic hepatitis C who had not been treated previously, and Davis et al. [7] studied patients with chronic hepatitis C who relapsed after IFN treatment. They reported that the rate of sustained virological response was higher among patients who received combination therapy (31–49%) than among patients who received IFN monotherapy (5–19%).

The mechanism of action of ribavirin is not clearly understood; however, various possible mechanisms have been proposed including: (1) ribavirin inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH)

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Table 1. Clinical and virological characteristics of the patients studied

Patient	Sex	Age years	Histo-pathological staging	Geno-type	Viral load, kIU/ml		
					pretreatment	4 weeks	end of treatment
IFN plus ribavirin therapy							
1	M	60	1[a]	1b	>850[b]	<0.5[b]	<0.5[b]
2	M	56	1[a]	1b	>850[b]	420[b]	450[b]
3	M	35	2[a]	1b	>850[b]	57[b]	190[b]
IFN therapy							
4	M	51	1[a]	1b	>850[b]	64[b]	(+)
5	M	57	1[a]	1b	>850[b]	>850[b]	>850[b]

[a] Staging of chronic hepatitis by Desmet et al. [21].
[b] Viral load was measured by the Amplicor HCV Monitor assay (version 2.0) (Roche, Tokyo, Japan).

and reduces the guanosine triphosphate (GTP) pool in hepatocytes; (2) ribavirin induces a T cell helper (Th)2 to Th1 bias in favor of a host antiviral response via either cytotoxic T lymphocytes (CTLs) or Th1 cytokines; (3) ribavirin inhibits HCV NS5B-encoded RNA-dependent RNA polymerase (RdRp), and (4) ribavirin acts as an RNA mutagen [for review, see 8]. Crotty et al. [9, 10] hypothesized that the antiviral effect of ribavirin is due to induction of nucleotide substitutions in the genome of RNA viruses forcing them into error catastrophe. They used a polio virus system to investigate the effect of ribavirin and demonstrated induction of nucleotide substitutions in the viral genome [9, 10].

The effect of ribavirin on HCV was examined using a replicon system [11, 12]. Contreras et al. [11] assayed mutation frequencies using a replicon system, and reported that ribavirin broadly increased error generation, particularly in otherwise invariant regions (5′ UTR and core). However, to our knowledge, no data are available about the effect of IFN and ribavirin combination therapy on HCV in humans. Sookoian et al. [13] investigated HCV quasispecies by SSCP analysis in hypervariable regions in patients who received ribavirin monotherapy, but they did not analyze nucleotide sequences or quasispecies. In the present study, we determined the HCV quasispecies in patients who received combination therapy of IFN-alpha-2b and ribavirin or IFN-alpha-2b monotherapy. We investigated five conserved and variable regions of the HCV genome including the 5′ untranslated region (UTR), EI, E2 (HVR1), NS5A and NS5B regions. The 5′ UTR was chosen because it plays important roles in key processes in viral infection such as replication of the viral genome and translation of viral protein. The E1 and E2 regions were also selected because they are variable regions as targets of the humoral immune response [14–16]. The NS5A region was studied because of its putative implication in IFN resistance [17, 18]. NS5B is a domain harboring the putative catalytic site (GDD) of the viral polymerase and is a putative target of nucleoside analogs, including ribavirin [19, 20].

Materials and Methods

Patients

Five male Japanese patients chronically infected with HCV genotype 1b who received antiviral therapy at the Department of Gastroenterology, Toranomon Hospital, were enrolled in this study. Three of these 5 patients (patients 1, 2 and 3) received IFN-alpha-2b plus ribavirin (800 mg/day) for 6 months. The remaining 2 patients (patients 4 and 5) were treated with IFN-alpha-2b alone (table 1). Serum samples for sequence analyses were collected just before the start of therapy and at the end of therapy. Informed consent was obtained from each patient and study protocol conformed the ethical guidelines of 1975 Declaration of Helsinki, and institutional approval was obtained.

Amplification of 5 HCV Genomic Regions by Reverse Transcription-Polymerase Chain Reaction

HCV-RNA was isolated from 100-μl serum samples using Sepa Gene RV-R (Sanko Junyaku Co., Japan). HCV-RNA was reverse transcribed with random primer and a reverse transcriptase according to the instructions provided by the manufacturer (ReverTra Ace [Toyobo Co., Osaka, Japan]). HCV cDNA was then amplified using primer sets specific for each region (table 2). For the first and second rounds of nested PCR, 35 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 1 min were performed after an initial denaturation step at 94°C for 5 min, followed by a final extension for 7 min at 72°C.

Table 2. Primers used for RT-nested PCR amplification of 5′ UTR, E1, E2, NS5A and NS5B regions

5′ UTR	outer sense primer	5′-CCT GTG AGG AAC TAC TGT C-3′	(32–50)[a]	144 bp[b]
	outer antisense primer	5′-CAA CAC TAC TCG GCT AGC AGT C-3′	(254–233)[a]	
	inner sense primer	5′-TTC ACG CAG AAA GCG TCT AGC-3′	(51–71)[a]	
	inner antisense primer	5′-TTT ATC CAA GAA AGG ACC-3′	(194–176)[a]	
E1	outer sense primer	5′-CAG CCC GGG TAC TAC CCT TGG C-3′	(561–579)[a]	706 bp[b]
	inner sense primer	5′-CTC GAA TTC GGC TTC GCC GAT CTC ATG G-3′	(705–732)[a]	
	antisense primer	5′-CTC GGA TCC CCG CCA GGA CTC CCC AGT G-3′	(1,383–1,410)[a]	
E2	outer sense primer	5′-CAA GAC TGC AAT TGC TCC ATC T-3′	(1,233–1,254)[a]	535 bp[b]
	outer antisense primer	5′-GGT GCC GGA TCC ATC GGT CGT CCC CAC-3′	(1,875–1,901)[a]	
	inner sense primer	5′-CTA CTC CGG ATC CCA CAA GC-3′	(1,383–1,357)[a]	
	inner antisense primer	5′-CAA CAG GGA TCC GAG TGA AGC AAT A-3′	(1,848–1,872)	
NS5A	outer sense primer	5′-TTC CAC TAC GTG ACG GGC ATG AC-3′	(6,624–6,646)[a]	418 bp[b]
	outer antisense primer	5′-CCC GTC CAT GTG TAG GAC AT-3′	(7,590–7,609)[a]	
	inner sense primer	5′-GGG TCA CAG CTC CCA TGT GAG CC-3′	(6,798–6,820)[a]	
	inner antisense primer	5′-GAG GGT TGT AAT CCG GGC GTG C-3′	(7,194–7,215)[a]	
NS5B	outer sense primer	5′-TGG GGT TCT CGT ATG ATA CC-3′	(8,230–8,249)[a]	372 bp[b]
	inner sense primer	5′-CGC TGC TTT GAC TCA ACG GTC AC-3′	(8,250–8,272)[a]	
	antisense primer	5′-CCT GGT CAT AGC CTC CGT GAA-3′	(8,601–8,621)[a]	

[a] Location of nucleotide sequences according to Kato et al. [22].
[b] Size of PCR products in base pairs.

Cloning and Sequencing

PCR products were electrophoresed in 2% agarose gels and purified using Geneclean (Qbiogene Inc., Carlsbad, Calif., USA). Purified DNA was ligated into the plasmid vector pGEM-T Easy Vector (Promega, Madison, Wisc., USA), and transformed into *Escherichia coli*-competent cells according to the instructions provided by the manufacturer. Transformants were grown overnight on LB/ampicillin/IPTG/X-gal plates, and 10 individual clones from each sample were sequenced with an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems Japan, Tokyo).

Phylogenetic Analysis and Evaluation of Genetic Distances

Nucleotide sequences were aligned using the Expansion of CLUSTAL W in DNA Data Bank of Japan (DDBJ). Genetic distances were calculated with the Kimura two-parameter method [23] using these nucleotide alignments. Phylogenetic trees were constructed with the help of MEGA2 software [24] with the neighbor-joining method [25]. Bootstrap resampling (1,000 replicates) was utilized as a pseudo-empirical test of the reliability of the tree topology [26].

Evolution of quasispecies was estimated as described by Pawlotsky et al. [18]. Within-sample genetic distances, before and after treatment, was calculated for the quasispecies in each of 5 patients by comparing the genetic distances of pairs of sequences. Between-sample genetic distances were calculated on the basis of distances between pairs of pre- and post-treatment sequences. These genetic distances were calculated using the Kimura two-parameter method using MEGA program and expressed as mean ± SEM.

Statistical Analysis

Distributions of continuous variables were analyzed by the Mann-Whitney U test. $p < 0.05$ was considered statistically significant. Comparisons of genetic distances were made with the t test.

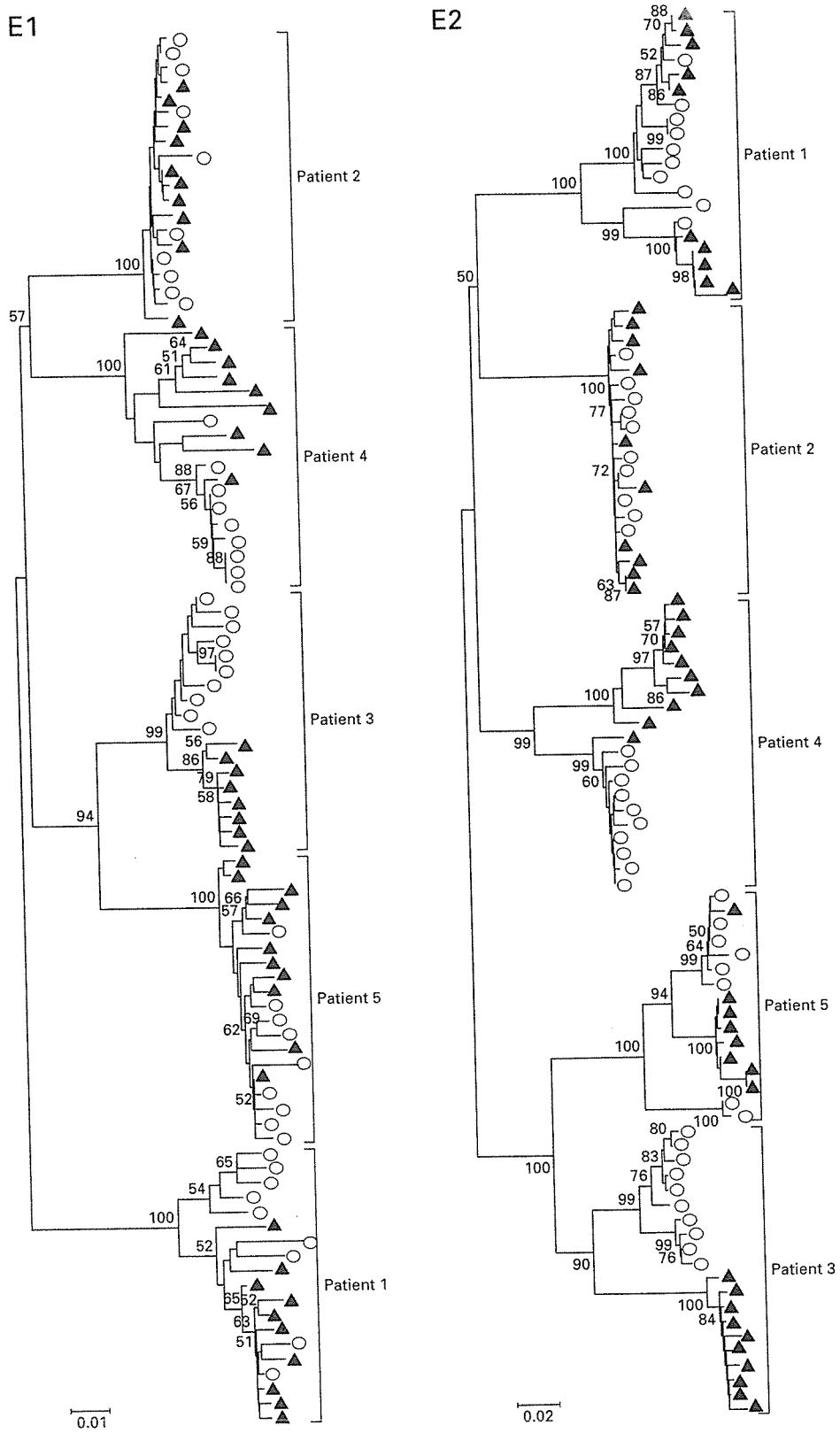
Results

Genetic Drift of HCV Quasispecies before and after Therapy

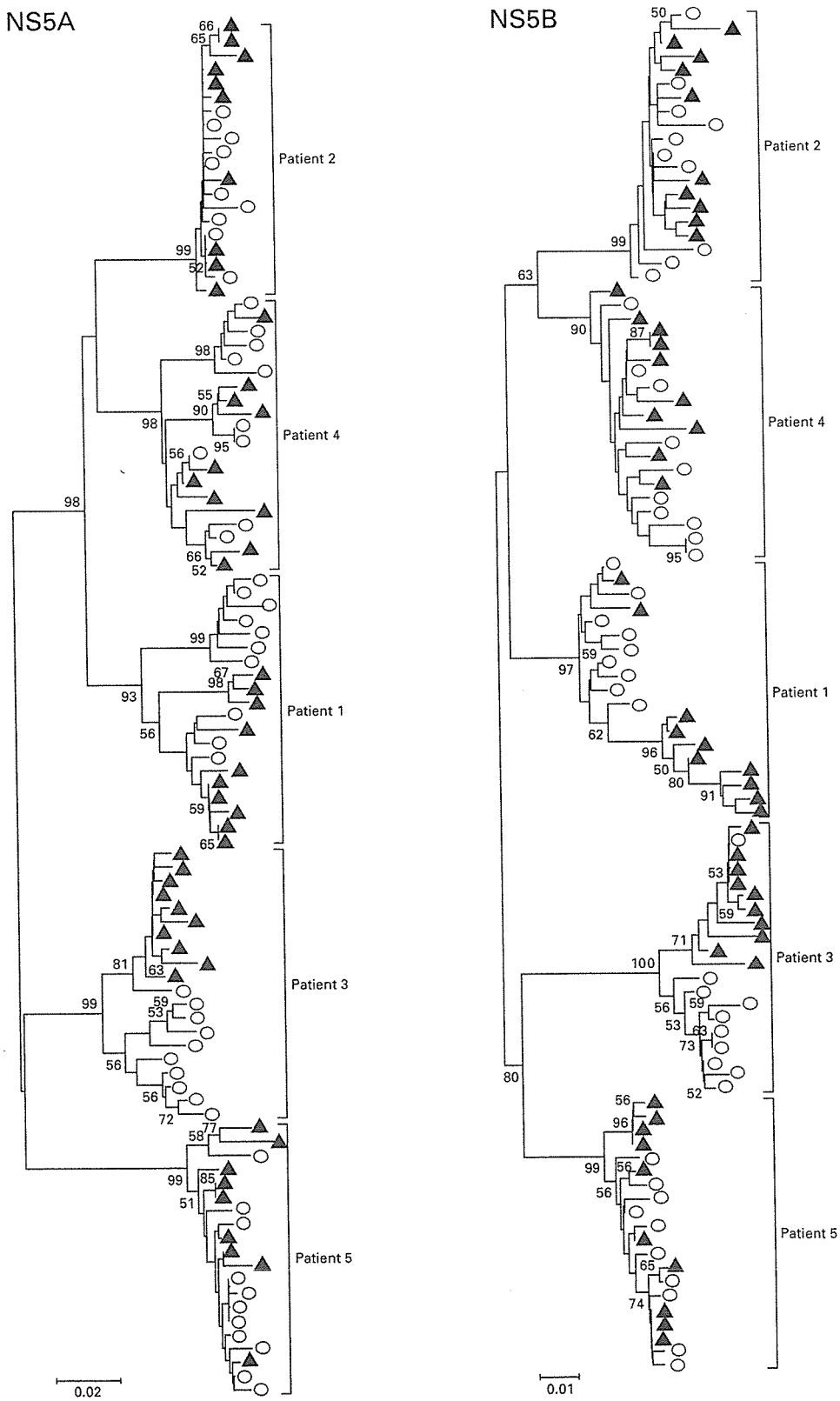
Nucleotide sequences of HCV clones in each region were aligned and phylogenetic trees were constructed (fig. 1). HCV evolution was observed in some patients in certain regions. Typical evolution, for instance, was seen in the phylogenetic tree of the E1 region in patient 3, the E2 region in patient 4, the NS5A region in patients 3 and 5,

(For figure see next pages.)

Fig. 1. Phylogenetic trees based on nucleotide sequences of E1, E2, NS5A and NS5B regions. Open circles represent clones obtained from serum samples extracted before therapy and closed triangles represent clones obtained after therapy. Figures on the branches of the trees represent bootstrap values. Bars represent nucleotide substitutions per site.


E1
Patient 2
Patient 4
Patient 3
Patient 5
Patient 1
0.01
E2
Patient 1
Patient 2
Patient 4
Patient 5
Patient 3
0.02

1


NS5A
Patient 2
Patient 4
Patient 1
Patient 3
Patient 5
0.02
NS5B
Patient 2
Patient 4
Patient 1
Patient 3
Patient 5
0.01

1

and the NS5B region in patients 1 and 3. To evaluate these evolutions, statistical analyses were performed using the MEGA program (fig. 2). To evaluate evolution during therapy, within-pretreatment sample genetic distances were compared with between-treatment sample genetic distances. If the between-treatment sample genetic distances were significantly greater than within-pretreatment genetic distances, the virus exhibited significant evolution. 5′ UTR analyses showed statistically significant evolution in only 1 of the 5 patients. Analyses of the E1 and E2 regions showed significant evolution in patients 3, 4 and 5. Since 2 of these 3 patients (patients 4 and 5) did not receive ribavirin, these evolutions are not related to ribavirin. Significant evolutions were seen in the NS5A and NS5B regions in patients 1 and 3, but not in patients 2, 4 and 5. These evolutions might be the effect of the combination therapy, or evolution of the virus to escape the effect of the therapy and develop resistance to it.

To evaluate whether the combination therapy induced errors in the HCV genome, we compared within-pretreatment sample genetic distances to within-post-treatment sample genetic distances (fig. 3). If the combination therapy induced nucleotide substitutions in the HCV genome, post-treatment sample genetic distances would exceed pre-treatment sample genetic distances. Post-treatment sample genetic distances in the 5′ UTR were significantly greater in 2 of the 3 patients who received combination therapy (patients 2 and 3; fig. 3). However, analyses of the other four regions of the HCV genome did not show such a tendency. The post-treatment genetic distances were smaller in 2 patients in E1. It was therefore difficult to detect error catastrophe from these genetic distance analyses.

Another possible mechanism of HCV evolvement is the acquisition of drug resistance. We compared nucleotide and amino acid sequences of HCV before and after therapy. There was no common amino acid substitution suggestive of resistance to the combination therapy (data not shown).

Discussion

Nucleotide substitutions during viral nucleic acid synthesis are important for viruses to survive under certain pressures of host immune responses and drugs. However, too many substitutions result in so-called error catastrophe. Ribavirin has been shown to induce nucleotide substitutions into RNA virus genomes and to reduce the virus load by inducing error catastrophe [9, 10, 27]. Induction of nucleotide substitutions by ribavirin has been shown in some in vitro systems. Crotty et al. [9, 10] reported that ribavirin induced nucleotide substitutions in the polio virus genome. Airaksinen et al. [27] observed a 10-fold increase in nucleotide substitutions in foot-and-mouth disease virus cultured with ribavirin. Contreras et al. [11] used a HCV full-length replication system and reported that ribavirin induced viral mutations. On the other hand, only limited in vivo data are available for the effect of ribavirin on the HCV viral genome. Querenghi et al. [28] analyzed nucleotide substitutions in the HVR1, NS5A and NS5B regions of HCV in patients treated with ribavirin monotherapy. They observed no significant effect for ribavirin on the amino acid sequence evolution in these regions. Furthermore, Sookoian et al. [13] analyzed HCV quasispecies of the hypervariable region, and concluded that the combination therapy did not affect HCV quasispecies. Since the hypervariable region is known to evolve very rapidly, we considered that analyses of different regions were necessary.

As shown in the phylogenetic tree depicted in figure 1, the apparent evolution of HCV during interferon and ribavirin combination therapy was observed in 2 of the 3 patients, particularly in the NS5A and NS5B regions in patients 3 and 5. These results are consistent with previous observations of Contreras et al. [11] who showed region-specific substitutions induced by ribavirin in vitro. However, investigation of the evolution of the E1 and E2 regions yielded different results. Statistical evaluation showed that not only patients who received combination therapy, but also patients who received interferon monotherapy showed significant evolution (fig. 2; patients 4 and 5). Since these regions encode the envelope protein, these substitutions might be induced by host immune pressure. In contrast, evolution in the NS5A and NS5B regions was seen predominantly in patients who received combination therapy. Such evolution might reflect induction of errors by ribavirin or the development of resistance against the therapy. To clarify this issue, we compared within-pretreatment sample genetic distances to within-post-treatment sample genetic distances. If the ribavirin-interferon combination therapy induced errors in the HCV genome, the post-treatment sample distances should have been greater than the pretreatment sample distances. However, an increase in genetic distance was observed in only limited patients and only in some regions.

We then examined the possibility that the virus developed resistance to the combination therapy. Typical

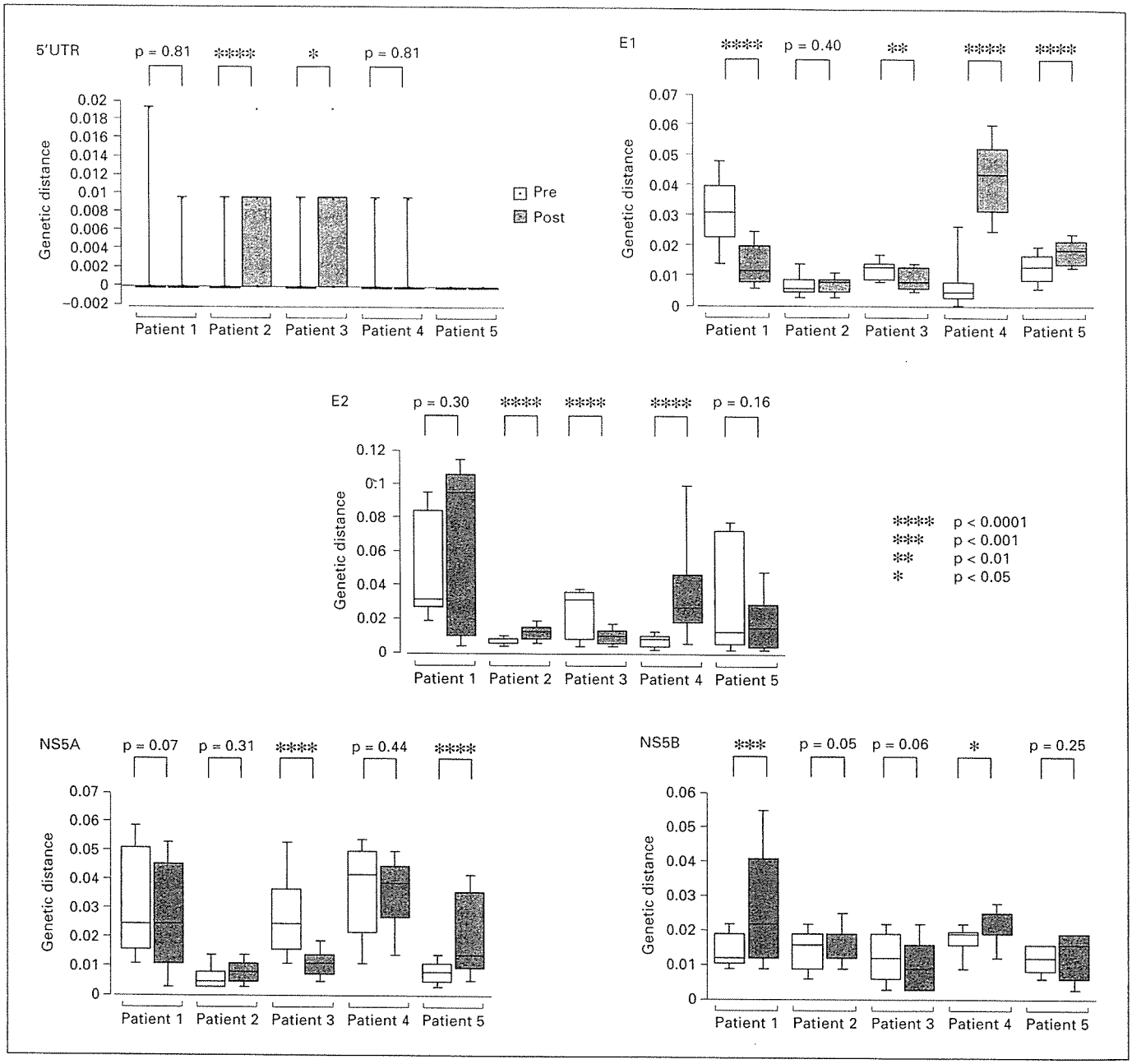


Fig. 2. Comparisons of pretreatment sample genetic distances and between-sample genetic distances. Open bars represent pretreatment sample genetic distances calculated by pairwise comparisons of nucleotide sequences of clones obtained before treatment. Closed bars represent between-sample genetic distances obtained by pairwise comparisons of clones obtained before and after treatment. Median genetic distances are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and the third quartiles.

nucleotide and amino acid substitutions that are related to resistance of the virus against nucleoside analogs are seen in human immunodeficiency virus and hepatitis B virus reverse transcriptase/polymerase. Amino acid substitutions of the methionine of the YMDD motif to leucine or valine induce strong resistance against lamivudine [29–32]. However, no specific nucleotide or amino acid changes suggestive of resistance to the therapy were

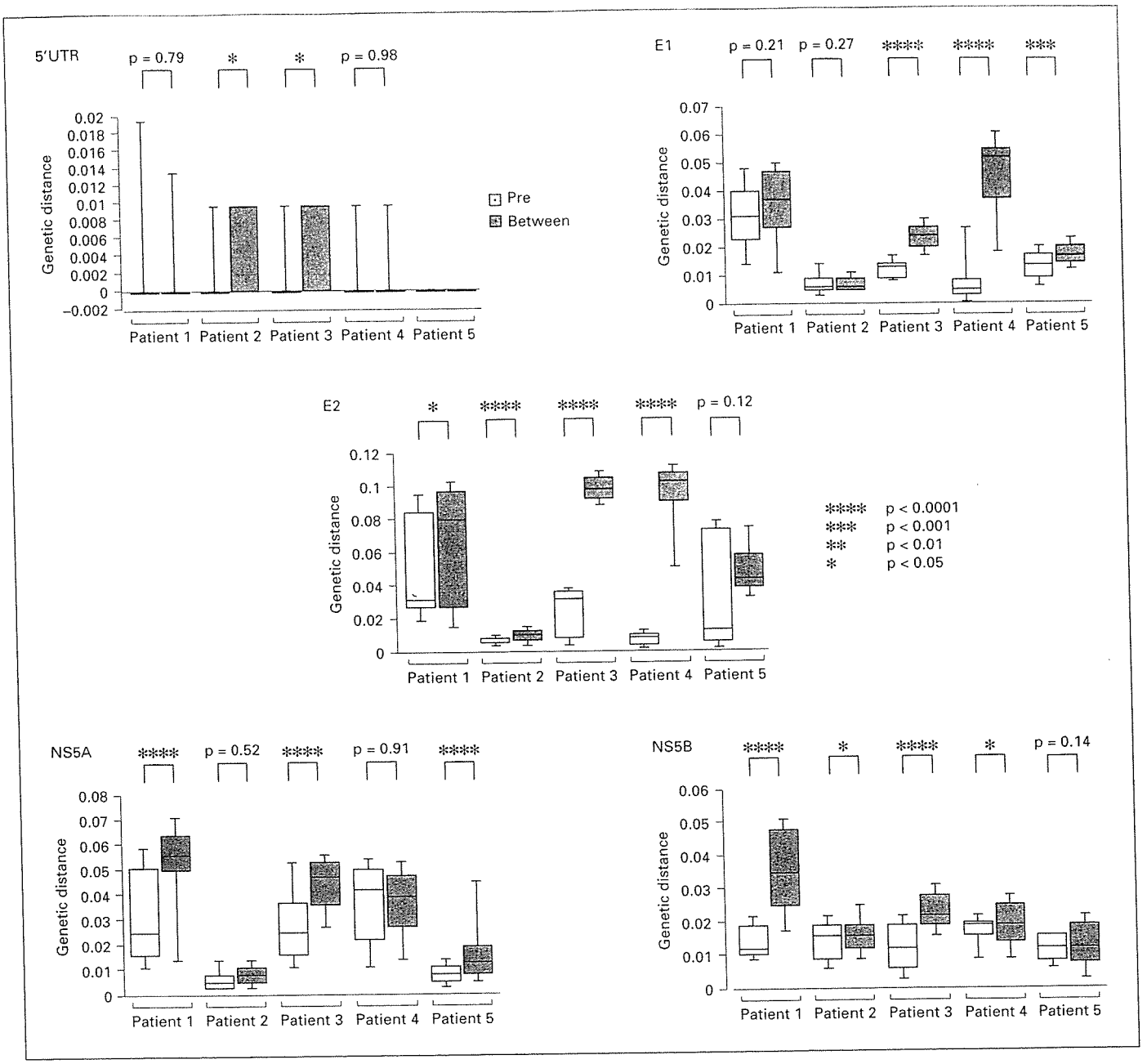


Fig. 3. Comparisons of pretreatment sample genetic distances and post-treatment sample genetic distances. Open bars and closed bars represent distances obtained by comparing nucleotide sequences of clones obtained before and after therapy, respectively. Median genetic distances are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and the third quartiles.

detected in this study. This finding was consistent with the observations of Lee et al. [33] who analyzed patients who received ribavirin monotherapy and observed no escape mutation of HCV. A possible escape mutation requires analysis in a larger number of patients with comparisons of sequences before and after combination therapy.

Although ribavirin is known to improve liver function without reducing the viral load, the mechanism of the additive effect of ribavirin to interferon therapy is not

yet clear [8]. Some possibilities have been proposed, but there is no definitive evidence to support each hypothesis. Although in vitro findings have suggested the induction of error catastrophe is likely to be the primary mechanism of action of the drug, no in vivo study, including this report, has yielded evidence in support of that hypothesis. One possible explanation for this discrepancy is that we were unable to observe virus with nucleotide substitutions because of the rapid turnover of the virus in vivo.

Clarification of the mechanism of action of these drugs in combination will be useful in developing new treatment strategies against HCV infection. The mechanism of ribavirin in reducing HCV in combination with interferon requires further investigation to enhance eradication of HCV and reduce liver-related deaths from this viral infection.

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Early decline of hemoglobin correlates with progression of ribavirin-induced hemolytic anemia during interferon plus ribavirin combination therapy in patients with chronic hepatitis C

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Background. The aim of this study was to examine the factors correlated with the progression of ribavirin-induced hemolytic anemia in patients with chronic hepatitis C treated by interferon and ribavirin combination therapy. ***Methods.*** This study was conducted on 505 patients by the Osaka Liver Disease Study Group. A decline of hemoglobin (Hb) concentration by 2 g/dl at the end of 2 weeks from the start of the treatment ("2 by 2" standard) was adopted as a predictive factor for progression to severe anemia. The ribavirin apparent clearance (CL/F) was also examined. ***Results.*** Of 482 patients whose Hb value was more than 12 g/dl before the treatment, 68 patients (14%) had to discontinue ribavirin owing to severe anemia. Patients in the "2 by 2"-positive group (Hb decline over 2 g/dl) and the group with lower CL/F were significantly more likely to discontinue ribavirin owing to severe anemia. Discontinuation was more common among patients aged 60 years or older than for those under 60 years old (21% vs. 9%, $P < 0.001$). Among patients aged 60 years or older, only the "2 by 2" standard was significantly associated with the discontinuance of ribavirin owing to severe anemia in a multivariate analysis (odds ratio, 4.18; $P < 0.001$). ***Conclusions.*** The "2 by 2" standard of Hb decline can be used to identify patients likely to develop severe anemia. The early reduction of ribavirin can help prevent progression to severe anemia, thus allowing ribavirin therapy to be completed even in older patients.

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Key words: chronic hepatitis C, interferon and ribavirin combination therapy, progression of anemia, "2 by 2" standard

Introduction

Hepatitis C virus (HCV) is estimated to infect up to 170 million people worldwide,[1] and two million people in Japan. Long persistence of HCV infection can lead to progression of liver fibrosis, causing liver cirrhosis and ultimately hepatocellular carcinoma.[2,3] Past studies have made clear that interferon (IFN) therapy is effective for eliminating HCV,[4,5] but the sustained viral response (SVR) rate of IFN monotherapy is not sufficient. The addition of the nucleoside analog ribavirin to IFN in the treatment of patients with chronic hepatitis C can significantly improve the SVR rate, and combination therapy with IFN or pegylated-IFN (Peg-IFN) has been recommended as a standard regimen worldwide.[6–10] However, additional side effects of ribavirin have been reported, such as hemolytic anemia, which have not been found with IFN monotherapy, leading to discontinuance of the treatment.[11–14]

In previous studies, the discontinuance rate of IFN and ribavirin combination treatment due to severe side effects has been reported to be 6%–13%.[6,7] Ribavirin-induced hemolytic anemia has been suggested to depend on a high plasma concentration of ribavirin.[15] The ribavirin apparent clearance (CL/F), which reflects the plasma concentration of ribavirin at 4 weeks after the start of combination therapy, has been used as a

predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[16–18] Furthermore, in the manufacturer's drug information for ribavirin,[19] a dose reduction is recommended when hemoglobin (Hb) levels decrease to less than 10 g/dl, and discontinuance of ribavirin is recommended when Hb levels fall to less than 8.5 g/dl during combination therapy with IFN and ribavirin. However, according to this guideline, not a few patients are forced to discontinue ribavirin because the dose reduction to avoid severe anemia does not occur in time.

What is needed is a convenient guideline for avoiding ribavirin discontinuance due to severe anemia. In this study, we evaluated the correlation of Hb decline at 2 weeks after the start of combination therapy with the discontinuance of treatment due to progression of ribavirin-induced hemolytic anemia. We also assessed the utility of an early decline of Hb in comparison with the CL/F standard for predicting the progression to severe anemia.

Patients and methods

Patients

The current study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Disease Study Group. The 505 patients with chronic hepatitis C included in this study were treated with a combination of interferon-α-2b and ribavirin between January 2001 and December 2005. All patients were anti-hepatitis C virus antibody positive, had HCV RNA detectable in their serum by the polymerase chain reaction method, and had elevated serum alanine transaminase (ALT) (above the upper limit of normal) within the 6 months prior to treatment. Excluded from this study were patients who were positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or those with other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis). Twenty-three patients whose Hb was under 12 g/dl before the treatment were also excluded because the aim of this study was to analyze the progression of anemia; patients with a low Hb level before treatment are known to have a tendency toward progression of anemia. The remaining 482 patients were followed in this study.

The baseline clinical features of the 482 patients are shown in Table 1. Their mean age was 55.2 ± 10.9 years, and 66% were men. Among the patients, 347 had HCV RNA with genotype 1 and high viral loads (1H group) and 130 had HCV RNA with genotype 2 or low viral loads (non-1H group). The mean ALT level was 100 ± 74 IU/l. In this study, a high viral load was defined as a serum HCV-RNA level of more than 10^6 equivalents/ml by branched DNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay.

Treatment schedule

Of the 482 patients treated with a combination of interferon-α-2b and ribavirin, 273 were IFN naïve and 209 were undergoing retreatment. All patients were scheduled to receive interferon-α-2b (Intron-A, Schering-Plough, Kenilworth, NJ, USA) at a dose of 6 (n = 371) or 10 (n = 111) MU intramuscularly every day for the first 2 weeks and three times a week thereafter. Ribavirin (Rebetol; Schering-Plough) was given orally twice a day for a total dose of 800 mg (n = 261), 600 mg (n = 215), or 400 mg (n = 6) per day. The IFN dose was decreased from 10 to 6 MU or from 6 to 3 MU when the

Table 1. Baseline characteristics of patients

Number	482	
Age (y.o)	55.2 ± 10.9	(21–75)
Sex (male/female)	320/162	
Body weight (kg)	62.3 ± 9.9	(35–94)
HCV serotype (1/2/unknown)	364/111/7	
(1H/non-1H/unknown)	347/130/5	
Fibrosis (0/1/3/4/unknown)	19/192/202/13/56	
WBC (/mm^3)	5184 ± 1531	(2100–13200)
RBC (×10^4/mm^3)	449 ± 42	(329–617)
Hb (g/dl)	14.4 ± 1.2	(12.0–19.2)
Plt (×10^4/mm^3)	15.4 ± 5.4	(4.4–36.1)
ALT (IU/l)	100 ± 74	(17–736)
Serum creatinine (mg/dl)	0.8 ± 0.2	(0.3–1.7)
Ribavirin dosage/body weight (mg/kg)	11.4 ± 1.5	(4.6–17.8)

Data are shown as means ± SD
HCV, hepatitis C virus; 1H group, patients with genotype 1 and high viral load; non-1H group, patients not in the 1H group; Fibrosis, Knodell's histological score (category4); WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Plt, platelets; ALT, alanine aminotransferase

white blood cell (WBC) count was below 1500/mm^3, the neutrocyte count below 750/mm^3, or the platelet (Plt) count below 5×10^4/mm^3. IFN was discontinued when the WBC count was below 1000/mm^3, the neutrocyte count below 500/mm^3, or the Plt count below 2.5×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dl, and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dl, in accordance with the manufacturer's drug information for ribavirin.[19] Ferric medicine or erythropoietin to prevent anemia was not administered. Ribavirin was scheduled to be administered for 24 weeks for all patients, and IFN for 24 weeks for 307 patients and for 48 weeks for 175 patients.

Patients with persistently undetectable HCV RNA 6 months after completion of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level and underwent hematological and biochemical tests just before therapy, at the end of week 2, and every 4 weeks thereafter during treatment. When treatment was completed, the patients were assessed every 4 weeks until 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar et al.,[17] CL/F at the start of the treatment was calculated as follows:

$$\text{CL/F (l/h)} = 32.3 \times \text{BW} \times (1 - 0.0094 \times \text{Age}) \times (1 - 0.42 \times \text{Sex})/\text{Scr},$$

where BW = body weight; sex = 0 for male and 1 for female; and Scr = serum creatinine.

Definition of "severe anemia" leading to discontinuance of ribavirin

In this study, "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dl or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dl from the start of combination therapy.

Liver histology

Hepatic fibrosis was assessed by Knodell's histological score (category 4).[20] Fibrosis stage was evaluated on a scale from 0 to 4: 0 = no fibrosis; 1 = fibrosis portal expansion; 3 = bridging fibrosis (portal–portal or portal–central linkage); 4 = cirrhosis.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, WBC count, red blood cell (RBC) count, Hb concentration, Plt, serum ALT levels, and Scr are expressed as means ± SD. The SVR rate was evaluated using an intention-to-treat (ITT) analysis. The differences in proportions were tested by the χ-squared test. For univariate and multivariate analyses, a logistic regression analysis was used to predict ribavirin-induced severe anemia. A value of $P < 0.05$ (two-tailed) was considered to indicate significance.

Results

Efficacy of the combination therapy with dose reduction or discontinuance of ribavirin

The relationship between dose reduction or discontinuance of ribavirin and the SVR rate on ITT analysis is shown in Fig. 1. The SVR rate was 20% (71/347) for all 1H patients and 72% (93/130) for all non-1H patients. Among the 1H patients, SVR was achieved for 24% (45/189) without dose reduction of ribavirin and for 26% (20/76) with dose reduction. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin treatment owing to adverse effects (7%, 6/82) in comparison with those with ($P < 0.01$) or without ($P < 0.01$) dose reduction. In the non-1H group, similar SVR rates were found with dose reduction of ribavirin [SVR rate without dose reduction, 83% (58/70), vs. SVR rate with dose reduction, 82% (23/28)], and the SVR rate of patients who had to discontinue ribavirin owing to adverse effects was significantly lower (38%, 12/32) than that for those with ($P < 0.001$) or without ($P < 0.0001$) dose reduction.

The same tendency was observed even in the 307 patients treated with IFN for 24 weeks. Among the 1H patients treated for 24 weeks, SVR was achieved for 19% (17/91) without dose reduction of ribavirin, 15% (6/41) with dose reduction, and 3% (2/75) with discontinuance. There were significant differences between the patients with discontinuance and those without ($P < 0.01$) or with ($P < 0.05$) dose reduction. Among the non-1H patients treated for 24 weeks, SVR rates were 85% (39/46) for the patients without dose reduction of ribavirin, 85% (17/20) for those with dose reduction, and 33% (10/30) for those with discontinuance. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin than for those with ($P = 0.05$) or without ($P < 0.05$) dose reduction.

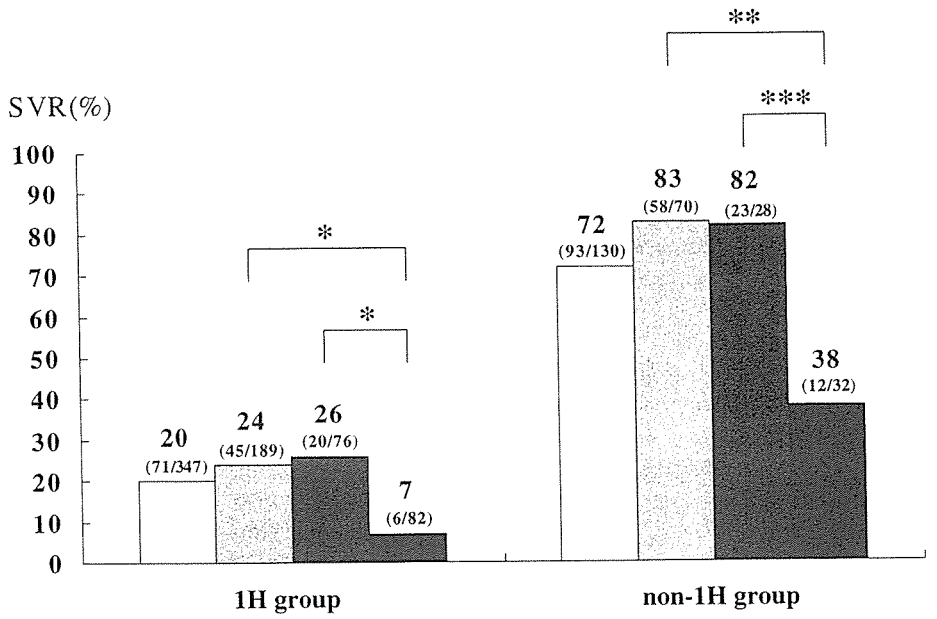

Fig. 1. Efficacy of combination therapy with dose reduction or discontinuance of ribavirin (intention-to-treat analysis). *1H group*, patients with genotype 1 and high viral load; *non-1H group*, patients not in the 1H group; *SVR*, sustained viral response. □ all patients; ■ patients without dose reduction of ribavirin; ■ patients with dose reduction of ribavirin; ■ patients with discontinuance of ribavirin. *, $P < 0.01$; **, $P < 0.0001$; ***, $P < 0.001$

Table 2. Rate of the ribavirin reduction or discontinuance due to adverse effects with different levels of CL/F

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
20 ≤ CL/F ($n = 45$)	94% (42/45)	2% (1/45)	4% (2/45)	0% (0/45)
15 ≤ CL/F < 20 ($n = 100$)	66% (66/100)	19% (19/100)	15% (15/100)	6% (6/100)
10 ≤ CL/F < 15 ($n = 179$)	54% (96/179)	24% (42/179)	23% (41/179)	14% (25/179)
CL/F < 10 ($n = 158$)	37% (58/158)	28% (44/158)	35% (56/158)	23% (37/158)

Frequency of and reasons for dose reduction or discontinuance of ribavirin during combination therapy

We examined the rate of discontinuance of therapy due to adverse effects up to the end of 24 weeks, because all cases of discontinuation occurred before the end of 24 weeks. Of the 482 patients, 401 patients completed 24 weeks of therapy, and 81 patients (17%) had to discontinue both IFN and ribavirin before the end of the 24 weeks. Of the 401 patients undergoing 24 weeks of therapy, the entire treatment schedule without reduction or discontinuance of either drug was completed by 262 patients (54%). The ribavirin dose was decreased for 106 patients (22%) and was stopped without discontinuance of IFN for 33 patients (7%). Overall, 114 patients (24%) discontinued ribavirin treatment. The reasons for dose reduction or discontinuance of ribavirin were anemia, general fatigue, digestive disorder, eczema, neutropenia, thrombocytopenia, or psychological disorder. Among the patients discontinuing ribavirin, the major reasons were anemia (14%), general fatigue (2%), or digestive disorder (2%).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients was 4.6–32.5 l/h. The mean CL/F was 13.0 l/h, and the median was 11.9 l/h. At the start of treatment, CL/F was less than 10 l/h for 33% (158/482) of patients, 10–15 l/h for 37% (179/482), 15–20 l/h for 21% (100/482), and more 20 l/h for 9% (45/486).

Table 2 shows the rates of dose reduction or discontinuance of ribavirin in relation to different levels of CL/F. The rate of discontinuance of ribavirin among all patients was 4% (2/45) for patients with CL/F ≥ 20, 15% (15/100) for those with 15 ≤ CL/F < 20, 23% (41/179) for those with 10 ≤ CL/F < 15, and 35% (56/158) for those with CL/F < 10. The rate of discontinuance of ribavirin due to severe anemia was 14% (68/482) among all pa-

tients. There was no discontinuance of ribavirin due to severe anemia among patients with CL/F ≥ 20, but the rate of discontinuance was 6% (6/100) among those with 15 ≤ CL/F < 20, 14% (25/179) among those with 10 ≤ CL/F < 15, and 23% (37/158) among those with CL/F < 10. The rate of continuance of ribavirin without dose reduction decreased in proportion to the decline of CL/F. In this study, we adopted two categories of CL/F, below 15 l/h (CL/F < 15) and below 10 l/h (CL/F < 10), to assess CL/F as a factor for predicting anemia progression.

We also analyzed the predictive factor of anemia progression according to patient age, because CL/F varies widely with patient age and tends to be lower among older patients. Among patients under 60 years old (n = 288), 17% (48/288) had CL/F under 10 l/h, 38% (109/288) had CL/F 10–15 l/h, 30% (86/288) had CL/F 15–20 l/h, and 16% (45/288) had CL/F over 20 l/h. On the other hand, among those 60 years old or older (n = 194), 57% (110/194) had CL/F under 10 l/h, 36% (70/194) had CL/F 10–15 l/h, 7% (14/194) had CL/F 15–20 l/h, and none had CL/F over 20 l/h. Thus, the majority (93%) of the patients 60 years old or older had a low CL/F (<15), whereas only 55% of those under 60 years old had CL/F < 15.

Early decline of Hb and progression of anemia during combination therapy

Figure 2 shows the decline of Hb from the start of combination therapy. We conducted this analysis for the 433 patients: those who did not need a dose reduction of ribavirin (n = 262), those who needed a dose reduction owing to a decrease of Hb to less than 10 g/dl (n = 103), and those who discontinued ribavirin due to "severe anemia" (n = 68). We excluded 49 patients from this analysis: 46 patients stopped combination therapy for reasons other than anemia, such as general fatigue or digestive disorder, and the other three patients were not responding to antiviral treatment and stopped therapy before 24 weeks without a dose reduction of ribavirin. Following the initiation of combination therapy, Hb concentration decreased rapidly until the end of the 4th week. At the end of 2 weeks, Hb had decreased by 0.9 ± 1.2 g/dl among the patients without dose reduction of ribavirin, by 1.8 ± 1.3 g/dl among those with dose reduction, and by 2.3 ± 1.4 g/dl among those who discontinued ribavirin. At the end of 4 weeks, Hb had decreased by 2.1 ± 1.5 g/dl among the patients without dose reduction of ribavirin, by 3.2 ± 1.5 g/dl among those with dose reduction, and by 3.9 ± 1.5 g/dl among those discontinuing ribavirin.

ΔHb [ΔHb = (Hb value just before treatment) − (Hb value during treatment)] both at the end of 2 weeks and at the end of 4 weeks were significantly larger among the patients discontinuing ribavirin than among those without dose reduction of ribavirin (P < 0.0001, P < 0.0001, respectively). In this study, we adopted the category of ΔHb at the end of 2 weeks because it allowed the progression of anemia to be estimated at an earlier phase of treatment than did ΔHb at the end of 4 weeks.

To establish the cutoff value of ΔHb at the end of 2 weeks, we used two categories of ΔHb: a decrease in Hb concentration at 2 weeks to 2 g/dl below the baseline (ΔHb2.0) or to 1.5 g/dl below the baseline (ΔHb1.5). We conducted this analysis for 480 patients, because two patients stopped combination therapy before 2 weeks for reasons other than anemia. With the ΔHb2.0 standard, the rate of discontinuance of ribavirin due to severe anemia was 10% (32/338) in the ΔHb < 2.0 group and 25% (36/142) in the ΔHb ≥ 2.0 group, with the difference being significant (P < 0.0001) (Table 3). With the ΔHb1.5 standard, the rate of discontinuance of ribavirin due to severe anemia was significantly higher

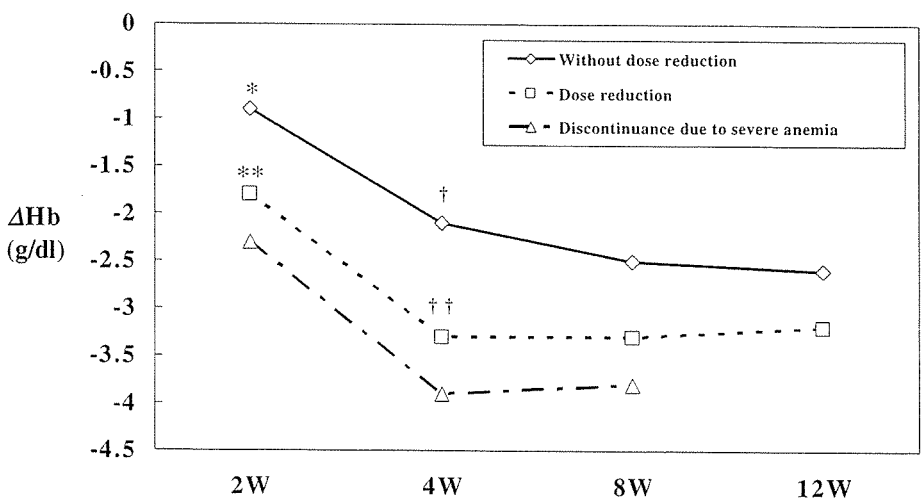

Fig. 2. Decline of hemoglobin according to dose reduction or discontinuance of ribavirin. *Significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); **significantly different from patients with discontinuance (P < 0.02); †significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); ††significantly different from patients with discontinuance (P < 0.01)