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A Comparison of the Exponential Decay Slope between PEG-IFN alfa-2b/Ribavirin and IFN alfa-2b/Ribavirin Combination Therapy in Patients with Chronic Hepatitis C Genotype 1b Infection and a High Viral Load

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Key Words
Chronic hepatitis C · HCV dynamics · PEG interferon · Ribavirin · Genotype 1

Abstract
Objectives: A high virological response rate can often be shown to be obtained with PEG-IFN α-2b and ribavirin combination therapy in chronic hepatitis C patients. Viral dynamics have been utilized for the evaluation of antiviral effects, especially the exponential second decay slope, which represents the elimination of infected cells. ***Methods:*** Forty-nine patients were randomly assigned to the IFN α-2b group (n = 26) or the PEG-IFN α-2b group (n = 23). Ribavirin was administered equally to both groups. Measuring the serum concentration of HCVRNA, the exponential viral decay during phase 1 and 2 was calculated. ***Results:*** The exponential decay slope in phase 2 during the first 2 weeks was greater in the IFN α-2b group than in the PEG-IFN α-2b group; however, from weeks 3 to 4, it was greater in the PEG-IFN α-2b group than in the IFN α-2b group. Interestingly, in the PEG-IFN α-2b group, the exponential decay slope was greater from weeks 3 to 4 after initiating combination therapy than during the weeks 1–2 ($p < 0.01$), despite administration of the same PEG-IFN α-2b dose (1.5 μg/kg once weekly). ***Conclusions:*** In PEG-IFN α-2b and ribavirin combination therapy, elimination of infected cells may be pronounced following an increase in serum ribavirin concentration in chronic hepatitis C patients with genotype 1b infection and a high viral load.

Introduction

Chronic hepatitis C viral (HCV) infection affects almost 4 million people in the United States, and 170 million worldwide. A large percentage of infected patients develop cirrhosis and more severe sequelae, including hepatocellular carcinoma [1, 2]. Pegylated interferon (PEG-IFN) is the most recent advance in the treatment of patients with chronic hepatitis C. PEG-IFN α-2b consists of a conjugate of straight-chain polyethylene glycol (molecular weight, 12 kD) and IFN α-2b in a 1:1 ratio [3, 4]. The main effects of pegylated proteins are to decrease clearance and prolong serum half-life, and they also reduce the immunogenicity of a number of proteins,

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which allows for once-weekly dosing and may be associated with increased efficacy [5]. In one study, PEG-IFN α-2b increased the sustained virological response rate to 25% compared with the 12% rate observed with standard IFN therapy [6]. Recently, PEG-IFN α-2b plus ribavirin has become the standard therapy for patients with chronic hepatitis C [7], and sustained virological response rates across hepatitis C genotypes ranged from 48% in patients infected with genotype 1 to 88% in patients infected with genotype 2 or 3 [7]. Ribavirin and IFN combination therapy gives a higher sustained response rate than IFN-α monotherapy, although there is little information regarding the mechanisms responsible for the increased efficacy seen with therapy using concurrent administration of different antiviral therapies in patients with chronic HCV [8]. Jen et al. [9] reported that an increase in serum ribavirin concentration after 4 weeks of treatment with IFN and ribavirin is an important factor in the improved sustained response rate.

Analysis of serum HCV dynamics has proven useful for elucidating the mechanisms of action of antiviral drugs, predicting clinical effects and optimizing treatment regimens [10]. Knowledge of vial dynamics in response to PEG-IFN α-2b and IFN α-2b may be important for understanding the mutual effect between ribavirin and IFN α-2b or PEG-IFN α-2b.

The current study assessed the viral dynamics of HCVRNA during 12 weeks of therapy with IFN α-2b or PEG-IFN α-2b plus ribavirin in patients with chronic hepatitis C with genotype 1b infection and a high HCV load. In the PEG-IFN α-2b group, the exponential decay slope was compared during the first 2 weeks and the following 2 weeks to elucidate the relationship between the viral decay slope and serum ribavirin concentration.

Materials and Methods

Patients

Forty-nine patients with biopsy-proven chronic hepatitis C at the Musashino Red-Cross Hospital from November 2001 to May 2002, who had genotype 1b infection and a high viral load (HCVRNA >100 kIU/ml by Amplicore Monitor assay; Roche Molecular Diagnostics Co., Tokyo, Japan), were included in this study. Patients with cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. None of the patients had HBs antigen or anti-human immunodeficiency virus antibody in their serum. No patients received immunomodulatory therapy before enrollment in the study. Additionally, no patient had a history of excess alcohol drinking (>80 g/day). After obtaining written informed consent, patients were randomly assigned to two groups as follows: one group received a combination therapy with IFN α-2b and ribavirin (n = 26) and the other group received: a combination therapy with PEG-IFN α-2b and ribavirin (n = 23). This study was approved by the ethical committee of Musashino Red-Cross Hospital in accordance with the Helsinki Declaration.

IFN and Ribavirin Treatment Regimen

The IFN patient group received intramuscular IFN α-2b (Intron, Schering-Plough, Kenilworth, N.J., USA), in combination with daily oral ribavirin (Schering-Plough). For the first 2 weeks of therapy, 6 MU of IFN α-2b was administered daily, after which time 6 MU was given 3 times a week for 46 weeks. Ribavirin was dosed at 600 mg daily for patients who weighed less than 60 kg, 800 mg for patients who weighed between 60 and 80 kg, and 1,000 mg for patients who weighed more than 80 kg. In the PEG patient group, 1.5 μg/kg of PEG-IFN α-2b was given once weekly for 48 weeks.

Quantitation of HCVRNA and Ribavirin in Serum

Blood was obtained from patients according to the following schedule: on day 0, blood was collected before administration of IFN and at 4 and 8 h after initiation of therapy. On days 1, 2, 4, 7, 8, 11, 14, 21, 28, 56 and 72, blood was collected before administration of IFN. Total RNA was extracted from serum, and serum HCVRNA levels were quantified at each time point by real-time detection PCR as reported previously [11, 12], using an ABI PRIZM 7700 Sequence Detection System (Applied Biosystems Japan, Chiba, Japan). The detection sensitivity of this assay is approximately 10 copies/ml, and this method was able to linearly measure HCVRNA from 1×10^1 to more than 10^8 copies/ml [11]. When HCVRNA drops below the detection limit, utilizing both the last detectable value and the date, the decay slope was calculated, because a very sensitive assay method was used in the present study which can measure 1×10^1 copies/ml. The serum concentration of ribavirin was measured by a validated high-performance liquid chromatography/tandem mass spectrometric assay using ^{13}C-ribavirin as an internal standard [13]. The lowest detection limit of quantitation for the assay was 50 ng/ml.

Statistical Analysis

Differences between the groups were assessed by the χ^2 test, Fisher's exact test, or Student's t test. One-way ANOVA and Fisher's PSLD were used for multiple group comparisons. $p < 0.05$ was considered statistically significant.

Results

Forty-nine patients (26 women and 23 men) with chronic hepatitis C (median age 54.5 years; range 38–63 years) who were infected with genotype 1 and who had a high serum HCVRNA level (>100 kIU/ml) and abnormal alanine aminotransferase levels (mean alanine aminotransferase level 78.8 IU/l; range 51–142 IU/l) were included. Of these patients, 26 were randomly assigned to receive IFN α-2b and 23 were assigned to receive PEG-IFN α-2b; patients in both groups also received ribavirin. Forty-six patients (24 IFN α-2b, 22 PEG-IFN α-2b) completed the entire treatment. Baseline characteristics of the patients in both groups are shown in table 1. All

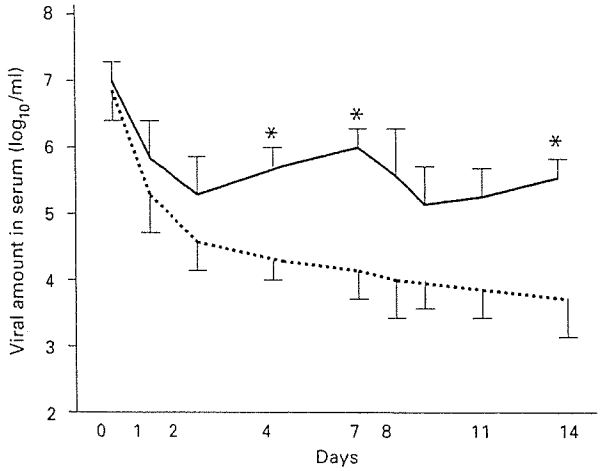

Fig. 1. Serum HCV dynamics during the initial 2 weeks between IFN α-2b (·····) and PEG-IFN α-2b (——) groups. The first sharp decline was similar between the two groups. From 48 to 168 h, a rebound increase in HCVRNA levels was observed in the PEG-IFN α-2b group. The exponential decay slope in the IFN α-2b group was greater than that of PEG-IFN α-2b group during the initial 2 weeks. * Statistically significant difference in HCVRNA levels between the two groups.

Table 1. Clinical characteristics of the patients

	IFN α-2b + RBV	PEG-IFN α-2b + RBV	p
Gender			
Male	12	11	
Female	14	12	n.s.
Age, years	54.0 ± 2.9	54.7 ± 3.4	n.s.
Treatment-naïve	18	13	
Retreatment (median)	8	10	n.s.
HCVRNA level	640	720	n.s.
NS5A			
Wild type	14	15	
Intermediate	10	7	n.s.
Mutant	2	1	
Fibrosis of the liver			
F1	13	8	
F2	7	12	n.s.
F3	6	3	
Activity of the liver			
A1	15	11	
A2 and A3	11	12	n.s.
Hemoglobin, g/dl	14.2 ± 0.4	14.0 ± 0.3	n.s.
Platelet, $\times 10^3$/mm^3	167 ± 14	160 ± 8	n.s.

RBV = Ribavirin; n.s. = not significant.

Table 2. Comparison of serum HCVRNA levels and exponential decay slope between patients treated with IFN α-2b with RBV and PEG-IFN α-2b with RBV

Exponential decay slope	IFN α-2b + RBV	PEG-IFN α-2b + RBV
Phase 1 (log_{10}/day)	2.21 ± 0.18	1.92 ± 0.25
Phase 2 (weeks 1–2) (log_{10}/day)	0.077 ± 0.0016	0.010 ± 0.011[a]
Phase 3 (weeks 3–4) (log_{10}/day)	0.016 ± 0.012	0.060 ± 0.011[b,c]

[a] $p < 0.05$ compared to phase 2 of IFN α-2b + RBV; [b] $p < 0.01$ compared to phase 3 of IFN α-2b + RBV; [c] $p < 0.01$ compared to phase 2 of PEG-IFN α-2b + RBV.

49 patients had undergone a liver biopsy within 2 months prior to therapy, and none of them showed cirrhotic lesions.

HCVRNA Dynamics

The mean baseline HCVRNA load was similar in both groups: 6.8 ± 1.3 log in patients who received IFN α-2b versus 7.1 ± 1.4 in PEG-IFN α-2b patients (p = not significant). HCVRNA levels greater than 850,000 IU/ml were detected in 23% of the IFN α-2b patients and 26% of the PEG-IFN α-2b patients.

HCVRNA dynamic results were analyzed in three phases: phase 1 encompassed the 1st day of therapy, phase 2 encompassed days 2–15, and phase 3 encompassed the 3rd and 4th week.

The serum HCV dynamics showed a biphasic pattern consisting of a rapid decrease within 24 h of initiation of treatment (phase 1), followed by a subsequent slow decrease during phase 2. At 24 h, a mean log 2.21 ± 0.18 reduction in HCVRNA was observed in the IFN α-2b group compared with a mean log 1.92 ± 0.25 reduction in the PEG-IFN α-2b group, which was not significant. From 48 to 96 h, HCVRNA levels decreased in the IFN α-2b group and increased slightly in the PEG-IFN α-2b group (0.42 ± 0.38 log, p = nonsignificant). By 168 h, HCVRNA levels gradually decreased in the IFN α-2b group, while in the PEG-IFN α-2b group, a rebound increase of HCVRNA was observed (fig. 1). Nevertheless, by the end of the 2nd week, both groups had HCVRNA levels lower than baseline with a decrease of 2.78 ± 0.34 log in the IFN α-2b group and 1.46 ± 0.27 log in the PEG-IFN α-2b group ($p < 0.05$) (table 2). The exponential decay slope in the IFN α-2b group was greater than that of the PEG-IFN α-2b group ($p < 0.05$).

HCVRNA Dynamics between Weeks 3 and 4

Between the 3rd and 4th week (phase 3), HCVRNA decreased by 0.78 ± 0.12 log in the IFN α-2b group compared with 1.4 ± 0.3 log in the PEG-IFN α-2b group ($p < 0.01$) (fig. 2). Using log-linear regression, we calculated the exponential decay slope and the viral RNA half-life in each group. The exponential decay slope from weeks 3 to 4 was greater for the PEG-IFN α-2b group than that for the IFN α-2b group ($p < 0.01$).

Interestingly, in the PEG-IFN α-2b group, the reduction of HCVRNA was greater between weeks 3 and 4 than weeks 1 and 2 ($p < 0.01$). The exponential decay slope was also greater from weeks 3 to 4 than from weeks 1 to 2 ($p < 0.01$) (table 2).

The Relationship between HCV Dynamics and Serum Concentration of Ribavirin

There was a significant positive correlation between the exponential decay slope of HCVRNA between weeks 3 and 4 and serum ribavirin concentration ($r = 0.58$, $p < 0.05$) (fig. 3). Otherwise, there were no significant associations between the viral decline slope and baseline clinical factors including gender, age, histological findings of the liver, initial viral load, platelet count and serum ALT levels (data not shown).

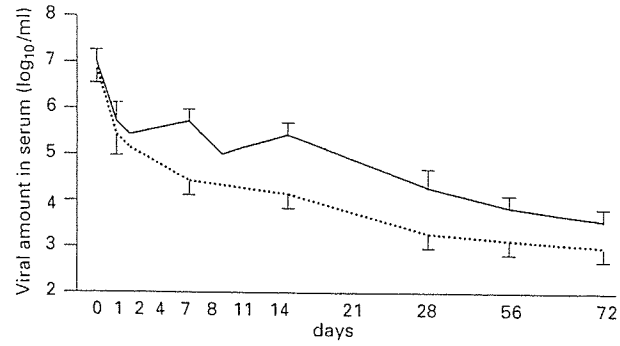

Fig. 2. Exponential decay slope from initiation of combination therapy to week 12. The exponential decay slope was greater in the PEG-IFN α-2b (——) group than in the IFN α-2b group (·····) ($p < 0.01$). In the PEG-IFN α-2b group, the exponential decay slope from week 3 to 4 was greater than that during weeks 1 and 2 ($p < 0.01$).

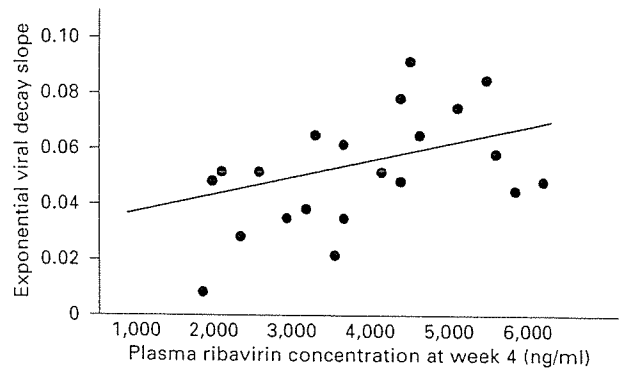

Fig. 3. Relationship between the exponential decay slope of HCVRNA during weeks 3–4 and the serum ribavirin concentration at week 4. There was a significant positive correlation ($r = 0.58$, $p < 0.05$).

Discussion

In the present study, by analyzing HCV dynamics after administration of PEG-IFN α-2b with concomitant administration of ribavirin, we demonstrated that the exponential decay slope in the PEG-IFN α-2b group was smaller during weeks 1–2 than during weeks 3–4, even though the same dosage of PEG-IFN α-2b was administered weekly. The exponential decay slope from weeks 3 to 4 was correlated with the serum concentration of ribavirin, indicating a delayed improvement in HCVRNA exponential decay of HCVRNA in the PEG-IFN α-2b group, which suggests that this is dependent on the slow increase in serum ribavirin concentration.

A previous report that evaluated HCV dynamics in the serum of patients treated with IFN-α alone described a biphasic kinetic pattern of HCVRNA decline [10, 12, 14–17]. According to the mathematical model proposed by Neumann et al. [10], the decrease of virus seen in phase 1 of clinical response in patients receiving daily IFN-α is believed to be dependent on the direct effect of IFN α-2b on virion production or release from infected target cells. The decrease of virus seen in phase 2 was proposed to reflect the presumably immune-mediated elimination of virally infected cells, in addition to the direct antiviral properties of IFN α-2b. It should be noted that the mathematical analysis of Zeuzem et al. [16] made the biological assumption that the clinical suppression of HCV seen with intermittent (i.e. 3 times/week) administration of IFN-α is associated with the suppression of de novo infection.

With regard to combination therapy, the results suggest that using ribavirin in concert with IFN therapy may

be effective for both increasing viral suppression in infected cells and preventing the reappearance of virus after the end of therapy by promoting the 'clearance' of HCV-infected cells. Although the molecular mechanisms involved in HCV inhibition by ribavirin are still poorly understood, there have been reports that ribavirin activates type 1 virus-specific cytotoxic T lymphocytes [18, 19] and endogenous effects of IFN [20]. Furthermore, peginterferon and ribavirin have been shown to enhance HCV-specific CD4+ T helper 1 responses in patients with chronic hepatitis C [21], suggesting that ribavirin – consistent with the beneficial results seen clinically – is likely to be therapeutically relevant.

Although ribavirin and IFN combination therapy has been known to give a higher sustained response rate than IFN-α monotherapy, there is little information regarding the mechanisms responsible for the increased efficacy seen with therapy using concurrent administration of different antiviral therapies in patients with chronic HCV [8]. Among the possible mechanisms for inducing a high rate of sustained response, a direct effect as a mutagen that induces error catastrophe has been postulated [22, 23]. Using the poliovirus model, ribavirin induces lethal mutational events to the poliovirus genome as a result of ribavirin triphosphate utilization by the viral RNA-dependent RNA polymerase, which induce misincorporation of ribavirin monophosphate into viral RNA [23]. The pseudobase of ribavirin (1,2,4-triazole carboxamide) can base-pair equally well with cytidine and uridine. This misincorporation of ribavirin into the viral RNA genome can promote transitions of A to G and G to A. This mutagenic effect has been shown to be dose-dependent [22], which suggests that ribavirin concentration plays an important role in its antiviral effect. Jen et al. [9] reported that an increase in serum ribavirin concentration after 4 weeks of treatment with IFN and ribavirin correlated with an improvement in the sustained response rate. Tsubota et al. [14] demonstrated that a gradual increase in the serum ribavirin concentration occurs until 4 weeks after initiating combination therapy. These facts suggest that the improvement of HCV decline observed in our study 3 weeks after starting combination treatment was due to an increase in serum ribavirin concentration. Finally, a sustained virological response was achieved in 11 patients, in whom the third phase decay slope of HCVRNA was better than that of nonresponders (0.079 ± 0.012 vs. 0.041 ± 0.010). In fact, there was a positive correlation between the exponential decay slope of HCVRNA during weeks 3 and 4 and the serum ribavirin concentration. On the other hand, the exponential decay slope in IFN α-2b group has become dull 3 weeks after starting combination treatment, and this has been suggested to be caused by a change dosing of IFN, from 6 to 3 times a week.

PEG-IFN α-2b induced a gradual decrease in HCVRNA levels for 3 weeks following the initiation of combination therapy with ribavirin, which has been suggested to be induced by an increase of ribavirin concentration.

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Changes of HCV quasispecies during combination therapy with interferon and ribavirin

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Abstract

Treatment of chronic hepatitis C virus (HCV) infection with interferon (IFN) and ribavirin improves the rate of eradication of HCV, but only about 13–14% of non-responders (NR) with HCV of genotype 1b previously treated with IFN achieve a sustained virological response (SVR). To determine whether HCV quasispecies diversity correlates with the outcome of therapy with IFN and ribavirin, we studied 13 patients undergoing combination therapy with IFN-α2b and ribavirin after failure of IFN monotherapy. HCV quasispecies diversity was assessed by cloning and sequencing before and during combination therapy. During therapy, quasispecies diversity diminished in NR patients, both in the hypervariable region (HVR) 1 of the envelope 2 (E2) domain and in the interferon sensitivity-determining region (ISDR) in the NS5A. Pre-treatment nucleotide quasispecies diversity was lower in SVR and end-of-therapy viral response (ETR) patients than in NR patients. Resistance to ribavirin was associated with high pre-treatment heterogeneity and the selection of quasispecies of the HCV genome. HVR quasispecies may be a predictor of efficacy of combination therapy with IFN and ribavirin.

Keywords: Chronic hepatitis C; Interferon (IFN); Ribavirin; Quasispecies; Hypervariable region (HVR); Interferon sensitivity-determining region (ISDR)

1. Introduction

Since its discovery in 1989, hepatitis C virus (HCV) has been recognized as a major cause of acute and chronic hepatitis, leading to liver cirrhosis and hepatocellular carcinoma [1,2]. The development of effective treatments to eradicate HCV and halt progression to cirrhosis and hepatocellular carcinoma is of great medical importance. Combination therapy with interferon (IFN)-α2b plus ribavirin results in a higher rate of sustained virological response (SVR) (35–45%) than IFN monotherapy, but only about 13–14% of non-responders and relapsers previously treated with IFN achieve a SVR [3,4]. It is important to address the question why combination therapy with IFN and ribavirin cannot eliminate HCV replication in these patients.

The precise mechanism of the action of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a purine nucleoside analogue, is not understood completely. To date, the enhancement of host immunity or direct antiviral mechanisms have been proposed as the modes of the action of ribavirin [5]. A number of studies have shown that ribavirin acts during the immune response as a modulator of the type 1/type 2 cytokine balance in favor of type 1 [6,7]. Monotherapy with ribavirin does not affect significantly HCV titers or HCV quasispecies [8], suggesting that ribavirin may enhance IFN antiviral activity through an immune modulatory mechanism [9,10]. Ribavirin also may have a direct anti-HCV effect through its incorporation into newly synthesized RNA transcripts by the NS5B polymerase [11]. More recently, Crotty et al. [12] proposed that ribavirin may act as an RNA mutagen that drives a rapid mutation of an RNA virus, leading to accumulation of defective viral genomes and suppression of viral replication or "error catastrophe" [13,14].

HCV is an RNA virus whose genome exhibits significant genetic heterogeneity as a result of the accumulation of mutations during viral replication. The resultant swarm of viruses with genetically heterogeneous but closely related

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genomes is referred to as a quasispecies [15–17]. Such quasispecies provide the flexibility for rapid adaptation of HCV to adverse environments, such as drug therapy and to evade host immune responses. The hypervariable region 1 (HVR1, amino acid position 384–410) of the envelope 2 (E2) domain of the HCV genome, which encodes the major neutralizing epitope, has been considered responsible for the generation of escape mutants [15–17]. It also has been reported that mutations in the IFN sensitivity-determining region (ISDR, amino acid position 2209–2248) in the NS5A gene are correlated closely with the response to IFN in patients with HCV 1b [18–22]. Therefore, determination of changes of HCV quasispecies in these regions from ribavirin-resistant patients should be helpful in elucidating the mechanism of action of ribavirin and resistance of HCV to ribavirin.

In this study, we examined the pre-treatment HCV quasispecies of E2–HVR and NS5A–ISDR, as well as whether the HCV quasispecies diversity decreased during combination therapy with IFN and ribavirin in patients with HCV 1b who were resistant to IFN monotherapy.

2. Patients and methods

2.1. Patients

The study group comprised 13 patients infected with HCV RNA of genotype 1b who had not responded to previous IFN monotherapy. They were negative for serum HBsAg (hepatitis B surface antigen), anti-HBc (hepatitis B core antibodies) and antinuclear antibodies and had no other causes of hepatitis, including excessive alcohol intake and hepatotoxic drugs. Liver biopsies were performed on all patients before therapy and the presence of chronic active hepatitis was confirmed histologically. Written informed consent was obtained from each patient for liver biopsy, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. They were treated with IFN-α2b (6 MU daily for the first 2 weeks followed by 6 MU three times a week for 22 weeks) and ribavirin (600–800 mg per day, according to body weight) in combination for 24 weeks. Five patients achieved a SVR with eradication of HCV, a further five patients showed an end-of-therapy virological response (ETR) but developed recurrent HCV viremia after treatment and the other three patients did not respond to the IFN–ribavirin combination therapy, with persistent viremia during treatment (no response) NR. Serum samples were obtained from each patient just before the commencement of combination therapy, and thereafter at several time points, and stored at −70 °C until use. We analyzed by cloning and sequencing the quasispecies of the HVR1 in E2 and the ISDR in NS5A in HCV RNA extracted from the serum samples taken from each patient just before therapy and 12 weeks later for the three NR patients.

2.2. RNA extraction

Serum RNA was extracted using a modified acid–guanidium–phenol–chloroform method. Briefly, 150 μl of serum were mixed with 700 μl of ISOGEN (Wako, Osaka, Japan), and the aqueous phase was extracted once with 140 μl of chloroform. The RNA was precipitated with isopropanol using 20 μg of glycogen (Boehringer Manheim, Manheim, Germany) as a carrier. The resultant RNA pellet was washed once with ethanol and finally dissolved in 10 μl of double distilled water and stored at −70 °C until use.

2.3. cDNA synthesis

Five μl of the reverse transcription mixture were adjusted to contain 1 μl of the RNA solution, 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen, Carlsbad, CA) diluted to the appropriate concentration, 10 U of RNase inhibitor (Promega, Madison, WI) and 50 pg of random hexamers (Takara, Shiga, Japan). The mixture was incubated at 37 °C for 45 min.

2.4. PCR and sequencing of cloned cDNA

The fragments of E2–HVR and NS5A–ISDR of HCV genome were amplified by nested PCR with the following primer sets. The nucleotide sequences of the primers were:

HVR 1st-sense: GCCATTTATCAGGTCACCGCATGGC;
HVR 1st-antisense: GCTCCGGGCACCCGGACGAGTTGAA;

HVR 2nd-sense: TGTAAAACGACGGCCAGT TGGTGGCGGGGGCCCACTGG;
HVR 2nd-antisense: CAGGAAACAGCTATGACC AACCTGTGTGTGTAGAACAG;

ISDR 1st-sense: TGGATGGAGTGCGGTTGCACAGGTA;
ISDR 1st-antisense: TCTTTCTCCGTGGAGGTGGTATTGG;

ISDR 2nd-sense: TGTAAAACGACGGCCAGT CAGGTACGCTCCGGCGTGCA;
ISDR 2nd-antisense: CAGGAAACAGCTATGACC GGGGCCTTGGTAGGTGGCAA.

We used an automatic hot start PCR with TaqStart antibodies (Advantage cDNA Polymerase Mix, CLONTECH, Alto, CA), according to the manufacturer's instructions. PCR schedules were as follows: for the first PCR, denaturing at 94 °C for 60 s, followed by 40 cycles of denaturing at 94 °C for 10 s, annealing at 55 °C for 10 s, and polymerization at 72 °C for 30 s. We transferred 1 μl of the first PCR product to the upper mixture of the second round PCR assay. Other conditions of the second round PCR were the same as for the first. The amplicon was purified using Mini

quickspin DNA columns (Roche, Mannheim, Germany) and ligated into the pGEM-T vector (Promega, Madison, WI). The plasmids with HCV cDNA inserts were transformed into competent *Escherichia coli* XL-2 blue cells and plated onto agar plates containing ampicillin (100 μg/ml) and incubated overnight at 37 °C. Ten clones on average per sample were picked and subjected to colony PCR.

Thereafter, both strands of the PCR products were cycle sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. The products were purified on Mini Quickspin DNA column (Roche) and sequenced using an automated DNA sequencer (model 373S, Applied Biosystems, Chiba, Japan). The nucleotide and deduced amino acid sequences were compared using the prototype sequence of HCV 1b, HCV-J [23].

Sequences were aligned and edited using CLUSTAL W [24]. Final fragments of E2–HVR were 281 bp in length (part of E1/E2; amino acids, aa, 354–446; 93 aa) including the HVR1 (located in the first 81 bp of the E2 region; aa 384–410; 27 aa) and those of NS5A–ISDR were 532 bp in length (aa 2139–2314; 176 aa) including the ISDR (aa 2209–2248; 40 aa). The consensus sequence is the sequence of amino acids the most frequently shown before and after combination therapy.

2.5. Definitions and statistical analysis

Quasispecies intrasample diversity was defined as the degree of homogeneity of sequences from the same sample. We calculated the average number of nucleotide or amino acid differences between every sequenced clone of each sample and their respective consensus sequence. First, we calculated the mean change rates (percentage changes per site per clone) of nucleotides or amino acids of the total fragments of E2–HVR and NS5A–ISDR and then we calculated the mean change rates (percentage changes per site per clone) of nucleotides or amino acids of fragments of E2–HVR only and NS5A–ISDR only. We observed that both change rates generated a similar pattern of intrasample diversity. We used the former method [25].

Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test for three groups, using StatView-J 5.0 software (Hulinks, Tokyo, Japan). A *P*-value <0.05 was considered to be statistically significant.

3. Results

The clinical features of the patients, in relation to the outcome of combination therapy with IFN and ribavirin after the failure of IFN monotherapy, are summarized in Table 1. There were no statistically significant differences between the groups in terms of age, sex, alanine aminotransferase (ALT) levels, platelet counts, liver histology, and the number of amino acid mutations in the ISDR. In the NR patients, HCV levels rapidly decreased to one-tenth to one-hundredth of pre-treatment levels 4 weeks after the start of combination therapy, and then remained stable. Also, the decrease in ALT levels was not sufficient in the NR patients. On the other hand, in the SVR patients, HCV RNA levels decreased immediately after the start of treatment and were below the limit of detection 12 weeks later. Finally, ALT levels normalized in all SVR patients and were sustained. As for the ETR patients, HCV RNA levels were below the limit of detection in four of the five patients 12 weeks later, and in one patient decreased under 100 international units (IU)/ml. In all ETR patients, HCV RNA became undetectable at the end of therapy but increased later.

Fig. 1 shows the pre-treatment amino acid consensus sequences of the HVR and ISDR for each patient. The HVR showed a variety of differences in each patient, but no distinct correlation between the sequences and the effectiveness of therapy was recognized. Meanwhile, the ISDR contained no amino acid changes (wild type) or fewer than two changes

Table 1
Clinical features of the patients in relation to the response to IFN–ribavirin therapy

	NR ($n = 3$)	ETR ($n = 5$)	SVR ($n = 5$)	*P*-value
Median age (range)	48 (32–52)	55 (52–62)	45 (35–60)	NS
Sex (male/female)	2/1	4/1	5/0	NS
Laboratory data				
Median serum ALT (IU/L) (range)	63 (31–72)	53 (45–160)	117 (43–327)	NS
Median platelet ($\times 10^4/mm^3$) (range)	18.0 (15.6–20.1)	16.0 (13.8–20.8)	16.9 (11.4–19.9)	NS
Median HCV RNA (kcopy/ml) (range)	850 (380–980)	690 (610–850)	780 (31–850)	NS
Liver histology before treatment				
Median activity score (range)	1 (0–1)	1 (1–2)	2 (1–2)	NS
Median fibrosis score (range)	2 (1–2)	1 (1–3)	1 (1–3)	NS
Median number of amino acid				
Changes in ISDR (range)	0 (0–2)	1 (0–2)	1 (0–1)	NS

Abbreviations: NR, no response; ETR, end-of-therapy virological response; SVR, sustained virological response; ALT, alanine aminotransferase; ISDR, interferon sensitivity-determining region.

```
                    384                          410
HCV-J HVR       GVDG HTHVTGGRVASSTQSLVSWLSQGPSQK IQLVNT

NR
 Patient 1      ---- S-Y---AAAGR--S-IA-LFTP-A--- ------
 Patient 2      ---- E-R-S--SQGRT-FR-TQFFTL--Q-- V--I--
 Patient 3      ---- D---S--TA-YNARG-STLF-F-A--- ------
ETR
 Patient 4      ---- Q-R----AA-FT-S--T-LF-P-SR-- ------
 Patient 5      ---- E-----AAAS-T--RFT-LF-L-SA-R ---I--
 Patient 6      ---- G-RI---QQ-RAASG-T-LFTP--T-- L--I--
 Patient 7      ---- E-YT---KAGRV-S-FT-LF----T-- ---I--
 Patient 8      ---- ---T---TA-HT-RG-T-LF-P----N ------
SVR
 Patient 9      S--- D-N-M--TAGKD-FGFA-LF-S-A--- ---I--
 Patient 10     ---- Q------N--RGA-G-N-LFAA----- ------
 Patient 11     ---- T---A--AAGRTAFR-A-IF-S-S--N ------
 Patient 12     ---- G-YT---TA-RT-RG-A-LF-S-AQ-- ---I--
 Patient 13     ---- R-YT---AQ-RT-SG-T-LF-T----- ---I--



                    2209                              2248
HCV-J ISDR      PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN

NR
 Patient 1      --------AR------------------------------
 Patient 2      ----------------------------------------
 Patient 3      ----------------------------------------
ETR
 Patient 4      ---------Y-G-----------------------------
 Patient 5      ---------R------------------------------
 Patient 6      ---------R------------------------------
 Patient 7      ----------------------------------------
 Patient 8      ---------R------------------------------
SVR
 Patient 9      ---------R------------------------------
 Patient 10     ---------H------------------------------
 Patient 11     ----------------------------------------
 Patient 12     --------I------------------------------
 Patient 13     ----------------------------------------
```

Fig. 1. Amino acid (aa) consensus sequences of the E2–HVR (aa 384–410) and the NS5A–ISDR (aa 2209–2248) before treatment of 13 patients infected with HCV 1b. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to those in HCV-J, the prototype strain of HCV 1b.

(intermediate type), and that is consistent with the clinical course of a poor response to previous IFN monotherapy [18].

The amino acid sequences of the E2–HVR quasispecies from the 13 patients are shown in Fig. 2. For the NR patients (Patient 1–3), the changes of quasispecies between pre-treatment (0W) and post-treatment (12W) samples are illustrated. Before treatment, several quasispecies were observed in each patient, but 12 weeks later the amino acid heterogeneity had decreased. In Patients 1 and 2, some HVR quasispecies were selected during combination therapy, and in Patient 3, the emergence of quasispecies quite different to those at pre-treatment was observed. Consequently, the HVR quasispecies became homogenous in each patient during treatment. However, the increase in amino acid changes, which leads to viral "error catastrophe" and is suggested to be one of the mechanism of action of ribavirin, was not seen in these patients. Pre-treatment (0W) amino acid sequences of 10 clones are shown for the ETR and SVR patients. Patients 6 and 7 of the ETR group and Patients 9, 12 and 13 of the SVR group had a variety of mutations in the HVR, and the other patients had relatively homogenous HVR quasispecies. In Patients 12 and 13, one and two amino acid insertions were observed, respectively. Collectively, there was no definite tendency for amino acid changes in the HVR in patients in the ETR and SVR groups.

Fig. 3 shows the amino acid sequences of in the NS5A–ISDR in the 13 patients. In Patient 1 of the NR group, several quasispecies were observed before treatment (0W) as seen in the HVR. The mutant type quasispecies with six or seven amino acid changes disappeared during combination therapy and the intermediate type quasispecies with one or two amino acid changes persisted. This was considered to be a phenomenon of selection of the ISDR quasispecies by the IFN treatment. Patients 2 and 3 of the NR group had homogeneous quasispecies before treatment and the amino acid differences reduced during treatment, but the type of the ISDR did not change (wild type). Consequently, the effect of acceleration of mutation by ribavirin

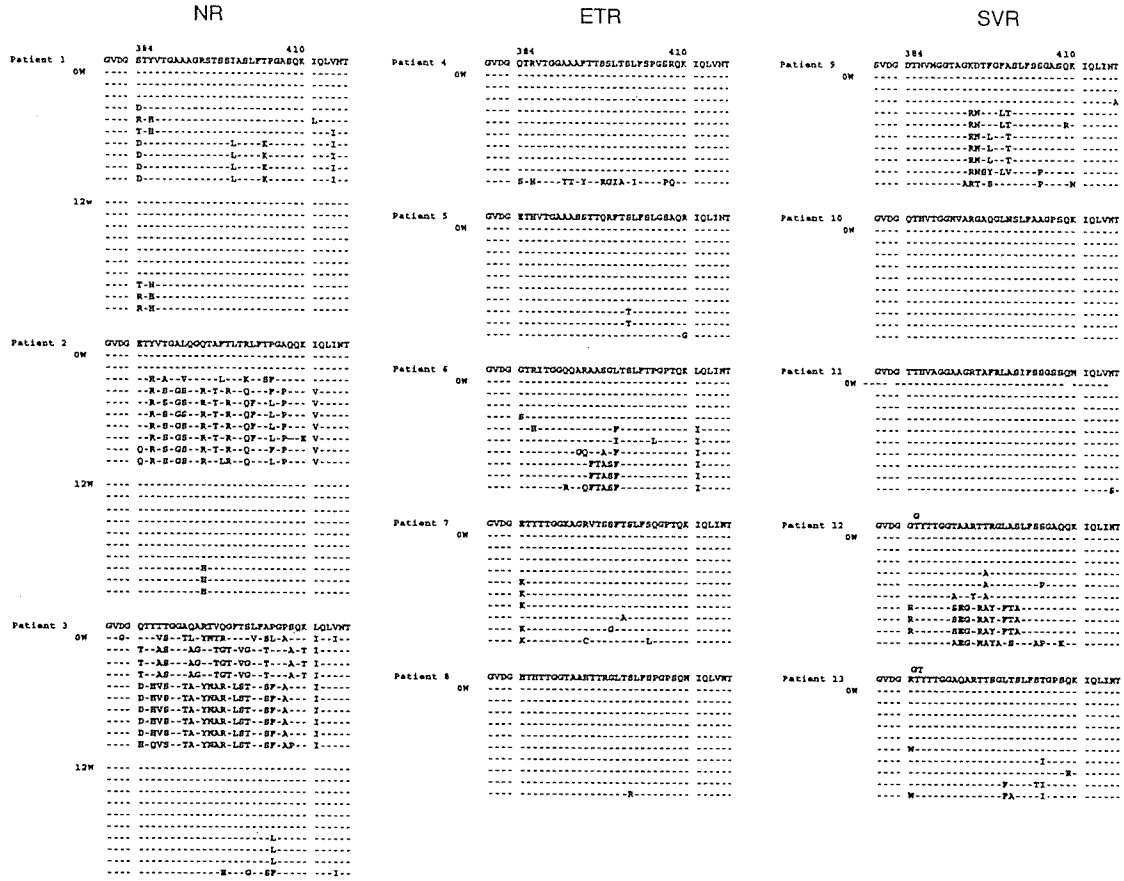

Fig. 2. Sequences of hypervariable region (HVR, aa 384–410) in 13 patients infected with HCV 1b and treated with interferon α-2b and ribavirin. The consensus sequences for Patients 1–13 are shown consecutively. The consensus sequence is the sequence of amino acids the most frequently shown before and after combination therapy. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to each consensus sequence. The v symbols above the consensus sequences of the Patients 12 and 13 indicate the positions of the insertions shown above.

was not observed and the amino acid heterogeneity around the ISDR reduced, as seen in the HVR. In the SVR and ETR patients, the variation in mutations in the ISDR was smaller than those in the HVR.

The mean rates of change of nucleotides or amino acids for each patient before and during combination therapy are summarized in Fig. 4. In the NR patients, the change rate of: (A) nucleotides and (B) amino acids for the E2–HVR reduced during combination therapy (12W) compared to pre-treatment (0W) (A, $P = 0.046$; B, $P = 0.049$). Similar trends also were observed in (C) and (D) of the NS5A–ISDR, although the results did not reach statistical significance (C, $P = 0.275$; D, $P = 0.275$). On the other hand, the pre-treatment rates of change of the HVR of the ETR and SVR patients were lower than those of the NR patients. This tendency is more distinct for the NS5A–ISDR. The pre-treatment rates of change of nucleotides in the HVR and ISDR in the ETR and SVR patients were significantly lower than those of the NR patients ($P = 0.014$ and 0.022) (Table 2). The rates of change of amino acids in both the HVR and ISDR did not differ significantly between the NR and the ETR plus SVR groups.

Table 2
Comparison of the change rates before combination therapy between the NR and the ETR plus SVR groups

	Rate of change (%)		
	NR median (range)	ETR + SVR median (range)	*P*-value
E2–HVR nucleotides	2.40 (2.1–5.1)	0.90 (0.2–2.1)	0.014*
E2–HVR amino acids	5.20 (2.8–8.3)	1.25 (0.2–5.1)	0.063
NS5A–ISDR nucleotides	1.40 (1.1–3.3)	0.55 (0.1–1.4)	0.022*
NS5A–ISDR amino acids	0.60 (0.5–3.6)	0.45 (0.2–1.5)	0.172

Change rate = percentage changes of nucleotide or amino acid per site per clone.

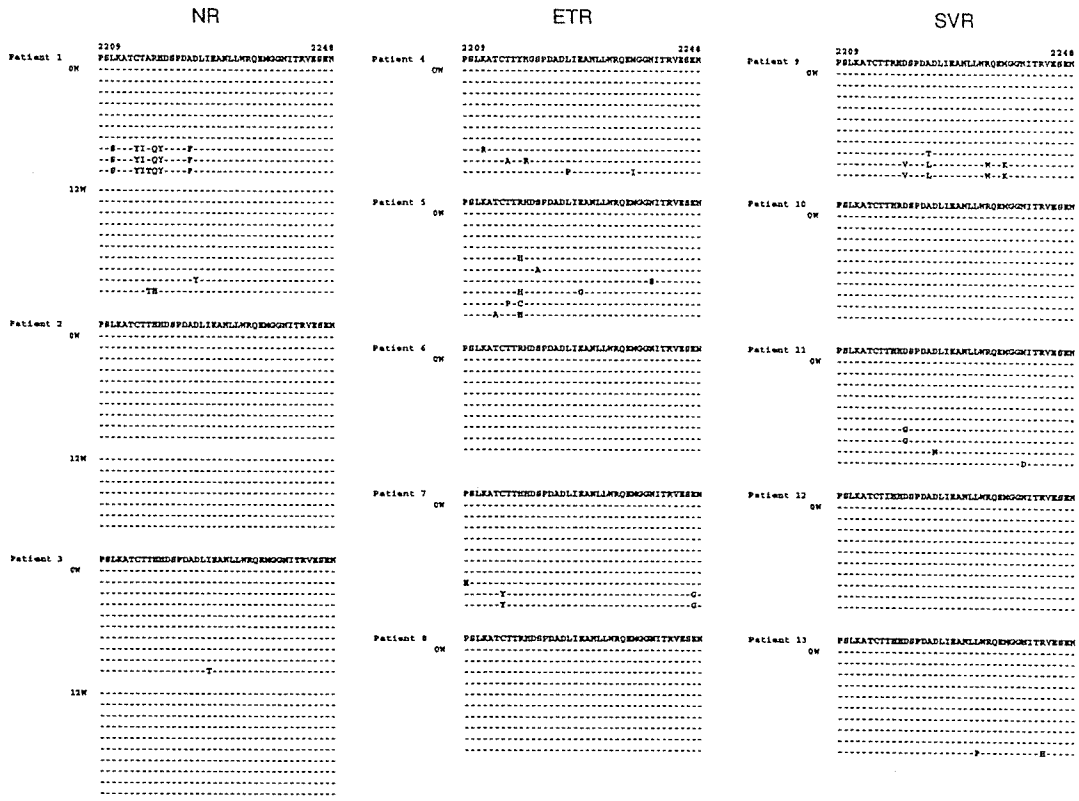

Fig. 3. Sequences of the interferon sensitivity-determining region (ISDR, aa 2209–2248) in 13 patients infected with HCV 1b and treated with interferon α-2b and ribavirin. The consensus sequences for Patients 1–13 are shown consecutively. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to each consensus sequence.

4. Discussion

In this study, we found that E2–HVR quasispecies diversity reduced significantly in NR patients during combination therapy with IFN and ribavirin. In addition, pre-treatment nucleotide changes in E2–HVR and NS5A–ISDR are more common in NR patients than SVR and ETR patients. These findings indicate that HCV quasispecies play an important role in resistance to combination therapy.

Reduction of HVR quasispecies diversity in NR patients indicates that quasispecies selection occurs during combination therapy. The HVR shows a high degree of variation in the amino acid sequences constituting a neutralizing epitope probably presented on the surface of the virion as a part of viral envelope, which is susceptible to various kinds of pressure such as host immune pressure or antiviral agents. Therefore, the changes in HVR quasispecies composition reflect the selection of escape mutants [10,26]. In fact, previous studies have also confirmed the reduction of HVR quasispecies during IFN monotherapy and the correlation between quasispecies diversity and IFN responsiveness [9,16,17]. Although the mechanism of action of combination therapy has not been elucidated completely, enhancement of a HCV-specific T-helper (Th)1 response [6,7], or more recently, the mutagenic effects of ribavirin on the HCV-RNA genome resulting in "error catastrophe" [12,14] have been postulated. Regarding the former possibility, enhancement of the immune response could accelerate the selection of immune escaping quasispecies, leading to reduced quasispecies diversity [5,6,10]. In the latter case, ribavirin sensitive quasispecies would be mutated and eliminated rapidly, because turnover of HCV replication in vivo is assumed to be less than several hours, so that detection of mutated HCV genomes by PCR cloning from serum may be difficult.

More importantly, HCV quasispecies that are resistant to the effects of ribavirin survive preferentially and selection will take place. In this case, HVR variation is merely a surrogate marker of various quasispecies with distinct genetic features outside the HVR. What kind of genetic features confer susceptibility to ribavirin has not been elucidated yet, but ribavirin is a nucleoside analogue acting during RNA synthesis and substantial evidences has been reported that the HCV–NS5B protein, which is the RNA-dependent RNA polymerase in HCV replication, has an affinity for ribavirin and ribavirin is incorporated into nascent RNA [11]. NS5B variations may be the primary determinant of ribavirin susceptibility for each HCV strain. In this regard, genetic analysis of the HCV–NS5B region and correlation with sensitivity to ribavirin is required.

On the other hand, a tendency for a reduction of ISDR quasispecies in NR patients was observed in our study. IFN monotherapy also influences the quasispecies as a result of

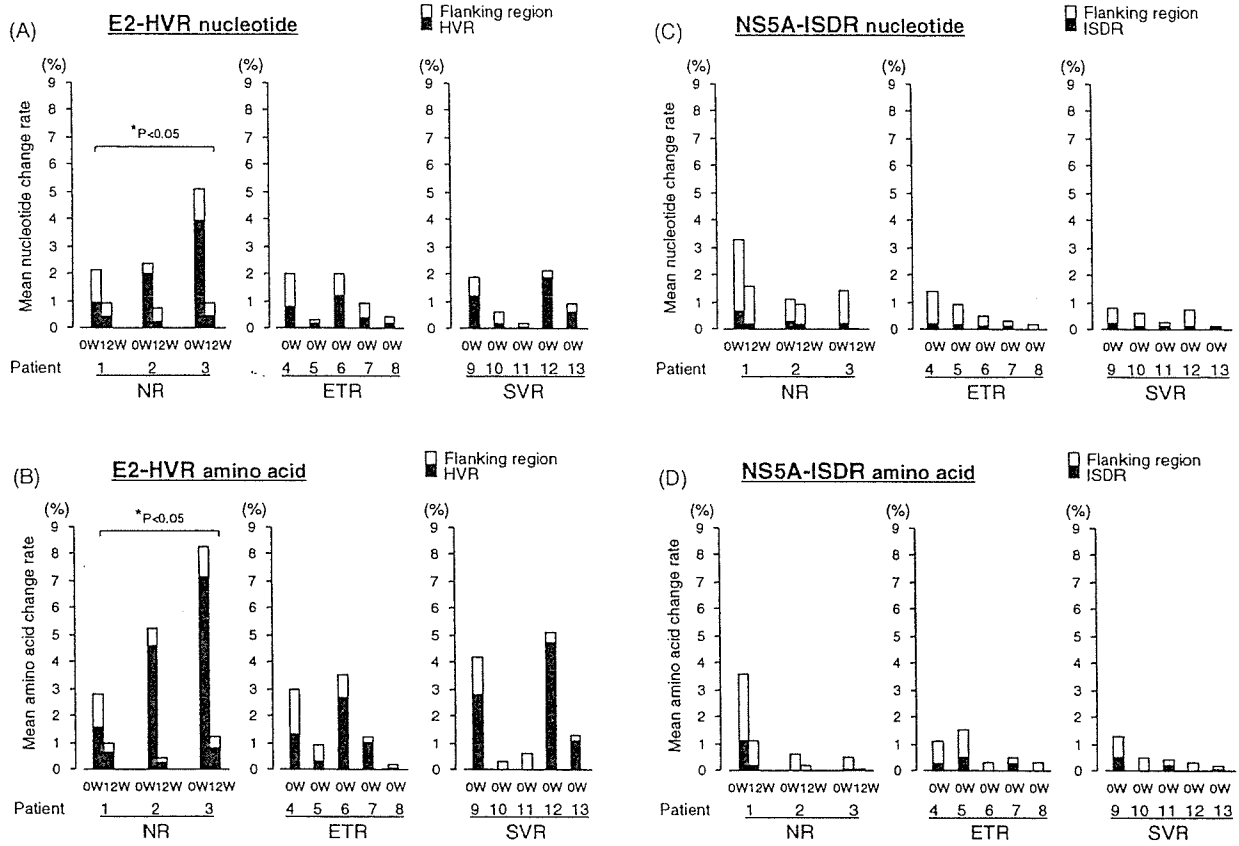

Fig. 4. Mean rates of change of nucleotides and amino acids of the E2–hypervariable region (HVR) and NS5A–interferon sensitivity-determining region (ISDR) for each patient. Change rate = percentage changes of nucleotide or amino acid per site per clone. The entire E2–HVR fragment is 281 bp in length (93 amino acids, aa, 354–446) including the HVR1 (aa 384–410) and the entire NS5A–ISDR fragment is 532 bp in length (aa 2139–2314; 176 aa) including the ISDR (aa 2209–2248). The flanking regions are excluding HVR or ISDR from the entire fragment. (A) The pre-treatment (0W) mean nucleotide change rates of E2–HVR were 2.1, 2.4 and 5.1% in the NR patients, and decreased to 0.9, 0.7 and 0.9% 12 weeks after the beginning of IFN–ribavirin therapy ($P = 0.046$). (B) The pre-treatment mean amino acid change rates of E2–HVR were 2.8, 5.2 and 8.3% in the NR patients, and decreased to 1.0, 0.4 and 1.2% 12 weeks after the beginning of IFN–ribavirin therapy ($P = 0.049$). (C) and (D) In the NR patients, mean rates of change of nucleotides and amino acids of the NS5A–ISDR also were decreased 12 weeks after the beginning of IFN–ribavirin therapy, but not significantly (C, $P = 0.275$; D, $P = 0.275$).

its direct antiviral and immunomodulatory activities [27,28]. It is well established that the ISDR in the NS5A protein is a determinant of IFN sensitivity [18,29]. A homogeneous viral population before combination therapy might be the result of prior selection of an IFN-resistant strain during the first cycle of IFN therapy. However, because no patients in this study responded to IFN monotherapy, these results indicate the additional effect of ribavirin. To investigate the effect of ribavirin, single-strand conformation polymorphism (SSCP) was used to analyze the evolution of the genetic heterogeneity of HCV in relation to the anti-HCV humoral response in patients treated with ribavirin alone [30]. The conclusion was that ribavirin monotherapy had no impact on the ISDR sequences. Collectively, resistance to combination therapy in NR patients results from the evasion of error catastrophe or enhanced immune pressure by some mechanism and the selection of the ISDR quasispecies.

We also showed that pre-treatment mean rates of nucleotides change in both the HVR and ISDR are higher in NR patients than SVR and ETR patients. This finding indicates that more complex quasispecies exist in the HCV population of NR patients and more strains with resistance to antiviral agents are present. No statistical significance in pre-treatment amino acids change rates means that synonymous substitutions were dominant in the process of selection. In addition to the well known pre-treatment variables associated with resistance to combination therapy, such as genotype 1, high serum HCV RNA levels, and severe fibrosis [3], the genetic heterogeneity of HCV may influence the response to treatment. Since all of the patients analyzed in the present study were non-responders to the previous IFN monotherapy and had HCV with IFN-resistant wild or intermediate ISDR, the HVR heterogeneity seems an important predictor of the response to combination therapy in such clinical settings.

The changes in quasispecies composition during combination therapy with IFN and ribavirin have been investigated clinically, principally by indirect methods, such as SSCP analysis [31] or heteroduplex tracking assay [32]. These studies reported somewhat conflicting data, but many of

them showed that no significant changes occur during combination therapy. The discrepancy between this study and those could be attributable to the different methods used. Our PCR cloning quantifies directly the quasispecies diversity, but other methods based on electrophoresis have a lower sensitivity because HCV fragments with different sequences may have the same electrophoretic pattern, especially in one-dimensional systems. Thus, these methods are merely qualitative surrogate analysis, underestimating the changes in quasispecies, and cloning and sequencing of each quasispecies, as in this study, is the gold standard. Only a relatively small number of patients were evaluated in this study, further studies of a large number of patients with a large number of serial serum samples are needed to draw firm conclusions about the impact of HCV quasispecies on IFN–ribavirin combination therapy.

In conclusion, during combination therapy with IFN and ribavirin the HCV quasispecies diversity diminished significantly in NR patients. This suggests that resistance to ribavirin is associated with the selection of HCV quasispecies under greater immune pressure with no incidence of error catastrophe. Also, the heterogeneity of HCV quasispecies could be a predictor of resistance to combination therapy.

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Development of Hepatocellular Carcinoma after Interferon Therapy in Chronic Hepatitis C

Is It Possible to Reduce the Incidence by Ribanirin and IFN Combination Therapy?

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Key Words
Chronic hepatitis C · Hepatocellular carcinoma · Interferon · Ribavirin

Abstract

Objectives: Although the incidence of hepatocellular carcinoma (HCC) has been shown to be reduced after interferon (IFN) monotherapy in chronic hepatitis C, the risk factors for the development of HCC have not been fully understood. The aim of this study is to investigate the risk factors for the development of HCC after IFN in chronic hepatitis C as well as whether the incidence of HCC will be reduced by ribavirin and IFN combination therapy or not. ***Methods:*** 495 patients with chronic hepatitis C and which received IFN monotherapy were followed and the incidence and risk factors for the development of HCC were examined. On the other hand, in the patients which received ribavirin and IFN combination therapy, the sustained response rate was assessed and the reduction rate of HCC development was predicted. ***Results:*** Multivariate analysis by the Cox proportional hazard model revealed that the risk factors for HCC development were age, male gender, severe fibrosis and outcome of IFN therapy. On ribavirin and IFN combination therapy, the sustained response rate reached 17.3% in genotype 1b and 74% in genotypes 2a and 2b infection, thus reducing 20% of the estimated incidence of HCC. ***Conclusion:*** To reduce the incidence of HCC in chronic hepatitis C, improvement of the sustained response rate is an essential issue, and ribavirin and IFN combination therapy shows to be promising.

Introduction

Hepatitis C virus (HCV) infection is a major risk factor for the development of both liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Recent epidemiological data highlight the fact that HCC associated with long-term HCV infection in a serious health care problem in regions such as Japan where HCV is widely endemic [2]. In Japan, HCV infection consists of 80% of the cause of hepatocellular carcinoma.

Interferon (IFN) monotherapy has been performed since 1992 in Japan for the treatment of hepatitis C which results in viral eradication in approximately 20–30% of the patients who received at least 6 months' treatment [3]. The viral eradication rate has been shown to be closely

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associated with genotype and viral load as well as viral mutation in genotype 1b infection [4]. An important question is whether IFN therapy is effective in reducing the incidence of HCC in the patients with chronic hepatitis C. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders; thus, improving the response rate is an essential issue to reduce the incidence of HCC.

Rivabirin and IFN combination treatment has been used in patients with chronic hepatitis C, which showed improvement of the sustained response rate from IFN monotherapy [6]. In Japan, this combination therapy is allowed for the treatment of patients with chronic hepatitis C at a limited duration of 24 weeks; however, the sustained response has been shown to improve especially in genotype 1b infection.

In the present study, the incidence and risk factors of the development of HCC after interferon therapy were examined. The reduction of occurrence in HCC was predicted after 24 weeks' treatment with ribavirin and IFN combination therapy.

Table 1. Clinical characteristics of the patients who received IFN monotherapy

Gender	
Male	282
Female	213
Age (mean ± SE)	52.3±0.57
Genotype	
1b	249
2a	63
2b	39
Unknown	141
HCVRNA level, kIU/ml (median 470)	1.1 to >850
Liver biopsy	
F1	132
F2	184
F3	123
F4	35
Total dose of IFN, MU (mean ± 53)	498±53
Outcome of IFN therapy	
SVR	155
NR	312
Development of HCC	
Yes	30
No	464

Patients and Methods

IFN Monotherapy Study

The first IFN monotherapy study included 495 consecutive patients with chronic hepatitis C in whom 24 weeks of IFN monotherapy was carried out from January 1994 to December 2001. The clinical characteristics of the patients are shown in table 1. The mean age is 52.3 years, and the HCV genotype was examined using the mixed-primer method [7]. Plasma level of HCVRNA was measured by amplicore monitor (version 2, Roche, Basel). The histological findings were classified according to established international criteria [8]. The median dosage of administered IFN was 640 MU, and sustained virological response (SVR) was defined as negative HCVRNA 6 months after interferon therapy and 155 patients achieved SVR. Otherwise, the patients were defined as non-responders. This study was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and written informed consent was obtained from all the patients included in this study.

The diagnosis of HCC was established by CT scan during hepatic arteriography (CTHA) and arterio-portography via the superior mesenteric artery as well as needle biopsy of the nodule. Development of HCC was observed in 30 patients during the observation period.

Ribavirin and IFN Combination Study

In 227 patients with chronic hepatitis C from December 2001 to November 2002, ribavirin and IFN combination therapy were carried out. Ribavirin was administered 800 mg per day in the patients having body weight 60 kg or more, and 600 mg with less than 60 kg. IFNα-2b of 6 MU was administered everyday during the initial two weeks followed by 3 times per week for remaining 22 weeks. The clinical characteristics are shown in table 2. The therapy was discontinued in 12 patients because of anemia, appetite loss, depression, and skin rash. Dose reduction of ribavirin was necessary in 21 patients because of anemia. Thus, the outcome of the combination therapy was assessed in 215 patients.

Statistical significance was assessed by Student's t test, χ^2 analysis with Yates' correction, and Kaplan-Meier method using the log-rank test as indicated. Multivariate analysis was carried out by the Cox proportional hazard model.

Results

The development of HCC was observed in 31 patients after IFN monotherapy. The clinical characteristics of the patients which developed HCC was evaluated by univariate analysis. A statistically significant difference was noted in age, gender, genotype, fibrosis of the liver and outcome of interferon therapy. The serum HCVRNA level before treatment and the serum ALT level were not different between the two groups (table 3).

The incidence of HCC after interferon therapy was compared according to the fibrosis score of the liver. The incidence of HCC was 0.3% per year in the patients with F1 and 1.1% per year in F2; however, it was 3.8% in the F3 groups. The development of HCC was significantly higher in the patients in the F3 and F4 groups than those in the F1 and F2 groups (Kaplan-Meier method, log-rank test, $p < 0.01$; fig. 1).

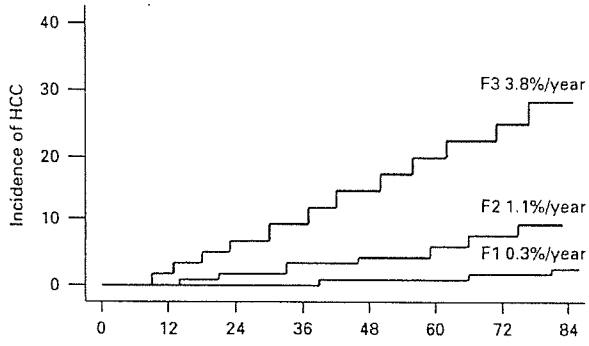

Fig. 1. Incidence of HCC was 0.1% in patients with fibrosis score F1, 1.1% in F2 and 3.8% in F3 (Kaplan-Meier method).

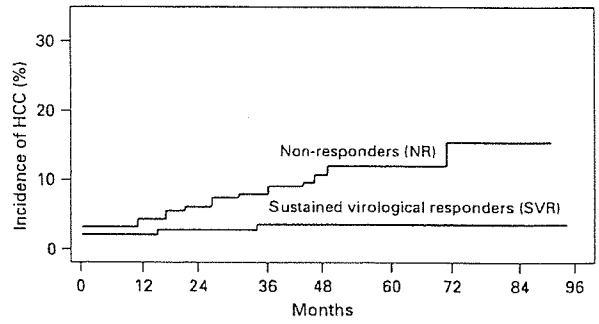

Fig. 2. Incidence of HCC was compared between sustained virological responder and non-responder patients. HCC development was significantly higher in the non-responders than in the sustained virological responders after IFN monotherapy.

Table 2. Clinical characteristics of the patients received ribavirin and IFN combination therapy

Gender	
Male	126
Female	101
Age (mean ± SE)	58.4 ± 1.2
Genotype	
1b	181
2a	30
2b	15
Mixed	1
HCVRNA level, kIU/ml (median 680)	67 to >850
Liver biopsy	
F1	86
F2	75
F3	64
F4	2
Outcome of IFN therapy	
SVR	61
NR	154
Withdrawal	12

Table 3. Comparison of the patients with or without development of HCC after IFN therapy (univariate analysis)

Development of HCC	Yes (n = 31)	No (n = 464)	p
Gender			
Male	22	260	<0.05
Female	9	204	
Age	60 ± 1.2	52 ± 0.6	<0.001
Genotype			
1b	22	228	<0.01
2a and 2b	1	102	
HCVRNA level, kIU/ml	512 ± 34	496 ± 18	n.s.
Liver biopsy			
F1 and F2	7	309	<0.001
F3 and F4	23	135	
ALT, IU/l	125 ± 8.3	118 ± 20	n.s.
Outcome of IFN therapy			
SVR	3	152	<0.01
NR	28	312	

The incidence of HCC was compared between sustained responders and non-responders. The incidence of HCC in sustained responders was 0.2% per year in the sustained responders; however, it was 3.9% per year in the non-responders. This difference was statistically significant (fig. 2).

Multivariate analysis using Cox hazard model was done. Age, gender, fibrosis of the liver and outcome of interferon were found to be independent risk factors (table 4). Among these risk factors, age, gender and fibrosis of the liver cannot be changed. Thus, to reduce the incidence of HCC, the improvement of sustained virological response is an important issue.

Since the end of 2001, ribavirin and IFN combination therapy for 24 weeks has been allowed in Japan, and 235 patients have been treated. The sustained virological response rate in genotype 1b dividing them according to their HCVRNA level before treatment. In the patient

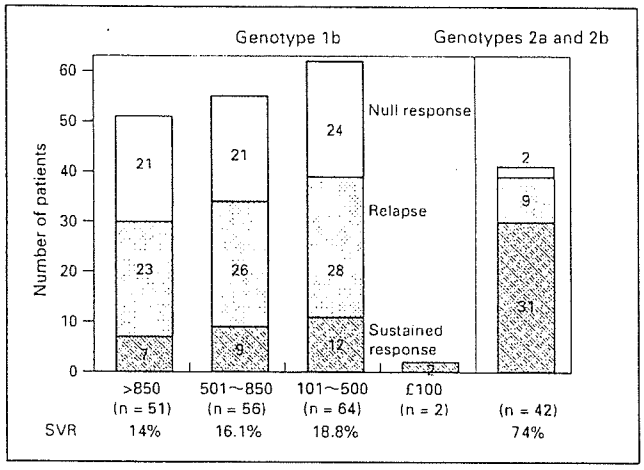

Fig. 3. Outcome of ribavirin and IFN combination therapy. ▨ = Sustained response; ▩ = relapse; □ = null response.

Table 4. Risk factors for the development of HCC after IFN monotherapy (Cox proportional hazard model)

Variable	Odds ratio	95% CI	p
Age (>56 vs. <55)	7.5	2.3–14.6	<0.005
Gender (male vs. female)	1.9	1.1–27.4	<0.05
Fibrosis (F3 and F4 vs. F1 and F2)	3.7	1.8–18.6	<0.01
Outcome of IFN (NR vs. SVR)	2.8	1.2–23.6	<0.05

group with a HCVRNA level higher than 850 kIU/ml, the sustained virological response rate was 0% by interferon monotherapy, while it was 14.0% by ribavirin and interferon combination therapy for 24 weeks. Similarly, it was 3.7% with a HCVRNA level from 500 to 850 kIU/ml on monotherapy, but it was 16.1% on combination therapy. The sustained virological response rate was 13.1% on monotherapy in those with a HCVRNA level from 100 to 500 kIU/ml, while it was 18.8% on combination therapy. However, a relapse rate, i.e. reappearance of HCVRNA after discontinuation of combination therapy, of 40–50% was observed in each group, and null response, i.e. no achievement of negative plasma HCVRNA during combination treatment, of around 30% was observed in each group. In the patients with genotype 2a and 2b infection, a sustained virological response was achieved in 74% (fig. 3).

Since the incidence of HCC reduced from 3.9% per year in non-responders to 0.2% per year in sustained virological responders, the incidence of HCC after treatment has been estimated to be reduced from 3.1 to 2.8% per year overall with 24 weeks' treatment with rivabirin and IFN combination therapy.

Discussion

HCC is the most life-threatening problem in the long-term course of chronic hepatitis C. The rising incidence of HCC has been pointed out not only in Japan but in the United States [9] and Europe [10]. Therefore, prevention of the development of HCC is an important issue in the clinical setting. In the present study, we analyzed the incidence and risk factors of HCC after IFN monotherapy in patients with chronic hepatitis C. The risk factors for the development of HCC were found to be age, male gender, fibrosis of the liver, and outcome of IFN therapy. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders, which is consistent with our data. They also reported that age, male gender and severe fibrosis of the liver were risk factors for the development of HCC. Imai et al. [11] reported similar risk factors for the development of HCC after IFN monotherapy in HCV-infected patients. Therefore, the liver fibrosis score is likely to be one of the most important risk factors for the subsequent development of HCC in HCV-infected patients, even following IFN therapy. Our data demonstrating that the degree of hepatic fibrosis is an independent risk factor for the development of HCC associated with HCV infection is certainly consistent with this supposition. Among these risk factors, age, male gender and fibrosis score of the liver cannot be changed before IFN therapy; thus, to reduce the incidence of HCC, improvement of the sustained response rate is an essential issue in patient care of HCV infection.

Recently, HCC-free survival could be obtained by IFN in patients with chronic hepatitis C, and the gain in HCC-free survival was greater when a patient was younger and fibrosis of the liver was more advanced [12]. The gain in HCC-free survival was calculated as difference between expected HCC-free survival with sustained virological response and that without. In this setting, improvement in achieving a sustained response is the central issue. Furthermore, the risk of death from liver-related disease was significantly reduced not only in sustained virological responders but also in biochemical responders in chronic hepatitis C [13].

Although the incidence of HCC has not been investigated after ribavirin and IFN combination therapy, HCC

development seems to be reduced by combination therapy by improving the sustained response rate, especially in genotype 1b infection. In the present study, the incidence of HCC is estimated to be reduced from 3.1 to 2.8% per year by combination therapy for 24 weeks. However, the sustained virological response rate has been shown to improve in genotype 1 infection by extended combination therapy for 48 weeks or by peginterferon-alfa-2b instead [14]. Thus, to reduce the incidence of HCC, extended treatment with ribavirin and IFN for 48 weeks is necessary in genotype 1b infection.

In the patients with HCV infection, the recurrence rate of HCC in the liver is as high as 20% per year, even after complete curative treatment was given to the primary HCC nodule [15]. The recurrence rate and prognosis was improved after elimination of hepatitis C virus RNA by IFN [16]. Furthermore, previous IFN therapy was shown to reduce the multicentric recurrence of HCC and improve the patients' survival in chronic HCV infection [17]. The rate of first recurrence of HCC was similar in patients treated with IFN and in untreated patients, but in the patients treated with IFN after curative treatment was given to the primary HCC nodule, the rate of second or third recurrence was lower than in the untreated group [15]. Moreover, IFN therapy enhanced patient survival after treatment of the HCC nodule.

From these results, it is concluded that IFN reduced the risk of the development of HCC when a sustained virological response was achieved in chronic hepatitis C. To reduce the risk of the development of HCC, it is an essential issue to improve the sustained response rate by prolonged ribavirin and IFN combination therapy.

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Rare Quasispecies in the YMDD Motif of Hepatitis B Virus Detected by Polymerase Chain Reaction with Peptide Nucleic Acid Clamping

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Key Words
Hepatitis B e antigen · Hepatitis B virus · Lamivudine · Peptide nucleic acid · YMDD mutant

Abstract
The emergence of drug-resistant mutants of hepatitis B virus (HBV) is a serious problem during antiviral therapy of patients with chronic hepatitis B. Lamivudine-resistant mutants with a mutation in the YMDD motif of reverse transcriptase of HBV emerge in approximately one half of the treated patients within 5 years. To date, the detection of YMDD mutants by polymerase chain reaction (PCR) with peptide nucleic acid (PNA) clamping is most sensitive. In this study, the performance of this method was evaluated in various clinical settings. The PCR-PNA method was able to detect the emergence of YMDD mutants 2–3 months earlier than the previously developed method involving restriction fragment length polymorphism. Further, rare quasispecies were detected by PCR-PNA in patients with chronic hepatitis B who were positive for hepatitis B e antigen (HBeAg). Many previously unrecognized mutants, such as those with YLDD and YMED, were found in them. Although precise sequence analyses of 10 patients identified YVDD and YIDD sequences in 6 of them, only 1 patient had a typical YVDD sequence that was identical with that in the reported lamivudine-resistant strain. All HBV mutants with the YIDD sequence accompanied stop codon(s) in the overlapping envelope (S) gene, suggesting that these strains would have no relevance as regards the emergence of lamivudine resistance. These results suggest that it would be difficult to detect lamivudine-resistant mutants before the therapy and that they would have a greater ability to evade the attack of antiviral drugs by frequent nucleotide substitutions than previously expected.

Introduction

Infection with hepatitis B virus (HBV) is a serious healthcare problem worldwide. Only interferon, lamivudine representing (–)-β-*L*-2′,3′-dideoxy-3′-thiacytidine and famciclovir standing for 2-[2-(2-amino-9H-purin-9-yl)ethyl]-1,3-propanediol diacetate have been approved for the treatment of chronic hepatitis B. Lamivudine reduces HBV DNA loads to almost undetectable levels in most patients who receive it and reduces inflammatory

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