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.

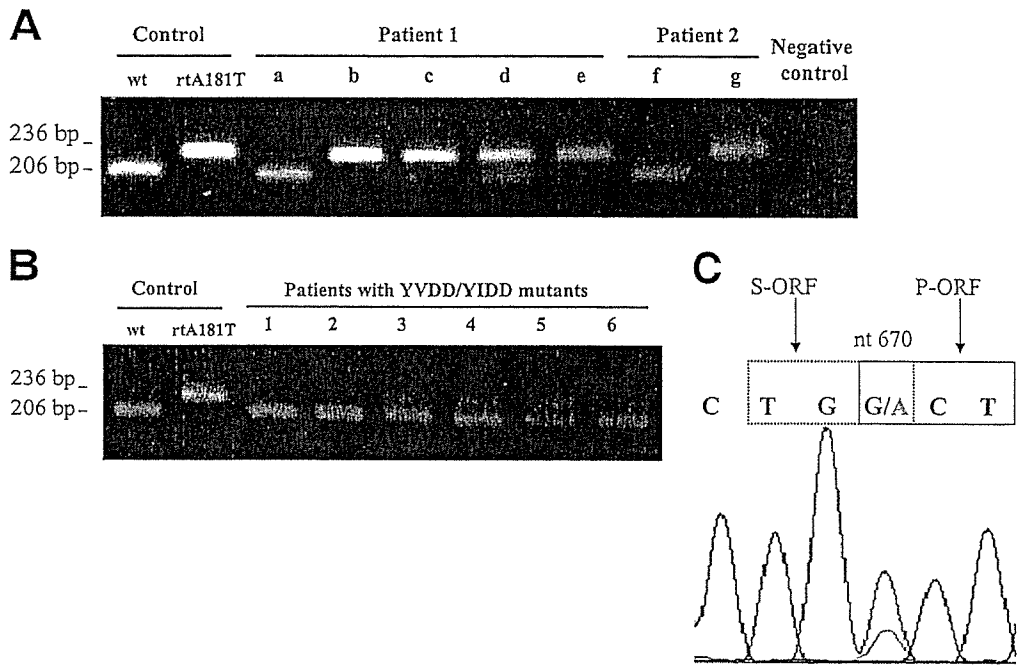

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. 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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Prolonged Negative HCV-RNA Status Led to a Good Outcome in Chronic Hepatitis C Patients with Genotype 1b and Super-High Viral Load

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Key Words
IFN-α_{2b} + ribavirin, combination therapy · Hepatitis C virus genotype 1b · High viral load · IFN-β, induction therapy

Abstract
Objective: We examined whether a sustained negative HCV-RNA status for 48 weeks affects the outcome in patients with genotype 1b and super-high viral load, and also investigated whether the outcome is affected by the induction therapy of twice-daily pre-administrated interferon (IFN)-β. ***Methods:*** 78 eligible patients were divided into four groups. 40 were patients assigned to the short treatment protocol. 13 patients received 3 MU IFN-β twice daily for 2 weeks followed by IFN-α_{2b} + ribavirin for 22 weeks (β-induction group: group 1). 27 patients received IFN-α_{2b} + ribavirin for 24 weeks (standard combination group: group 2). 38 patients were assigned to the maintenance treatment protocol. All of the 13 in the β-induction group (group 3) and 21 of 25 patients in the standard combination group (group 4) who were negative HCV-RNA PCR at week 24 had IFN monotherapy to maintain a negative HCV-RNA result for 48 weeks. ***Results:*** An HCV-RNA-negative status at week 24 was observed in 96% (25/26) of groups 1 and 3 versus in 79% (41/52) of groups 2 and 4 ($p < 0.01$). The sustained virological response (SVR) was 38% (5/13) in group 1 and 11% (3/27) in group 2 ($p < 0.05$). In the maintenance treatment, SVR was observed in 46% (6/13) of group 3 and 32% (8/25) of group 4 (NS). ***Conclusions:*** A sustained negative HCV-RNA status for 48 weeks might be associated with viral elimination in patients with genotype 1 and super-high viral load.

Introduction

Hepatitis C virus (HCV) infection is estimated to affect 170 million individuals worldwide [1], including 2 million people in Japan [2]. Chronic HCV infection often progresses into liver cirrhosis including the development of associated complications such as gastroesophageal varices and hepatocellular carcinoma over the course of 20–50 years [3–6]. Interferon (IFN) is the only effective treatment for HCV infection, and is widely used. The beneficial effects of IFN in patients with chronic HCV infection have been clearly defined and include decreases in serum transaminase concentration, eradication of the virus, and improvement of liver histology [7–10]. However, a sustained virological response (SVR) is rarely obtained by

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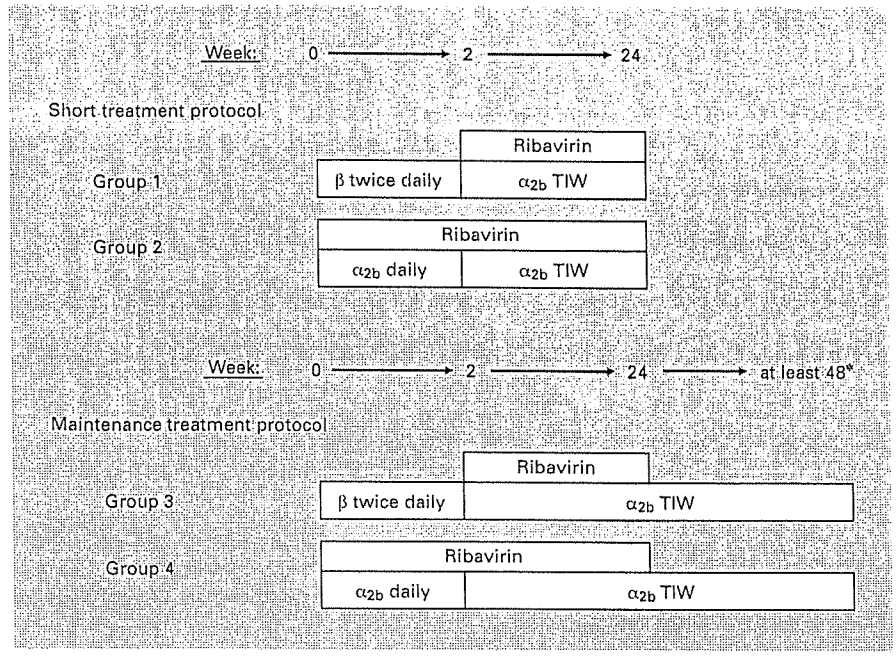

Fig. 1. Study design showing the two different protocols of IFN therapy. * Maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

IFN monotherapy in poor response categories (cirrhosis, high viral load, genotype 1/4) [11–16]. Recent advances of various IFN treatments such as consensus IFN, ribavirin combination, and pegylated IFN can achieve a relatively high SVR in those patients [17–25].

In Japan, the patients with genotype 1 and high viral load is most prevalent [26]. The oral administration of ribavirin has been permitted for only 24 weeks by medical insurance until December 2004 [27]. Because the relapse rate is higher in combination therapy only for 24 weeks [20, 22], we conducted prolonged IFN monotherapy after ribavirin combination. Recently, it was reported that not only the treatment duration but also the duration of therapy with an undetectable HCV-RNA load are associated with the probability of a long-term antiviral response during pegylated IFN/ribavirin combination therapy, and that patients infected with genotype 1 would require a continuous non-detectable viral load in serum for 36 weeks to attain 90% probabilities of SVR [28]. In this study, we designed a clinical trial consisting of combination therapy followed by prolonged IFN monotherapy, which was continued for 48 weeks from the time of the first negative HCV-RNA PCR result for HCV genotype 1 patients with high viral load. We also investigated whether the outcome of IFN therapy is affected by the induction therapy of twice-daily pre-administered IFN-β.

Materials and Methods

Patients

A total of 78 adult patients were recruited for this study. All patients were infected with HCV genotype 1b and had super-high viral load (>500 KIU/ml) as determined by Amplicor HCV monitor assay (Roche Molecular Diagnostics Co., Tokyo, Japan). The detection range of the assay was between 0.5 and 500 KIU/ml (a standard sample containing 10^5 copies/ml of HCV was assigned a titer of 10^5 IU/ml). Patients eligible for study participation were required to satisfy the following criteria: (1) aged from 20 to 65 years; (2) a recent liver biopsy within 3 months of the start of therapy; (3) diagnosis of chronic hepatitis by the conventional classification; (4) positive for HCV-RNA of genotype 1b in serum within 3 months in titers of >500 KIU/ml by the Amplicor HCV monitor assay; (5) abnormal serum alanine aminotransferase levels for >6 months; (6) leukocyte count >3,000/mm^3, platelets >100,000/mm^3; (7) serum bilirubin <2.0 mg/dl; (8) lack of liver cirrhosis, hepatocellular carcinoma, autoimmune hepatitis, alcoholic liver disease and any other chronic liver diseases (positive for serological markers of hepatitis B virus); (9) lack of psychiatric illnesses, including depression, or conditions affecting the bone marrow, alimentary, cardiovascular or pulmonary systems, and (10) no immunosuppressive or antiviral therapy within 6 months prior to entry.

IFN Protocol

Patients were treated with the combination therapy of IFN and ribavirin: 6–10 million units (MU) of IFN-α_{2b} subcutaneously administered three times weekly; oral ribavirin administered twice daily at a total dose of 600 or 800 mg for patients whose weight was less or more than 60 kg, respectively. The IFN therapy protocol is described in figure 1. At the start of the therapy, the physicians in

charge explained the purpose and method of the clinical trial as well as potential adverse events during the twice-daily IFN-β induction. The physicians also explained the information including the result of clinical trials of combination therapy for 48 weeks in other countries, such as the SVR rate, HCV-RNA relapse rate and adverse events. After giving sufficient informed consent, the patients themselves decided whether or not to be treated by twice-daily IFN-β induction and also decided whether or not to be treated by additional IFN monotherapy to sustain a negative HCV-RNA result for 48 weeks. According to the patients' decision, four therapeutic groups were divided as follows:

Short Treatment Protocol: 40 patients were treated by this protocol for 24 weeks. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy for 22 weeks (group 1). 27 patients were treated by the standard combination therapy for 24 weeks (group 2).

Maintenance Treatment Protocol: 38 patients were treated by this protocol. 13 patients were treated by 3 MU of IFN-β twice-daily administered for 2 weeks followed by the combination therapy (group 3). 25 patients were treated by the standard combination therapy (group 4). For consistency with current guidelines, patients who were HCV-RNA-positive by PCR at month 6 were removed from the study and considered as non-responders. The patients who had an undetectable HCV-RNA load in serum at month 6 had an additional minimum of 24 weeks' IFN monotherapy as maintenance treatment. The maintenance treatment was designed to sustain a negative HCV-RNA PCR result for 48 weeks.

The study was approved by the Institutional Review Boards of the participating clinical sites before study initiation, and the study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Virological Response to IFN

The virological response to IFN was determined by measuring serum HCV-RNA levels with the Amplicor HCV monitor assay at days 2, 3, 8, 15, 29 and every 28 days thereafter. Negative samples on the Amplicor HCV monitor assay were re-examined by the Amplicor qualitative assay, which has a detection limit of HCV-RNA of 0.2 KIU/ml. SVR was defined as a negative serum HCV-RNA during the 6 months following completion of IFN administration. All patients other than those with SVR were considered to be non-responders.

Histological Analysis

All patients underwent liver needle biopsy under sonographic guidance in the 3 months prior to the start of IFN administration. Baseline liver histology of chronic hepatitis was classified, based on the extent of fibrosis, into five stages (F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis)), and based on activity into four grades (A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity)), according to the method of Desmet et al. [29].

Statistical Analysis

Baseline clinical characteristics were compared between the treatment groups using Fisher's exact test or the Mann-Whitney U-test. Treatment efficacy was analyzed by Fisher's exact test. p values <0.05 were considered statistically significant.

Results

Characteristics of the Patients

There were no significant differences in the general characteristics of the patients in demographic, biochemical, virological and histological features between the β-induction group (group 1) and standard combination group (group 2) in the short treatment protocol. There were no significant differences in the background characteristics between the β-induction group (group 3) and standard combination group (group 4) in the maintenance treatment protocol. Among the four therapeutic groups, background characteristics were also not significant, except the history of previous IFN monotherapies: the rate of previous IFN monotherapies in the short standard combination group (group 2) was significantly lower compared with other therapeutic groups ($p < 0.05$) (table 1).

HCV-RNA Clearance

HCV-RNA negativity and the week after starting therapy are shown in table 2. 96% (25/26) of the β-induction group (groups 1 and 3) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. In comparison, 79% (41/52) of the standard combination group (groups 2 and 4) had undetectable HCV-RNA load in serum 24 weeks after starting therapy. There was a significant difference in the HCV-RNA status at 24 weeks between the β-induction group (groups 1 and 3) and the standard combination group (groups 2 and 4) ($p < 0.05$). Of the patients who received maintenance IFN monotherapy, 39% (5/13) in the β-induction group (group 3) and 43% (9/21) in the standard combination group (group 4) had detectable HCV-RNA during IFN monotherapy (breakthrough). The residual patients completed IFN monotherapy to sustain a negative HCV-RNA PCR profile for 48 weeks. In the patients with a negative HCV-RNA status for 48 weeks, 25% (2/8) in the β-induction group (group 3) and 33% (4/12) in the standard induction group (group 4) had re-appearance of HCV-RNA after IFN monotherapy. The periods of IFN maintenance monotherapy were 32.4 ± 6.2 weeks in the β-induction group (group 3) and 38.5 ± 6.9 weeks in the standard combination group (group 4) ($p < 0.05$).

HCV-RNA Dynamics and the Time of HCV-RNA Negativity

The first and second phase of HCV-RNA dynamics are shown in figure 2. An early significant decline in HCV-RNA was observed in the β-induction group (groups 1

Table 1. Baseline characteristics of the patients according to four therapeutic groups

	Short treatment protocol (n = 40)		Maintenance treatment protocol (n = 38)		p value
	β-induction group (group 1, n = 13)	standard combination group (group 2, n = 27)	β-induction group (group 3, n = 13)	standard combination group (group 4, n = 25)	
Mean age, years[a]	55.8 ± 5.6	54.6 ± 10.3	54.0 ± 9.2	56.7 ± 10.4	n.s.
Male:female	7:6	13:14	10:3	18:7	n.s.
Basal WBC, $\times 10^3/mm^3$	4.7 ± 1.4	4.5 ± 1.5	4.7 ± 1.3	4.9 ± 1.6	n.s.
Basal Hb, g/dl	14.4 ± 1.3	14.6 ± 1.0	15.2 ± 1.0	14.8 ± 1.1	n.s.
Basal ALT, IU/l[a]	72.4 ± 36.1	68.9 ± 31.7	73.8 ± 40.1	62.7 ± 26.2	n.s.
Platelets, $\times 10^4/mm^3$ [a]	16.4 ± 4.7	14.4 ± 4.4	16.7 ± 5.8	15.2 ± 3.7	n.s.
Serum HCV-RNA, KIU/ml	>500	>500	>500	>500	n.s.
Histological findings[b]					
Staging 0	0	0	0	0	n.s.
Staging 1	5	10	7	13	n.s.
Staging 2	4	7	3	8	n.s.
Staging 3	4	10	3	4	n.s.
Staging 4	0	0	0	0	n.s.
Grade 0	0	0	0	0	n.s.
Grade 1	4	9	4	10	n.s.
Grade 2	8	17	8	13	n.s.
Grade 3	1	1	1	2	n.s.
History of previous IFN monotherapies	6	5*	7	13	n.s.

[a] Data are mean ± SD. [b] Classified by the method of Desmet et al. [29]. n.s. = Not significant.
* The rate of previous IFN monotherapies in short standard combination group was significantly lower compared with other therapeutic groups ($p < 0.05$).

Table 2. HCV-RNA disappearance and the week after starting therapy

Weeks	β-Induction group (groups 1 and 3, n = 26)	Standard combination group (groups 2 and 4, n = 52)	p value
2	12% (3/26)	4% (2/52)	0.191
4	35% (9/26)	10% (5/52)	<0.01
8	62% (16/26)	29% (15/52)	<0.01
12	73% (19/26)	52% (27/52)	0.059
16	96% (25/26)	69% (36/52)	<0.01
20	96% (25/26)	77% (40/52)	<0.05
24	96% (25/26)	79% (41/52)	<0.05

and 3) on days 7 and 14 in the standard combination group (groups 2 and 4). Twice-daily administration of IFN-β accelerated HCV-RNA decline in the second phase against IFN/ribavirin combination therapy. As a result of early viral decline, HCV-RNA disappearance was attained in a shorter period in the β-induction group (groups 1 and 3) (table 2). That was significant with the standard combination group (groups 2 and 4) at weeks 4, 8, 16, 20 and 24. The mean time to the first negative HCV-RNA PCR result was 8.4 ± 6.2 weeks in the β-induction group (groups 1 and 3) and 14.5 ± 6.9 weeks in the standard combination group (groups 2 and 4) ($p < 0.01$).

Virological Response

Table 3 shows SVR rates. In patients who received the short treatment protocol, SVR was observed in 5 of 13 patients (38%) in the β-induction group (group 1) and in 3 of 27 patients (11%) in the standard combination group (group 2) ($p < 0.05$). In the patients who received the maintenance treatment protocol, SVR was observed in 6 of 13 patients (46%) in the β-induction group (group 3) and in 8 of 25 patients (32%) in the standard combination group (group 4) (NS).

Adverse Events

Table 4 summarizes the laboratory abnormalities and adverse events recorded for 24 weeks after initiation of IFN therapy. There were no patients with leukocyte counts <1,000/mm^3, hemoglobin concentrations

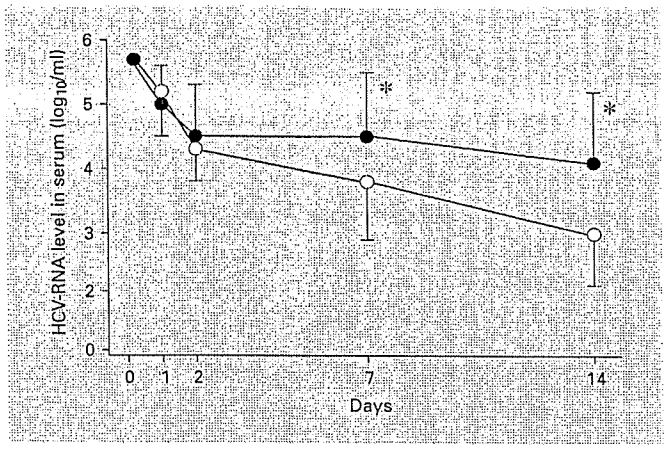

Fig. 2. Mean (±SD) of viral load in the serum during the first 14 days of treatment for patients chronically infected with HCV genotype 1b and super-high viral load (>500 KIU). The two treatment groups included: (1) twice-daily administration of IFN-β (groups 1 and 3) (○) and (2) combination therapy with ribavirin and daily IFN-α_{2b} (groups 2 and 4) (●). * $p < 0.05$.

Table 3. Sustained virological response rate to two different antiviral regimens with or without β-induction

	β-Induction group (groups 1 and 3, n = 26)	Standard combination group (groups 2 and 4, n = 52)	p value
Short treatment protocol	38% (5/13)	11% (3/27)	<0.05
Maintenance treatment protocol	46% (6/13)	32% (8/25)	n.s.

n.s. = Not significant.

Table 4. Number of patients who had laboratory abnormalities or adverse events

	β-Induction group (groups 1 and 3, n = 26)	Standard combination group (groups 2 and 4, n = 52)
Leukocytes <1,000/mm^3	0	0
Hb <10 g/dl	6	19
Hb <8.5 g/dl	0	0
Platelets <50,000 /mm^3	2	2
Albumin <3.5 g/dl	14	0
Albumin <3.0 g/dl	0	0
Proteinuria/day		
<1 g	7	0
1–3.5 g	10	0
>3.5 g	8	0
ALT elevation[a]	9	3

[a] ALT elevation was considered positive when ALT of anytime during IFN therapy increased more than twofold of the baseline ALT.

<8.5 g/dl and serum albumin level <3.0 g/dl. Incidences of hypoalbuminemia (<3.5 g/dl) and proteinuria were observed only in patients treated with the β-induction. A total of 8 patients had severe proteinuria (>3.5 g/day). ALT elevation (twofold against the baseline) was significantly higher in the β-induction group (groups 1 and 3) (9/26 vs. 3/52; $p < 0.01$). However, 2 weeks of β-induction therapy was completed in all patients and these adverse events recovered after the completion of β-induction. During maintenance IFN monotherapy (groups 3 and 4), the laboratory abnormalities and adverse events were not observed. No patients discontinued because of these adverse events during the therapeutic periods.

Discussion

Genotype 1 is the most prevalent genotype of HCV in most geographical areas, including Japan. Recent studies have revealed important information about viral dynamics following initiation of IFN therapy [30–32]. For genotype 1 patients, the antiviral effectiveness of IFN (blocking virus production, free virion clearance rate, and HCV-infected cell death rate) has been shown to be significantly lower than that for non-genotype 1 patients [32]. Failure to clear the virus can be observed at three different phases: during the initial treatment period (nonresponse), during maintenance treatment after an initial response (breakthrough), and after treatment discontinuation (relapse) [20, 22]. In IFN-resistant patients, a high prevalence of those three reactions was observed. Thus to obtain a high SVR rate, high prevalence of undetectable HCV-RNA and low rates of breakthrough and relapse would be desirable.

In the present study, an HCV-RNA-negative status at 24 weeks was significantly high and early in the β-induction group, while an HCV-RNA-negative status at 24 weeks was obtained in 79% of the patients in the standard combination group. While ribavirin combination achieved similar results even for 48 weeks [20, 22], the early disappearance and high rate of an HCV-RNA-nega-

tive status was obtained in the genotype-1-infected patients of super-high viral load (>500 KIU) by the induction of twice-daily administration of IFN-β. Twice-daily administration of IFN-β is associated with early virus elimination [33–37]. However, adverse events during administration of IFN-β can include marked elevation of serum alanine aminotransferase, decreased platelet count, and proteinuria especially in the patients treated with twice-daily administration [33–38]. To take advantage of the antiviral efficacy, the upper limit of duration of twice-daily administration of IFN-β needs to be established. Some reports demonstrated that about 70–85% of the patients treated with twice-daily administration of IFN-β could tolerate continuing treatment for 4 weeks [33, 34]. In our study, all patients treated with the twice-daily IFN-β induction protocol could tolerate continuing induction treatment for 2 weeks. The patients treated with β-induction had a relatively high SVR rate with or without IFN monotherapy. Although the significance of induction therapy remains unclear, our results suggest that induction therapy might be beneficial for genotype-1-infected patients of super-high viral load (>500 KIU).

A relatively high rate of breakthrough (approx. 40%) might be caused by the short duration of ribavirin usage or background of super-high viral load (>500 KIU). In Japan, the oral administration of ribavirin has been permitted for only 24 weeks by medical insurance until December 2004 [27]. Thus, it was our design for this study that prolonged IFN monotherapy would be continued for 48 weeks from the time of first negative HCV-RNA PCR result. As a result, we obtained a relatively low prevalence of relapse. The relapse rates were 33% in patients treated with standard combination therapy and 25% in those treated with IFN-β induction therapy. These low rates of relapse were similar to the result of pegylated IFN/ribavirin combination therapy for 48 weeks [23–25]. We obtained a relatively high SVR rate for the patients with genotype 1 and super-high viral load (>500 KIU) by the limited treatment with a 6-month course of ribavirin. In addition, the SVR rates were higher in the β-induction group than in the standard combination group with or without IFN monotherapy. In particular, patients treated with IFN monotherapy followed by combination therapy with twice-daily pre-administration of IFN-β had a SVR rate of 46%. Moreover, no patients discontinued because of adverse events during the treatment protocol.

Generally, the beneficial effect of induction therapy remains controversial. In non-1b patients, high rates of SVR are obtained without induction [32]. In patients with genotype 1b and a high viral load, various studies including induction-dosing trials showed greater rates of early viral clearance. However, there were a few reports suggesting that early viral clearance was associated with a high prevalence of SVR [37, 39, 40]. Vrolijk et al. [41] demonstrated that daily induction therapy might be beneficial for IFN-resistant patients, but only when combined with adequate maintenance therapy of long duration. Drusano and Preston [28] demonstrated that not only the treatment duration but also the duration of therapy with an undetectable HCV-RNA load are associated with the probability of a long-term antiviral response during pegylated IFN + ribavirin combination therapy, and that patients infected with genotype 1 require a continuous non-detectable viral load in serum at least for 32 weeks. Indeed, some reports described that a sustained negative status of HCV-RNA for 2 or more years by long-term IFN therapy correlated with SVR in patients with genotype 1b and high viral load. However, a limitation was found in the patients with viral load over 3 Meq/ml or 500 KIU who were treated with IFN monotherapy [42, 43]. Long-term IFN therapy can be associated with an increased risk of development of adverse effects. In the present study targeted for the patients with genotype 1b and super-high viral load (>500 KIU), relatively high rates of SVR were obtained by combination therapy for 24 weeks followed by prolonged IFN monotherapy for an average of totally 56 weeks with twice-daily pre-administration of IFN-β as induction. The SVR rate of prolonged group was not inferior to 48 weeks of pegylated IFN/ribavirin combination therapy [23–25]. However, the significance of induction therapy was diluted in the maintenance protocol, because a prolonged negative HCV-RNA status led to a decrease in the relapse rate in the standard combination group.

The reason for the importance of a sustained long-term negative HCV-RNA status is unclear. One line of speculation suggests that after the disappearance of HCV-RNA in serum, HCV persists in hepatocytes. In the presence of IFN, which blocks viral production, newly infected hepatocytes would not be observed. Although the considerable variation in infected cell half-life could reflect individual differences in cellular immunity against HCV, immune control through faster killing of infected cells may have an important role in successful IFN treatment [30]. If the killing of infected cells by cytotoxic T lymphocytes functions adequately, removal of infected cells would be completed and SVR would be observed. Even if cytotoxic T lymphocytes do not function due to a quasi-species diversity and high viral load [14, 15], we could attain SVR to be sustained for 48 weeks from the time of the first

negative HCV-RNA PCR result. As normal hepatocytes turn over every 1 year and in chronic inflammation, the duration would be shorter [44, 45].

Although pegylated IFN/ribavirin combination is now available and a most promising therapy, undetectable HCV-RNA at the end of treatment is obtained in about 80% of patients with genotype 1 and high viral load [23–25]. Pegylated IFN/ribavirin combination has the advantage of a low rate of breakthrough or relapse. However, SVR would never been achieved in a residual HCV-RNA-positive patient. Thus to obtain a further high SVR rate, more high prevalence of undetectable HCV-RNA would be necessary. In the present study, a high rate (96%) of HCV-RNA-negative status was obtained by the induction of twice-daily administration of IFN-β followed by ribavirin combination therapy. Although a too small number of patients was enrolled and complicated protocols were included, our data may indicate that twice-daily administration of IFN-β followed by pegylated IFN/ribavirin combination therapy could obtain a further high rate of HCV-RNA-negative status in patients with genotype 1 and high viral load.

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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.

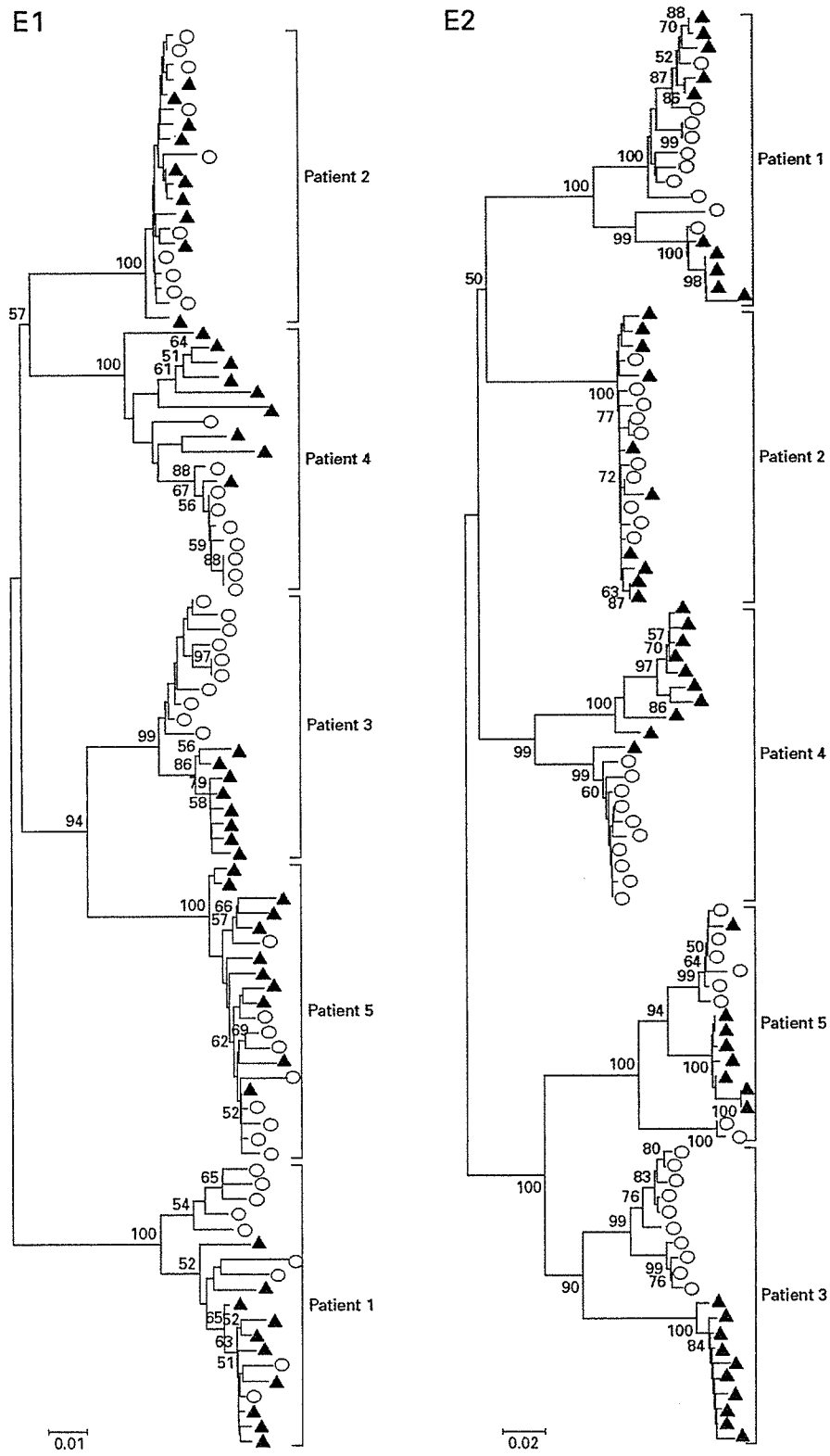
Results

Genetic Drift of HCV Quasispecies before and after Therapy

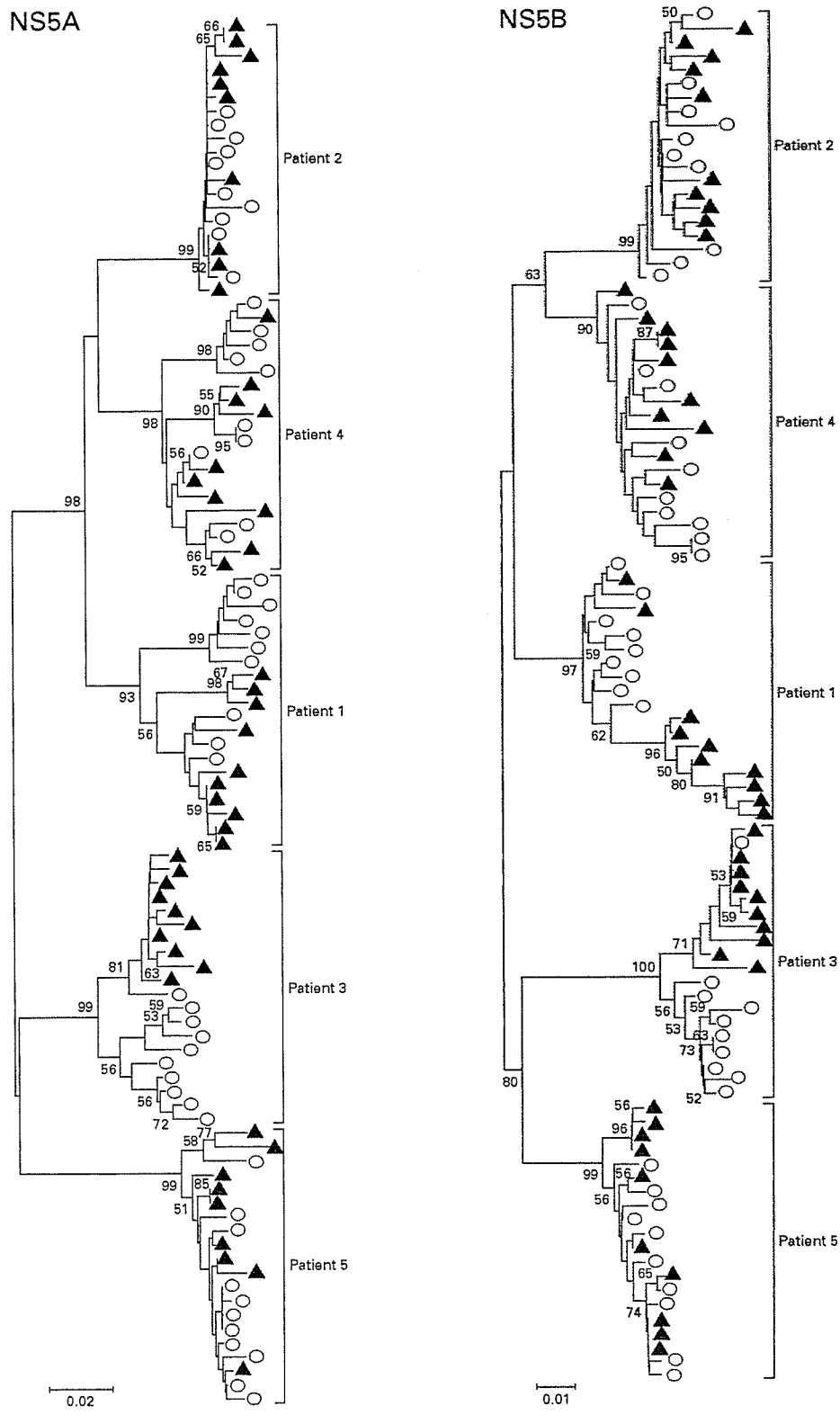
Nucleotide sequences of HCV clones in each region were aligned and phylogenetic trees were constructed (fig. 1). HCV evolution was observed in some patients in certain regions. Typical evolution, for instance, was seen in the phylogenetic tree of the E1 region in patient 3, the E2 region in patient 4, 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
NS5B
Patient 2
Patient 4
Patient 1
Patient 3
Patient 5
0.02
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

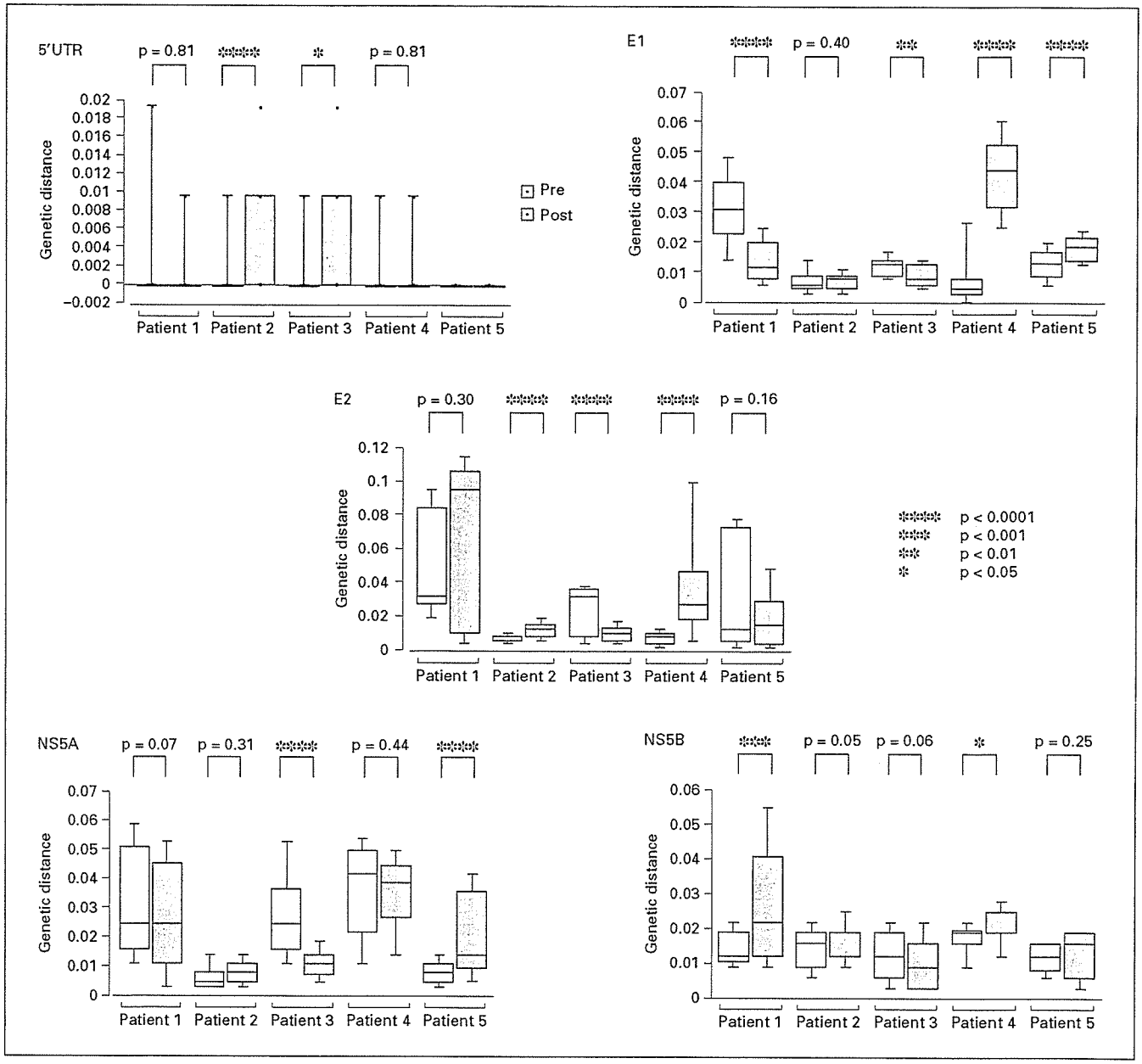


Fig. 2. Comparisons of pretreatment sample genetic distances and between-sample genetic distances. Open bars represent pretreatment sample genetic distances calculated by pairwise comparisons of nucleotide sequences of clones obtained before treatment. Closed bars represent between-sample genetic distances 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

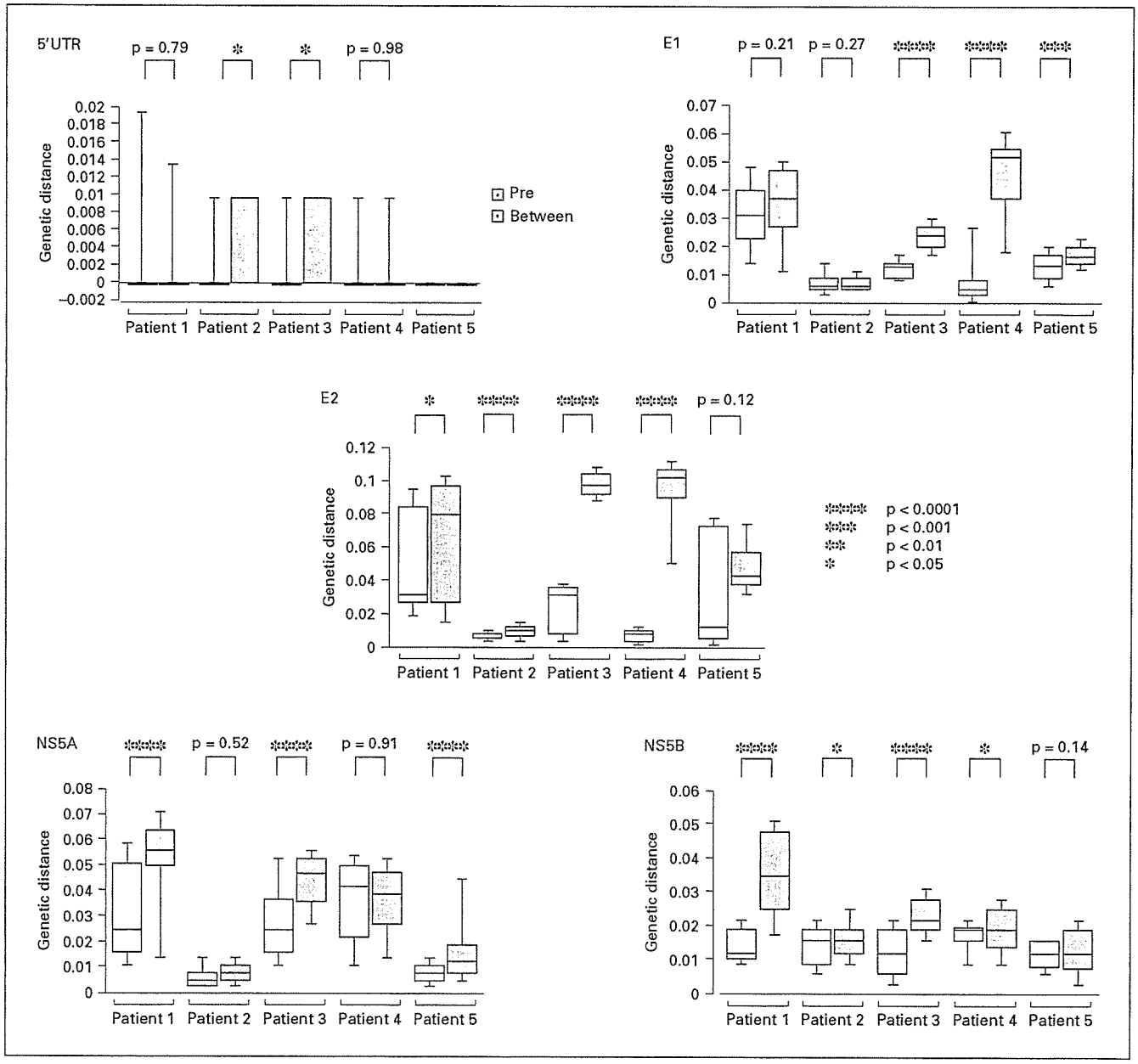


Fig. 3. Comparisons of pretreatment sample genetic distances and post-treatment sample genetic distances. Open bars and closed bars represent distances obtained by comparing nucleotide sequences of clones obtained before and after therapy, respectively. Median genetic distances are indicated with horizontal 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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