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Original Paper

Intervirology

Intervirology 2006;49:352–361
DOI: 10.1159/000095155

Received: August 25, 2005
Accepted after revision: October 26, 2005
Published online: August 21, 2006

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.

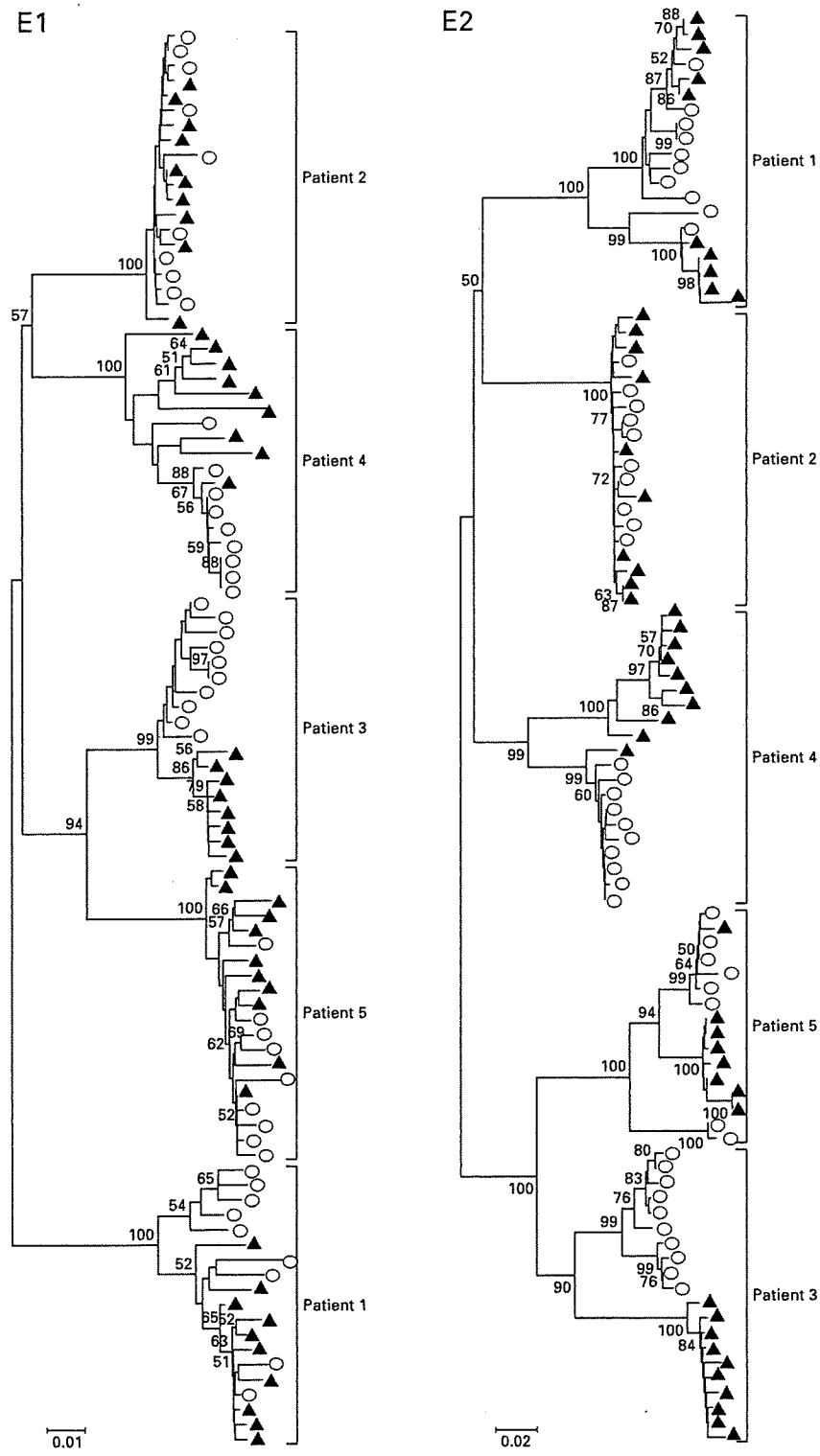
Results

Genetic Drift of HCV Quasispecies before and after Therapy

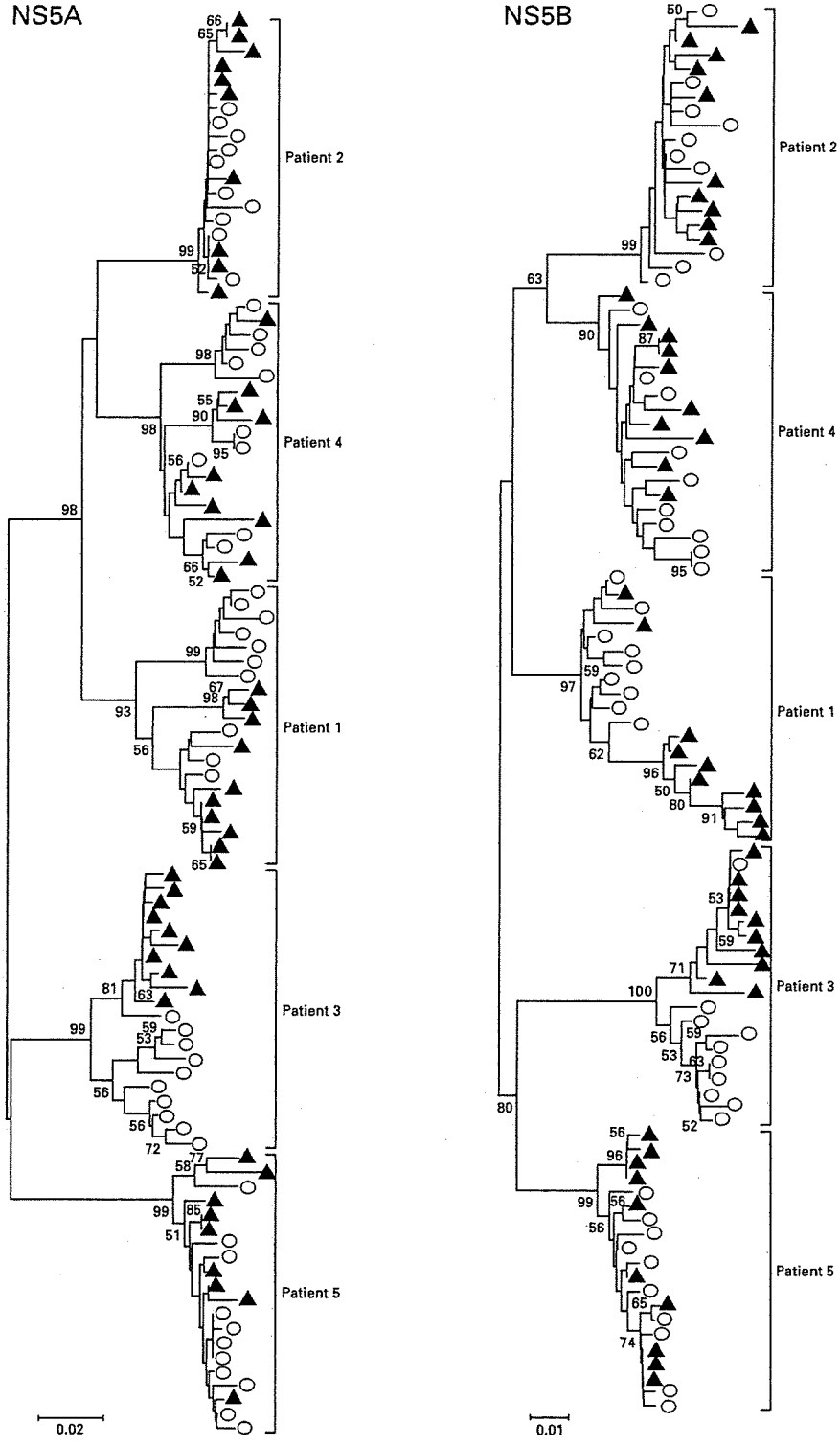
Nucleotide sequences of HCV clones in each region were aligned and phylogenetic trees were constructed (fig. 1). HCV evolution was observed in some patients in certain regions. Typical evolution, for instance, was seen in the phylogenetic tree of the E1 region in patient 3, the E2 region in patient 4,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

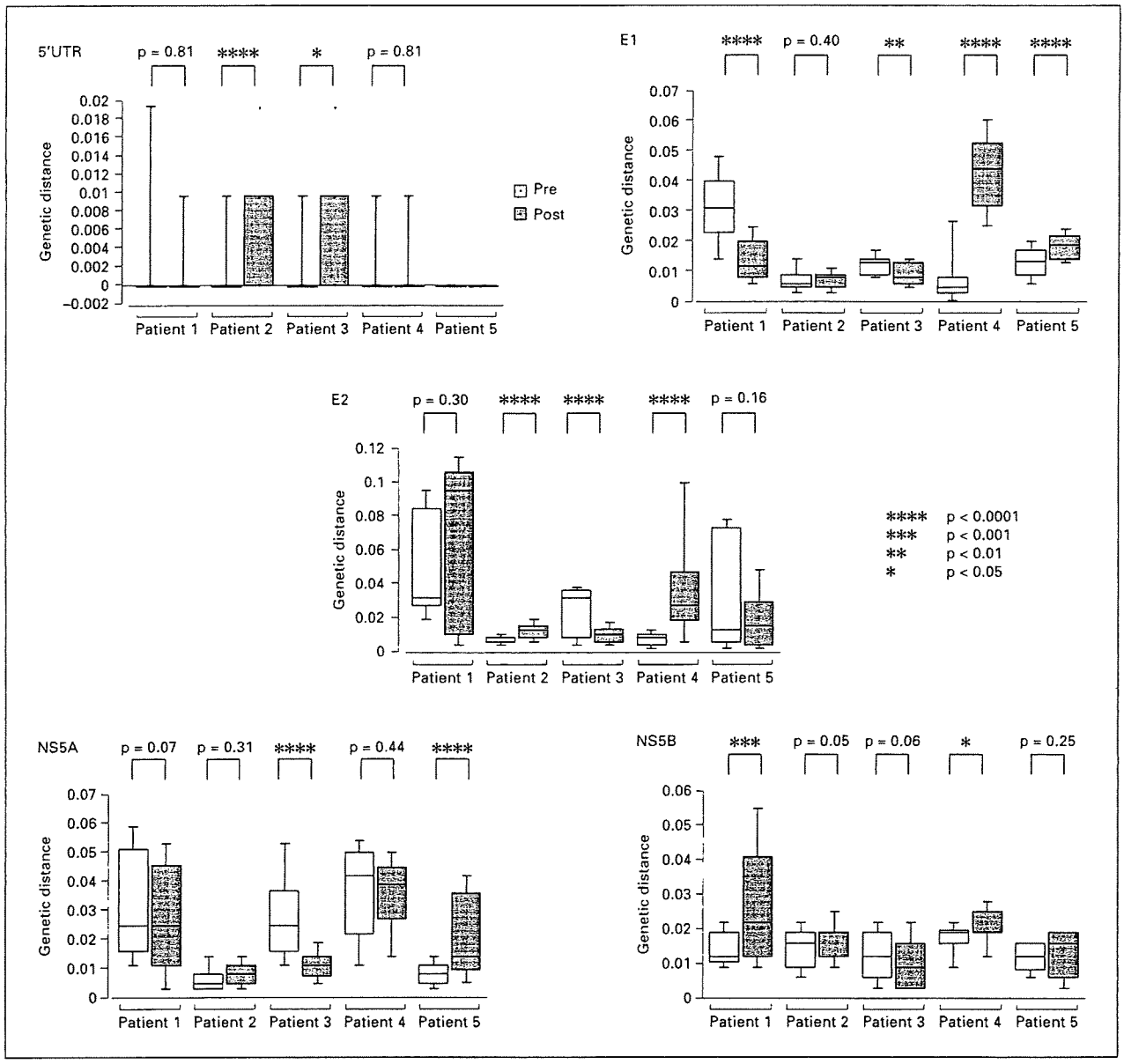


Fig. 2. Comparisons of pretreatment sample genetic distances and between-sample genetic distances. Open bars represent pretreatment sample genetic distances calculated by pairwise comparisons of nucleotide sequences of clones obtained before treatment. Closed bars represent between-sample genetic distances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

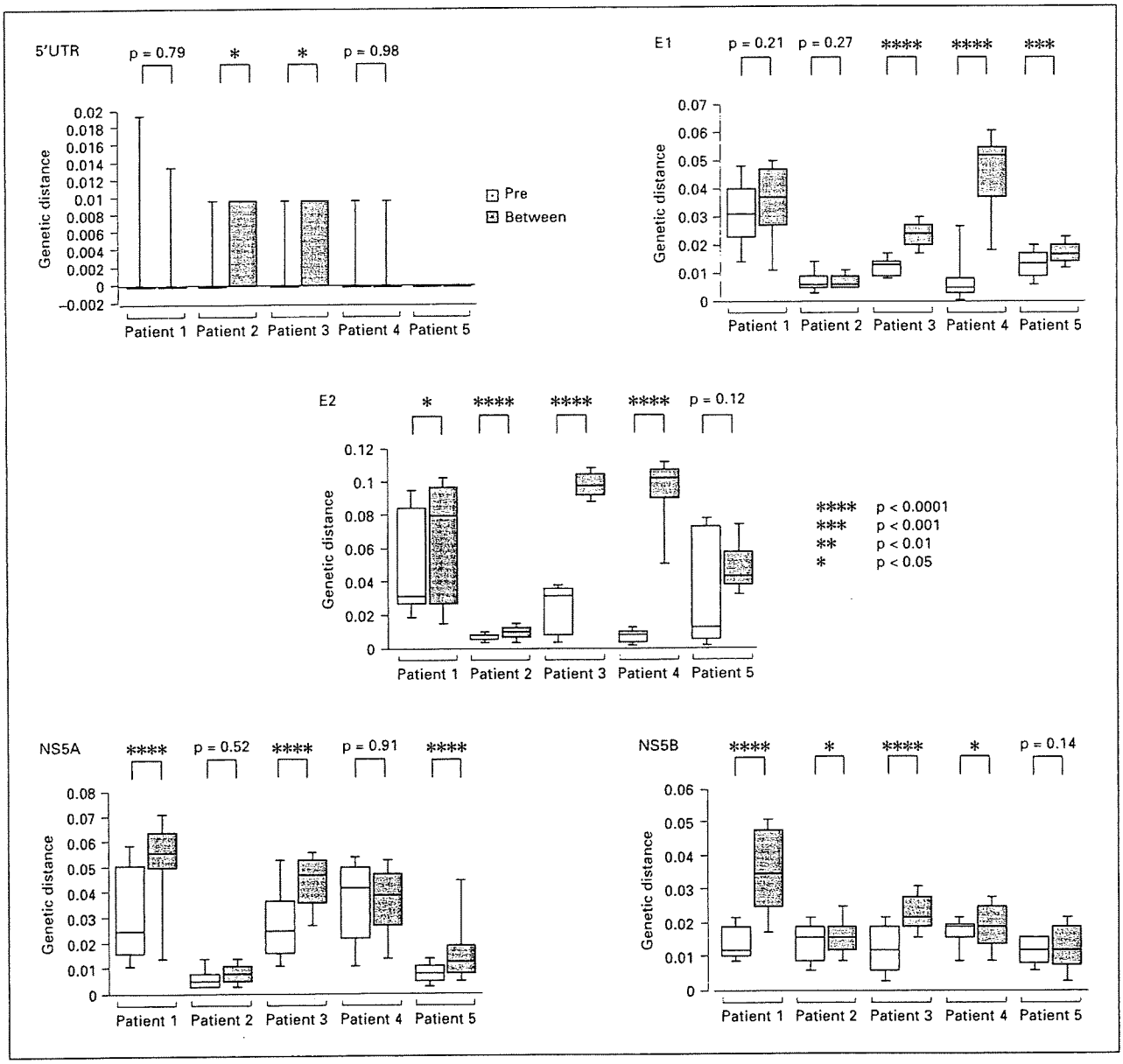


Fig. 3. Comparisons of pretreatment sample genetic distances and post-treatment sample genetic distances. Open bars and closed bars represent distances obtained by comparing nucleotide sequences of clones obtained before and after therapy, respectively. Median genetic distances are indicated with horizontal 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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Liver International 2006: 26: 90–96

Liver International
DOI: 10.1111/j.1478-3231.2005.01200.x

Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Received 19 June 2005,
accepted 8 August 2005

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

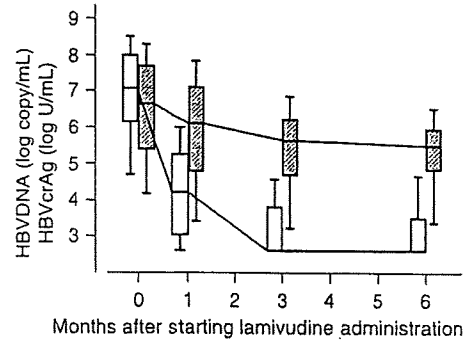

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

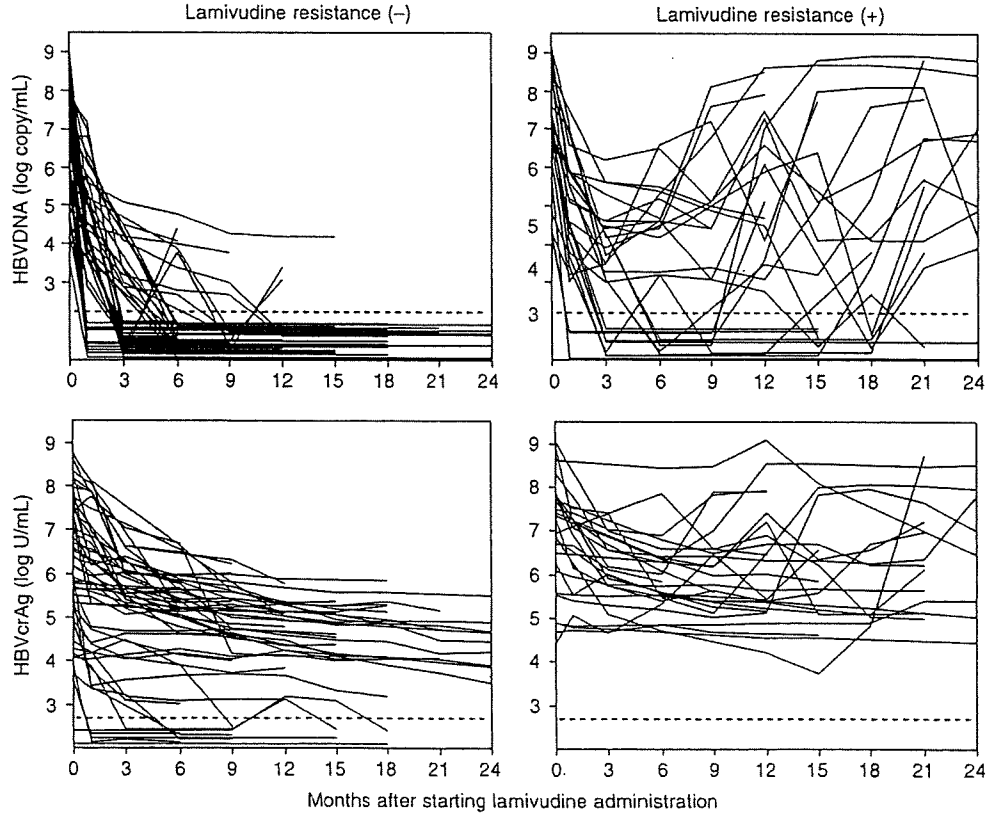

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

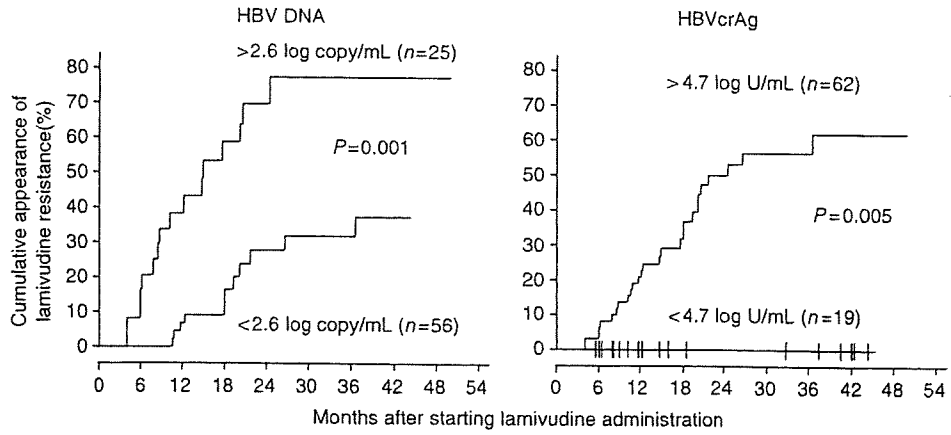

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

Acknowledgements

This research was supported in part by a research grant on hepatitis from the Ministry of Health, Labour and Welfare of Japan.

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Original Paper

Intervirology

Intervirology 2006;49:319–326
DOI: 10.1159/000095151

Received: August 8, 2005
Accepted after revision: August 18, 2005
Published online: August 21, 2006

Variations in the Viral NS5B Region in Japanese Patients with Chronic Hepatitis C Virus Genotype 1b Infection

No Specific Amino Acid Substitution Was Identified as Determinants of Treatment Response to Interferon/Ribavirin Combination Therapy

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Key Words
Hepatitis C virus · Nucleotide · Amino acids, substitutions · Interferon · Ribavirin · Genotype

Abstract
Objective: A recent study suggested that the substitution of amino acid 415 of HCV NS5B from phenylalanine to tyrosine in patients with HCV genotype 1a infection is induced by ribavirin and responsible for resistance to ribavirin therapy. The aim of this study was to evaluate whether specific variations in the HCV NS5B sequence in Japanese patients with HCV genotype 1b (HCV/1b) infection are associated with treatment response or selected by treatment with interferon-α/ribavirin combination therapy. ***Methods:*** Eighteen Japanese patients with HCV/1b infection receiving interferon-α/ribavirin combination therapy for 24 weeks were studied. Five patients treated with interferon-α monotherapy for 24 weeks were also studied as controls. The entire HCV NS5B sequence before and after therapy was determined. ***Results:*** All HCV isolates had tyrosine at position 415 of NS5B before and after therapy. Further analysis showed that no specific amino acid substitutions were identified to associate with clinical response and no specific amino acid substitutions were induced/selected by the clinical treatment. ***Conclusion:*** No specific HCV NS5B nucleotide/amino acid sequence variations, including amino acid 415 of NS5B, were identified as being associated with clinical treatment response or selected by the combination therapy in Japanese patients with HCV/1b infection.

The sequences reported in this paper have been deposited in the GenBank/DDBJ/EMBL databases (accession numbers AB189078–AB189119).

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Introduction

Hepatitis C virus (HCV) is a single-strand RNA virus of approximately 9,500 nucleotides and is a major etiology of parenteral non-A non-B hepatitis [1, 2]. Natural history studies have shown that a significant proportion of patients with chronic hepatitis C will eventually develop cirrhosis and hepatocellular carcinoma [3]. In Western countries, HCV-related end-stage liver disease is a major indication for liver transplantation [4]. In 1998, the United States Food and Drug Administration approved the interferon-α/ribavirin combination therapy for patients with chronic hepatitis C. A different treatment regimen of interferon-α/ribavirin combination therapy was also approved by the Japanese Health Authority in 2002.

Ribavirin, a synthetic guanosine analog, is an antiviral drug approved for the treatment of respiratory syncytial virus [5] and, in combination with interferon-α, for the treatment of clinically compensated chronic hepatitis C. Ribavirin monotherapy has been shown to reduce serum alanine transaminase (ALT) levels, but no significant reduction in serum HCV RNA levels was observed in most treated patients. Most patients who responded to ribavirin monotherapy relapsed biochemically after cessation of therapy [6]. Subsequently, it was shown that the combination of interferon-α and ribavirin has a much better efficacy than interferon-α monotherapy which was the gold standard of therapy for patients with chronic hepatitis C in the late 1990s [7–9]. However, in patients with HCV genotype-1 infection and high viral load, the response rate to interferon-α/ribavirin combination therapy is still at the 20–30% level, in contrast to a much higher response rate of 80% in patients infected with HCV genotypes 2 and 3 [10].

The non-structural (NS) genomic region of the HCV genome encodes the viral RNA-dependent RNA polymerase, an essential viral replicating enzyme [11]. A recent study suggested that ribavirin treatment in HCV genotype 1a infection might exert selective pressure in favor of a HCV variant with tyrosine (Y) instead of phenylalanine (F) at the 415 position of NS5B. The investigators suggested that this amino acid substitution may lead to viral resistance to ribavirin treatment, as corroborated by their in vitro studies [12].

In Japan, most patients had HCV genotype 1b infection with a high viral titer. The aim of the present study was to evaluate whether variations in the HCV NS5B region play a role in determining the clinical response to interferon-α/ribavirin therapy in Japanese patients with HCV genotype 1b infection.

Patients and Methods

In a clinical study comparing interferon-α/ribavirin combination therapy with interferon-α monotherapy, 70 Japanese patients with HCV genotype 1b infection were recruited in our centers, including 64 patients receiving the combination therapy and 6 patients receiving interferon-α monotherapy. All patients gave written informed consent. All patients were seropositive for anti-HCV and HCV RNA, and genotyping showed HCV genotype 1b infection in all patients. They were all seronegative for HBsAg and anti-HIV, and other causes of liver disease were excluded using standard clinical and laboratory criteria. All patients received treatment in Nagoya City University Hospital and its affiliated hospitals from January 2001 to March 2003. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Treatment regimen consisted of intramuscular injection of interferon-α2b (Schering KK, Osaka, Japan) at a dose of 6 million units (MU) daily for 2 weeks, followed thrice weekly injection for 22 weeks, combined with oral ribavirin 600–800 mg/day (Schering KK) based on body weight for patients with combination therapy. Patients were followed for an additional 24 weeks after cessation of therapy to determine the long-term clinical response. The 24-week treatment protocol was approved by the Japanese Health Authority back in 1999. More recently, the Japanese Health Authority has approved studies based on 48 weeks of treatment.

Clinical treatment response was classified into (1) sustained virologic response (SVR), defined as undetectable serum HCV RNA and normal ALT at week 24 after cessation of the treatment; (2) relapsers (Rel), defined as converting to negative for serum HCV RNA during therapy but relapsed to positive serum HCV RNA within 24 weeks after cessation of treatment, and (3) non-responders (NR), defined as patients who retained serum HCV RNA at the end of treatment. Of the 64 patients who received interferon-α/ribavirin combination therapy, only 45 completed the treatment without a dose reduction/discontinuation, the others had a dose reduction or treatment discontinuation due to side effects. Of the 45 patients who completed their therapy, a SVR was observed in 7 patients (15.6%), 25 patients were Rel (55.6%), and 13 patients were NR (28.9%). There were no significant differences in gender distribution, age, pretreatment ALT level, serum HCV RNA level, blood hemoglobin level, and platelet count between patients in the SVR, Rel and NR groups.

Eighteen patients with different clinical response profiles (SVR 4, Rel 8, NR 6) were randomly selected for this study. Five of the 6 patients who received interferon-α were also included to serve as controls (SVR 1, Rel 1, NR 3) (table 1). As the Japanese Health Authority did not authorize the ribavirin monotherapy arm in any clinical protocol due to a poor benefit to risk ratio, there was no access to ribavirin monotherapy for Japanese patients as controls. Serum samples before and after therapy were used for this study. The serum samples were collected and serum separated from clots within 3 h and stored at –80°C.

All patients gave written informed consent and this study was approved by the local institutional review board.

Methods

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. The extracted RNA was reverse-transcribed to cDNA using SuperScript II RNase H⁻