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

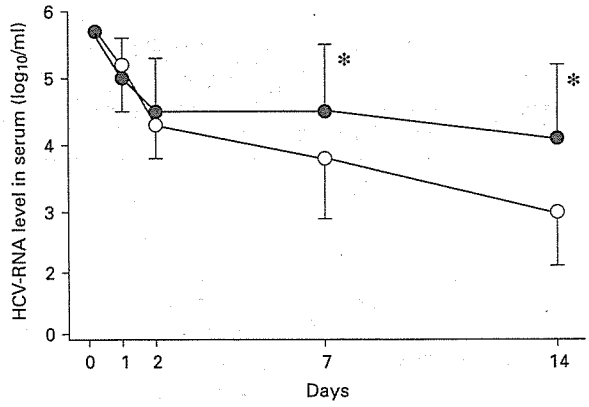

Fig. 2. Mean (±SD) of viral load in the serum during the first 14 days of treatment for patients chronically infected with HCV genotype 1b and super-high viral load (>500 KIU). The two treatment groups included: (1) twice-daily administration of IFN-β (groups 1 and 3) (○) and (2) combination therapy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

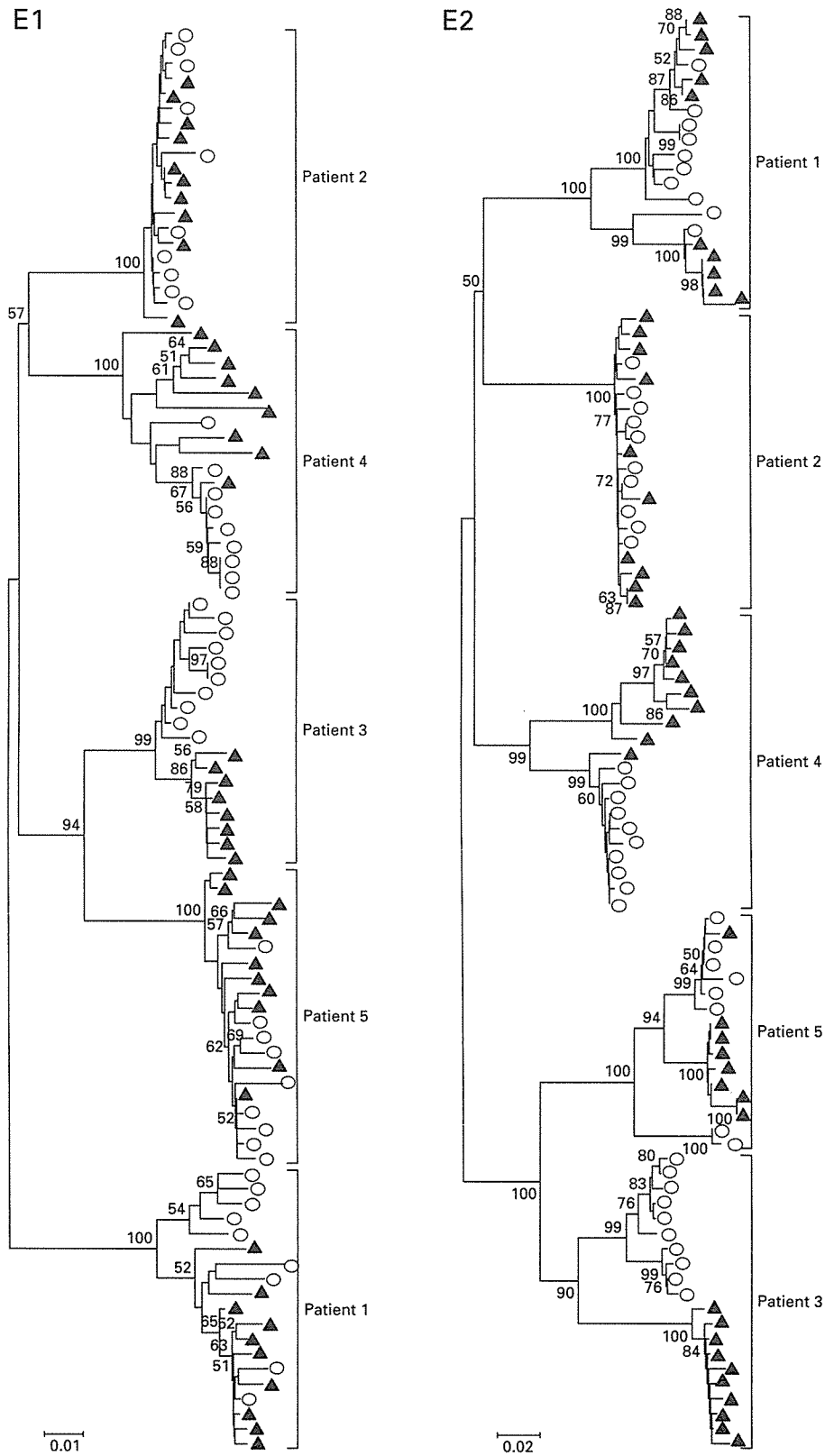
Results

Genetic Drift of HCV Quasispecies before and after Therapy

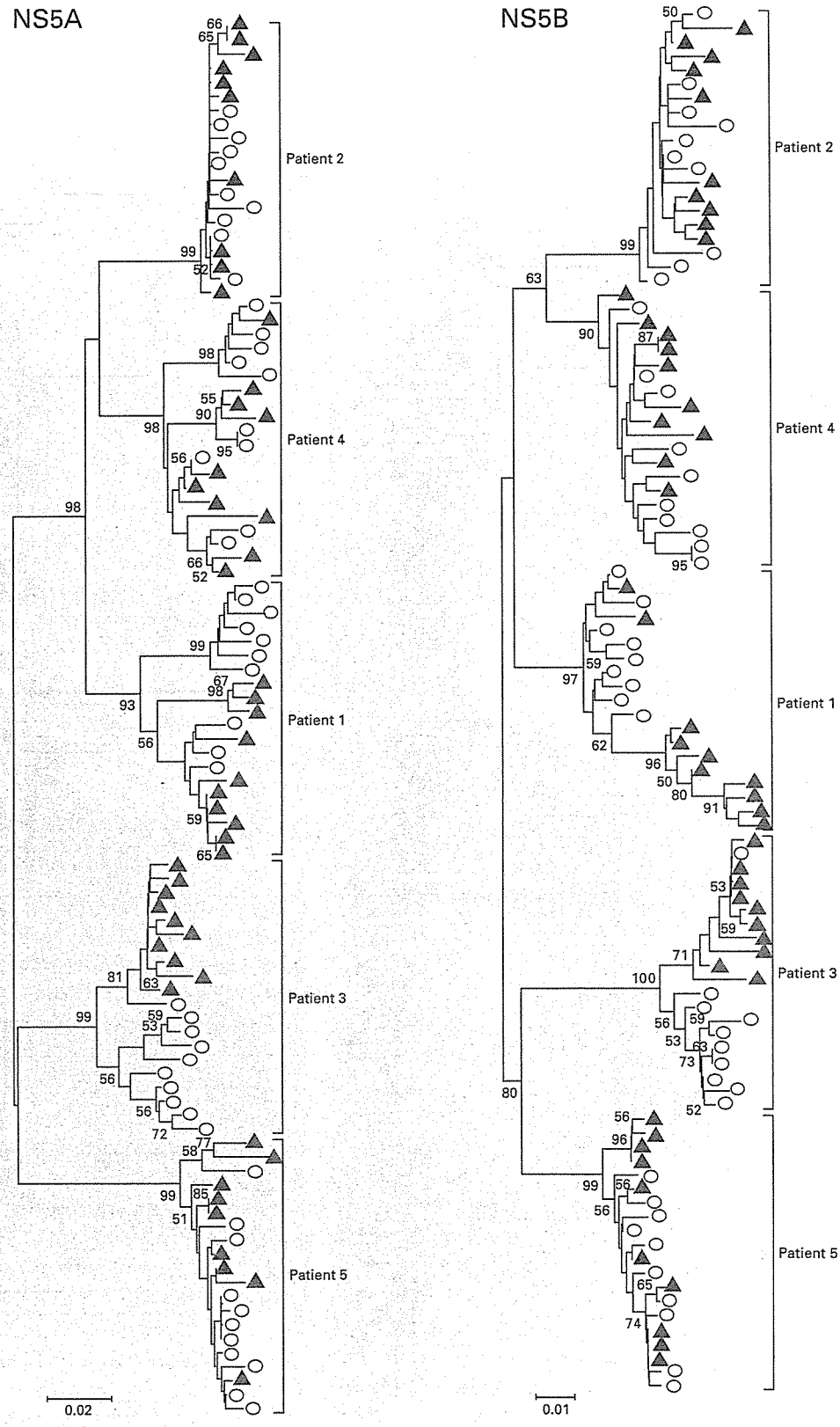
Nucleotide sequences of HCV clones in each region were aligned and phylogenetic trees were constructed (fig. 1). HCV evolution was observed in some patients in certain regions. Typical evolution, for instance, was seen in the phylogenetic tree of the E1 region in patient 3, the E2 region in patient 4,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

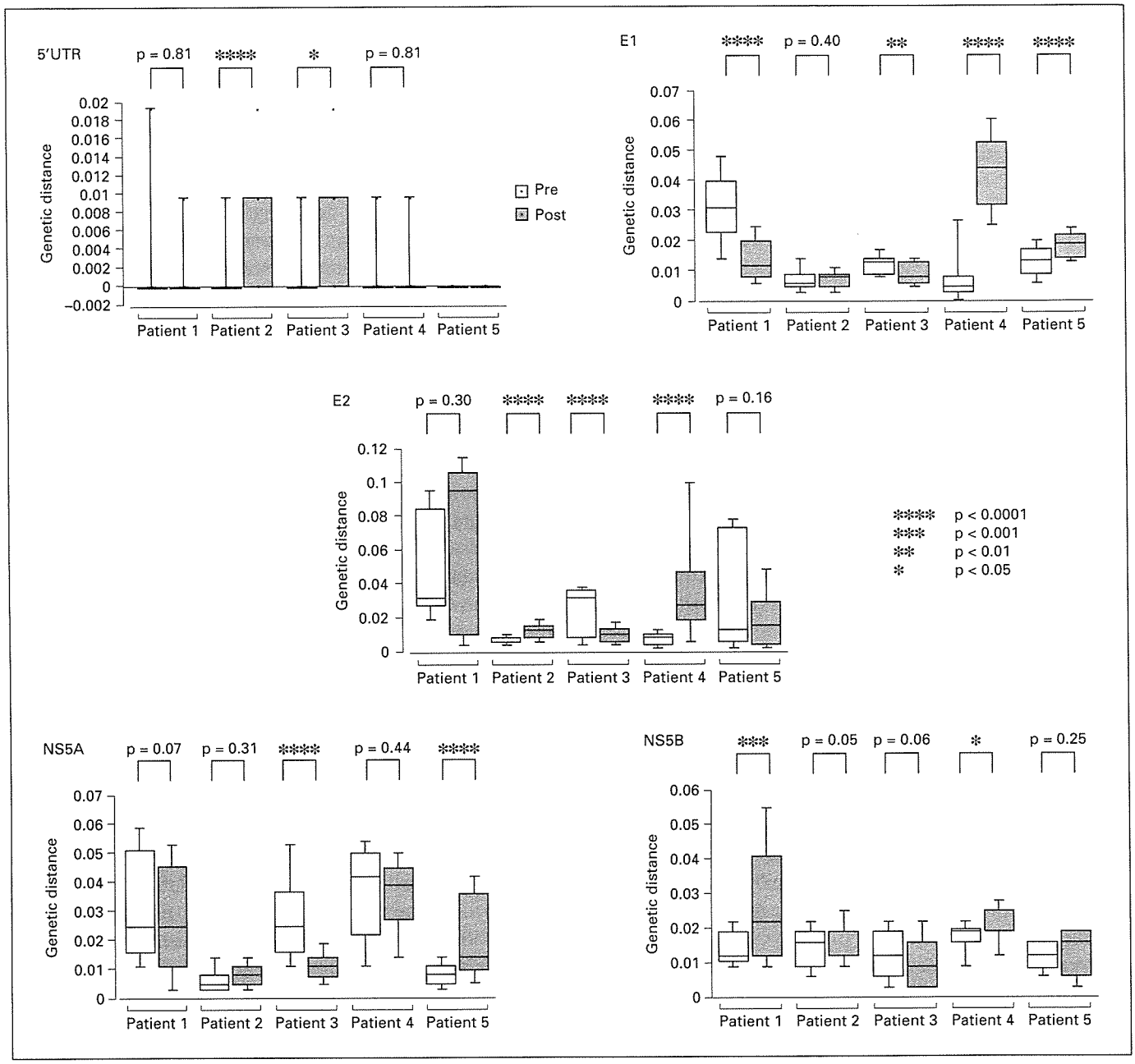


Fig. 2. Comparisons of pretreatment sample genetic distances and between-sample genetic distances. Open bars represent pretreatment sample genetic distances calculated by pairwise comparisons of nucleotide sequences of clones obtained before treatment. Closed bars represent between-sample genetic distances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

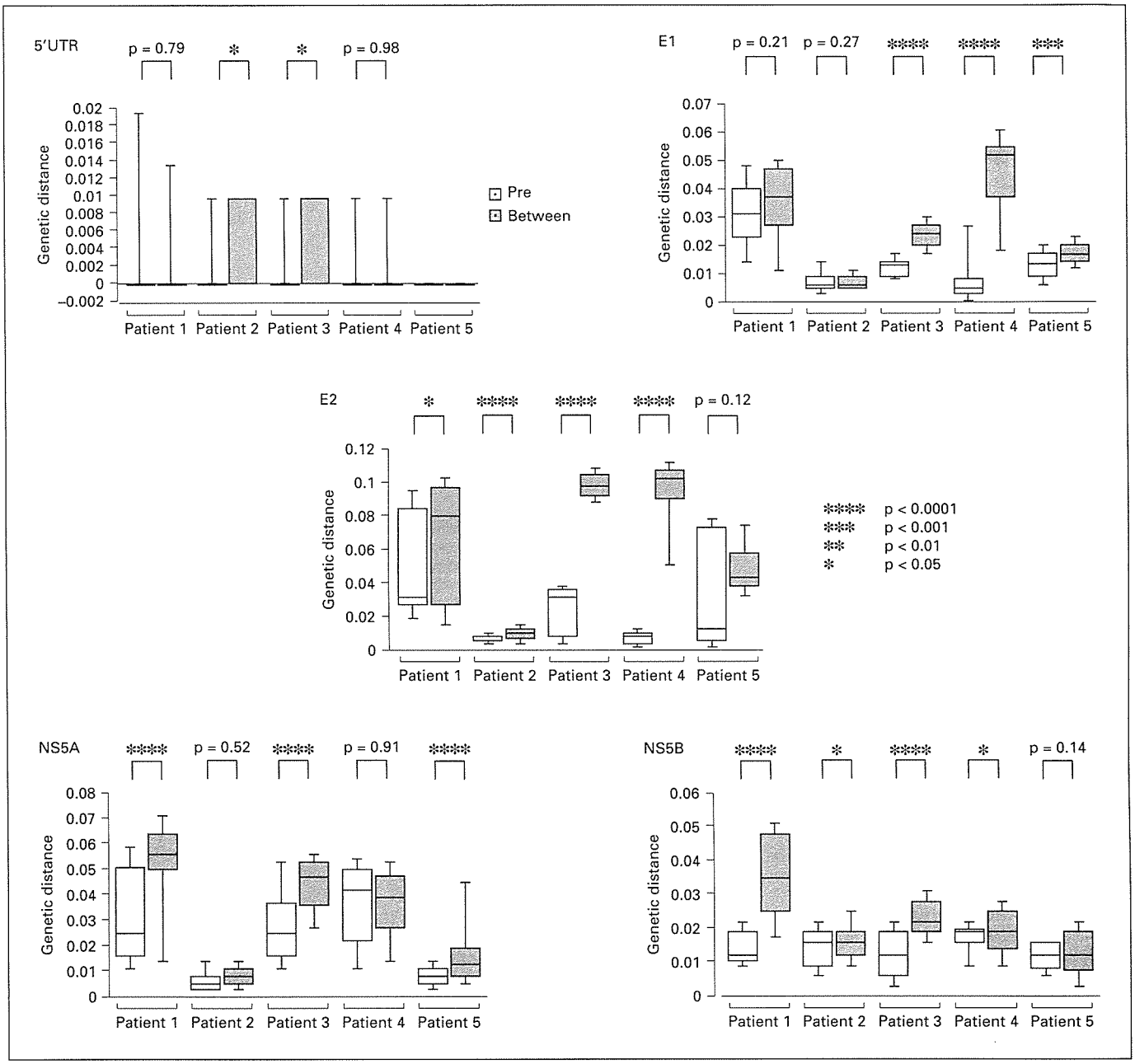


Fig. 3. Comparisons of pretreatment sample genetic distances and post-treatment sample genetic distances. Open bars and closed bars represent distances obtained by comparing nucleotide sequences of clones obtained before and after therapy, respectively. Median genetic distances are indicated with horizontal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

高橋祥一　茶山一彰*

要旨

- B 型慢性肝炎に対する治療は，若年者かつ肝線維化が軽度な症例を除き，核酸アナログの投与が第一選択となり，新規発売の entecavir は強力な抗ウイルス効果と低い耐性株出現率により，初回投与例の第一選択となる可能性が高い．
- B 型急性肝炎では，本邦に存在しなかった genotype A の感染が著明に増加している．
- C 型慢性肝炎の治療は，2004 年 12 月にはじまった pegIFNα-2b＋ribavirin の併用療法が，genotype 1b 高ウイルス量症例には 48 週間，2b 高ウイルス量症例には 24 週間の投与で，従来より高い著効率が得られている．
- 肝細胞癌の治療は，早期例には経皮的ラジオ波焼灼術，高度進行例にはリザーバー肝動注化学療法，肝予備能低下例には肝移植術，と種々の治療オプションが開発されている．

はじめに

最近，肝疾患に関する世の中の注目度が高くなり，B 型肝炎，C 型肝炎，肝癌に関する記事がマスコミに取り上げられることも多くなっている．

本稿では 2000 年以降に新たに開発，投与開始され，さらに今後展開すると思われる治療薬，治療法を中心に解説する．

B 型慢性肝炎

B 型慢性肝炎の治療目標は，① HBe 抗原陽性から HBe 抗体陽性への seroconversion，② HBV DNA の陰性化，③ トランスアミナーゼの正常化，と考えられる．しかし，B 型慢性肝炎では，ウイルスの完全な排除がむずかしいため，HBV DNA の増殖を持続的に抑制していくこと，肝炎を沈静化させて肝病変を改善し，肝硬変や肝癌への進展を防いでいくことが重要になる．

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B 型慢性肝炎治療については「B 型および C 型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究」班（厚生労働科学研究補助金　肝炎等克服緊急対策研究事業）より，B 型慢性肝炎治療の新ガイドライン 2006 が発表されている（Table 1）[1]．35 歳未満の若年症例において，HBe 抗原陽性例に対しては，自己の免疫力により HBe 抗原の陰性化や肝炎の収束が期待されるため，インターフェロン（IFN）長期間欠投与を基本的に行い，HBe 抗原陰性例に対しては原則的に経過観察を行うが，肝病変進行例では lamivudine または entecavir 投与も考慮する．一方 35 歳以上の中高年者に対しては，lamivudine または entecavir 投与が原則となる．HBe 陽性で HBV DNA が 7 LGE/m*l* 以上の高ウイルス量症例に対しては，IFN 長期間欠投与も選択される場合がある．以下に各治療法の特徴および注意点を示す．

1．IFN 長期間欠療法

B 型慢性肝炎に対する IFN 治療の保険適用は

Table 1. B 型慢性肝炎治療ガイドライン 2006

35 歳未満	HBV DNA≧7 LGE/ml	HBV DNA<7 LGE/ml
HBe 抗原陽性	IFN 長期間欠	IFN 長期間欠
HBe 抗原陰性	経過観察	経過観察
35 歳以上	HBV DNA≧7 LGE/ml	HBV DNA<7 LGE/ml
HBe 抗原陽性	① lamivudine(entecavir) ② IFN 長期間欠	lamivudine (entecavir)
HBe 抗原陰性	lamivudine (entecavir)	lamivudine (entecavir)

厳しく制限され，対象は HBe 抗原陽性例で投与期間は連日 4 週間であったため，効果は決して満足できるものではなかった．2000 年 4 月から，6ヵ月間の長期投与が可能となり，治療成績の改善が報告されるようになってきた．なお現在も HBe 抗原陽性，HBV DNA polymerase 陽性の慢性肝炎のみが保険適用となっており，HBe 抗体陽性例，肝硬変例は保険適用になっていない．またペグインターフェロン(peg-IFN)は B 型肝炎に対しては保険適用になっていない．

自然経過による seroconversion(SC)率は年率 8〜15% 程度であるが，わが国の IFN 4 週投与の治療成績では，治療終了 1 年後，2 年後の HBe 抗原陰性化 seronegative(SN)率は 29%, 55%, SC 率は 12%, 29% であり，自然経過による SC をやや早めていると考えられる[2]．IFN 4 週投与と 24 週投与の比較では，治療 6ヵ月後の SN 率は 4 週投与の 4% に対し 24 週投与の 26%，SC 率は 4 週投与の 4% に対し 24 週投与の 20% と，IFN の長期投与の有効性が報告されている．

これまでに IFN の治療効果が高くなる背景因子として，30 歳未満の若年者であること，投与前の HBV DNA が低値であること，ALT 値が正常上限の 2 倍以上に上昇してピークを超えた直後で，HBV DNA が低下傾向の時期に IFN 投与を開始すると有効率が高くなると報告されている[3]．

現在欧州では peg-IFN の 12ヵ月投与が行われ，さらに高い効果が報告されていることより[4]，今後さらに IFN の長期投与や，核酸アナログ製剤とのコンビネーション療法の成績などの報告が予想されるが，臨床的治癒の状態に制御するためには各種薬剤の長所短所を理解したうえで使用することが重要である．

IFN の副作用としては，① インフルエンザ様症状，② 汎血球減少(以上必発)，ときに ③ 間質性肺炎，④ 精神神経症状(IFNα 製剤で多い)，⑤ 甲状腺異常，⑥ 糖尿病の増悪，⑦ 蛋白尿(IFNβ で多い)，⑧ 眼底出血，⑨ 脱毛(IFNα 製剤に多い)など種々の疾患があり，間質性肺炎，精神疾患は死亡例があるため，とくに注意が必要である．

2．核酸アナログ製剤

核酸アナログとは，細胞内で宿主のデオキシリボ核酸と競合し，ウイルス由来の逆転写酵素によるウイルス DNA の合成を阻害し，ウイルスの複製を抑える働きをする逆転写酵素阻害薬のことである．もともと HIV の治療薬として開発された経緯があるが，HBV でもその増殖を抑えることがわかり，臨床応用されるようになった．

lamivudine(LMV)は，2001 年から本邦でも認可されたシトシンの核酸アナログで，4 週間投与で HBV DNA を約 2 log，8 週間投与で 3 log 低下させることが示されている．また HBe 抗原陽性例を含めて，1 年間の投与で HBe 抗原の陰性化率が 20〜30%，ALT 値の正常化率が 70〜80%，HBV DNA 陰性化率が 30〜40% と，短期間投与では非常に高い有効率を示した．しかし，投与を中止すると大部分の症例で肝炎が再燃すること，また，長期投与を行うと YMDD 変異株(YIDD,

YVDD)と呼ばれるlamivudine耐性株が出現し，5年間で半数以上の症例に耐性株が出現したという報告もある．とくにHBe抗原陽性，HBV DNA高値症例に耐性株の出現が多くみられる[5]．

adefovir(ADV)はアデニンの核酸アナログで，2004年から本邦で発売されている．adefovirはlamivudineと比較して抗ウイルス効果が若干弱く，腎障害を起こすという副作用があるものの，耐性株が出現しにくいため，lamivudineによる耐性株が出現したときの肝炎対策として，lamivudineと併用することを条件に認可されている．欧米ではadefovir単独でも十分な抗ウイルス活性があることが示されており，現在国内ではadefovir単独投与の治験中である[6]．

グアニンの核酸アナログであるentecavir(ETV)は，国内で臨床第Ⅱ相試験が行われたところ，entecavir 0.5mg/day投与でlamivudine 100 mg/dayより有意なHBV DNAの低下が認められ，entecavirの強い抗ウイルス効果が認められた．また海外での報告において，entecavirの長期投与での耐性株の出現率はlamivudineより明らかに低く，adefovirとほぼ同等であった[7]．entecavirは2006年9月から発売予定である．

いずれの核酸アナログも短期投与では強力な抗ウイルス効果を有し，非常に有効であるが，投与中止によりほぼ全例が再燃し，放置すれば劇症化することもあるため，確実に内服を継続するよう服薬指導が重要である．またadefovir，entecavirはlamivudineほどではないが，やはり耐性株の出現の報告がある．これら3種類の核酸アナログの使用法(組み合わせ方など)については，今後国内外の学会等で検討されるものと思われる．また海外では，HIV製剤であるtenofovir(TDF)やエントリシタビン(FTC)のHBVに対する有用性も報告されている．

B型急性肝炎○

成人でのB型急性肝炎の発症は，ほぼ全例が性感染と考えて差し支えなく，HBV母児感染予防事業の普及により，将来的にHBV感染はなくなると考えられていた．しかしながら，近年急性B型肝炎症例が増加傾向にあり，さらに急性肝炎症例のgenotypeは，わが国に存在しなかったgenotype Aであることが明らかになってきた．

平成17年度の熊田らによる厚労省肝炎等克服緊急対策研究事業報告によると，全国13施設のB型急性肝炎321例のgenotypeは，A：26%，B：12%，C：58%であった．これは本邦のHBVキャリアのgenotype分布において，genotype Cが約70%，genotype Bが20数%，genotype A，Dがごくわずかという結果とは異なり，genotype Aの急性感染例が著明に増加してきていることがわかる．genotype Aは本来わが国には存在しなかったtypeであり，わが国の国際化を反映した現象と考えられる．またgenotype Aはgenotype B，Cに比して慢性化しやすいことが知られている．熊田らの報告によると，genotype Aの慢性化率は14.6%であり，genotype Bの5.4%，genotype Cの4.8%に比して高率であった．したがって今後は垂直感染によるHBVの慢性化に代わり，genotype Aの水平感染による慢性化，HBVの蔓延が危惧される[1]．

一方genotype AのHBVの感染は，男性間のhomosexual transmissionでしばしば起こることが知られており，ときとしてHBVとHIVの重複感染がみられる．抗ウイルス薬であるlamivudineはもともと抗HIV薬として開発されたが，HIVに対するlamivudineの常用量はHBVに対するそれの3倍である．また現在の抗HIV療法の基本は多剤併用であり，単剤投与では容易に薬剤耐性株が出現することが知られている．したがってHBV/HIV重複感染を知らずに，抗HIV薬としては1/3量であるlamivudineの単独投与を行うと，簡単にHIVは薬剤耐性を獲得する．さらにわるいことにlamivudine耐性を獲得したHIVは，同時に他の核酸系逆転写酵素阻害薬に対する耐性を獲得してしまうのである．このためHBVの初感染例に対しては，患者の承諾を得てHIV感染の有無を確

認しておくべきである.

C 型慢性肝炎◎

C 型慢性肝炎治療と B 型慢性肝炎治療の根本的な違いは，C 型慢性肝炎治療ではウイルスの完全消失が期待できるということである．わが国では 1992 年に IFN 療法が認可されたが，当初の著効率は約 3 割程度であった(著効：IFN 投与終了 6ヵ月後の HCV RNA 陰性[SVR：sustained viral response])．のちにこの理由は，genotype による IFN への反応性の違いと，HCV ウイルス量の違いによることが判明し，genotype 1B，HCV RNA 100KIU/m*l* 以上のいわゆる難治性の HCV に対しては，IFN 単独療法の著効率は 10% 以下で，成績は非常にわるかった.

しかし 2001 年 IFN α-2b＋ribavirin の併用療法の開始，2003 年の peg-IFNα-2a 単独療法の開始を経て，今日，世界標準ともいえる peg-IFNα-2b＋ribavirin の併用療法がわが国で認可されたのが，2004 年の 12 月である．genotype 1B，高ウイルス量の患者に対し，わが国の全国治験のデータでは対象 254 人で 47.6% の著効例が得られた．これは従来の IFN＋ribavirin 併用療法における著効率：約 20% や peg-IFNα-2a 単独投与における著効率：約 28% と比較して大きな進歩といえる．また，peg-IFN は週 1 回の投与であり，従来型の IFN に比して発熱等の自覚症状としての副作用の出現頻度が低く，患者の QOL も大きく改善されている.

また，治験のデータでは以前 IFN 療法を受けて，一度はウイルスが消失したにもかかわらず，最終的にはウイルスが再出現した「再燃群」では著効率が 62.6% と高値であり，とくに一過性に IFN が効果を示した症例については著効が期待できることが示された．ウイルス陰性化時期別に著効率をみると，治療開始 4 週目までにウイルスが陰性化した症例については著効率 100%，治療開始 4〜12 週目までにはじめてウイルスが陰性化した症例は，著効率 71.1%，治療開始 12〜24 週目までにはじめてウイルスが陰性化した症例は，著効率 36.4% であった．すなわちウイルスがいったん血液検査上陰転化してから，さらにいかに長く治療継続するかが，著効が得られるためのポイントになると思われる．また薬剤の減量中止と著効率の関係は，peg-IFN と ribavirin の両方を減量なく完遂した症例は 62.5% と高値であったが，どちらか一方を減量した症例で 52〜53%，両剤減量例でも 45.7% と比較的高値であり，投与中止例の著効率 19.2% と比して著明に高値であり，減量してでも 48 週間継続することがまず肝要であるということが示されている[8].

この C 型慢性肝炎に対する標準的な治療法についても，「B 型および C 型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究」班より，C 型慢性肝炎治療の新ガイドライン 2006 が発表されている(Table 2)．このガイドラインによると genotype 1 の高ウイルス量の患者に対しては，上述のごとく peg-IFNα-2b＋ribavirin の併用療法の 48 週間投与，genotype 2 の高ウイルス量症例に対しては peg-IFNα-2b＋ribavirin の併用療法の 24 週間投与が推奨されており，低ウイルス量症例に対しては genotype に関係なく，従来型の IFN 単独療法 24 週間または peg-IFNα-2a 単独療法の 24〜48 週間投与が推奨されている.

IFN の治療効果判定は，投与終了 6ヵ月後の HCV RNA の消失をもって著効(SVR)としており，2004 年 12 月に投与開始となった症例の効果判定が 2006 年 6 月であるため，市販後の著効率はようやく最近になって判明しはじめてきている．全国規模での peg-IFNα-2b＋ribavirin の併用療法の著効率については今後の報告を待たねばならないが，おそらく genotype 1B 抗ウイルス症例に対する著効率は，40〜50% とほぼ治験と同等のデータが示されるものと思われる．欧米の報告では，投与開始後 12 週目でのウイルス陰性化が得られない症例は，著効が得られる可能性が低いため，投与を中止するよう勧告されているが，結局はウイルス陰性化が得られてから，いかに長く投