Table 2
Mutation rates during the non-treatment observation period and during subsequent ribavirin monotherapy

	NS5A	NS5B
During non-treatment observation period		
Total	0.6×10^{-2}	0.24×10^{-2}
SVR	1.4×10^{-2} ‡	0.46×10^{-2}
NR	0.28×10^{-2} ‡ *	0.14×10^{-2} †
During subsequent ribavirin monotherapy		
Total	2.9×10^{-2}	1.3×10^{-2}
SVR	8.8×10^{-2} §	2.2×10^{-2}
NR	0.38×10^{-2} §	0.96×10^{-2}

Note that the difference in NS5A mutation rate between SVR and NR patients was greater during ribavirin treatment than that during the non-treatment observation (control) period (23-fold vs. 5-fold). *$P=0.02$ (paired t test). †$P=0.02$ (paired t test). ‡$P=0.04$ (unpaired t test). §$P=0.0005$ (unpaired t test).

the same patients (Table 2, $P=0.02$ for both NS5 regions). Of all nucleotide mutations, 72.1% in the NS5A region and 85.5% in the NS5B region were transition mutations. Percentages of transition mutations for all mutations are detailed in Table 3. Mutations from C to T and from G to A were frequent (Table 3). Synonymous mutations occurred more frequently than non-synonymous mutations in both regions, comprising 80.6% of all NS5A mutations and 73.5% of all NS5B mutations. No significant correlation was found between gene mutation rates and the subject's serum ribavirin concentration at the end of four weeks of ribavirin monotherapy.

Next, the relationship between mutations during ribavirin therapy and virological response to combination therapy was evaluated. The sustained viral response rate was 24.9% (10/34) (Table 1). The proportion of patients who had mutations in the NS5A region during ribavirin monotherapy was significantly higher in SVRs than in NRs (8 out of 10 vs. 2 out of 24 patients, respectively, $P<0.0001$, Fisher's exact test). Correspondingly, gene mutation rates in the NS5A region were significantly higher in SVRs than in NRs: 8.8×10^{-2}/site/year vs. 0.38×10^{-2}/site/year, respectively ($P=0.0005$) (Table 2). In the NS5B region, although statistically significant differences were not observed, the gene mutation rate tended to be higher in SVRs than in NRs: 2.2×10^{-2}/site/year vs. 0.96×10^{-2}/site/year, respectively (Table 2). The proportion of patients with mutations in the NS5B region did not significantly differ between SVRs and NRs.

Table 3
Transition mutations during the non-treatment observation period and during subsequent ribavirin monotherapy

	C to T	T to C	G to A	A to G	Others[a]
During non-treatment observation period					
NS5A (%)	22.2	33.3	22.2	11.1	11.2
NS5B (%)	28.5	57.1	0	14.3	0
During subsequent ribavirin monotherapy					
NS5A (%)	18.6	9.3	25.6	18.6	27.9
NS5B (%)	38.5	20.5	10.2	16.3	14.5

Data are expressed as a percentage of all mutations observed in each region.
[a] Transversion mutations were included in this column.

3.3. Non-synonymous mutations and virological response to IFN/ribavirin combination therapy

In the NS5A region, non-synonymous mutations were found in 19.4% of all nucleotide mutations observed during ribavirin monotherapy. Alterations in deduced amino acid residues by non-synonymous mutations are illustrated in Fig. 2, which shows all 10 patients who had nucleotide mutations during ribavirin monotherapy. In a pairwise comparison between pre- and post-ribavirin monotherapy, amino acid alterations were found in 5 of these 10 patients. Non-synonymous mutations were found exclusively in SVRs. In other words, 5 out of the 8 patients who achieved SVR status had 1 or 3 non-synonymous mutations; 2 of these 5 patients had amino acid alterations accompanied with an increase in the number of amino acid mutations in the interferon sensitivity determining region (ISDR). In contrast, all nucleotide mutations detected in NRs were synonymous mutations. dN/dS tended to be higher in SVRs than in NRs ($P=0.25$, Fig. 3).

In the NS5B region, non-synonymous mutations were detected in 3 out of the 10 SVRs and in 2 out of the 24 NRs. The proportion of patients with non-synonymous mutations did not significantly differ between the two response groups. dN/dS also did not significantly differ between the SVRs and the NRs ($P=0.77$, Fig. 3). Of the 9 amino acid mutations detected in the NS5B region, two were located in the functional domain of the RdRp, one was located in domain C (D310N), and one was located in domain D (R345S). As shown by the crystal model of the NS5B-RdRp in Fig. 4, amino acid mutations were primarily located on the molecular surface; none occurred in the nucleotide groove or in the tunnel.

3.4. Genetic changes during the non-treatment observation period

Gene mutation rates in the NS5A and NS5B regions during the non-treatment observation period were calculated as 0.60×10^{-2}/site/year and 0.24×10^{-2}/site/year, respectively, rates which were significantly lower than the mutation rates observed during ribavirin monotherapy (Table 2). Next, the relationships between viral mutation rates during

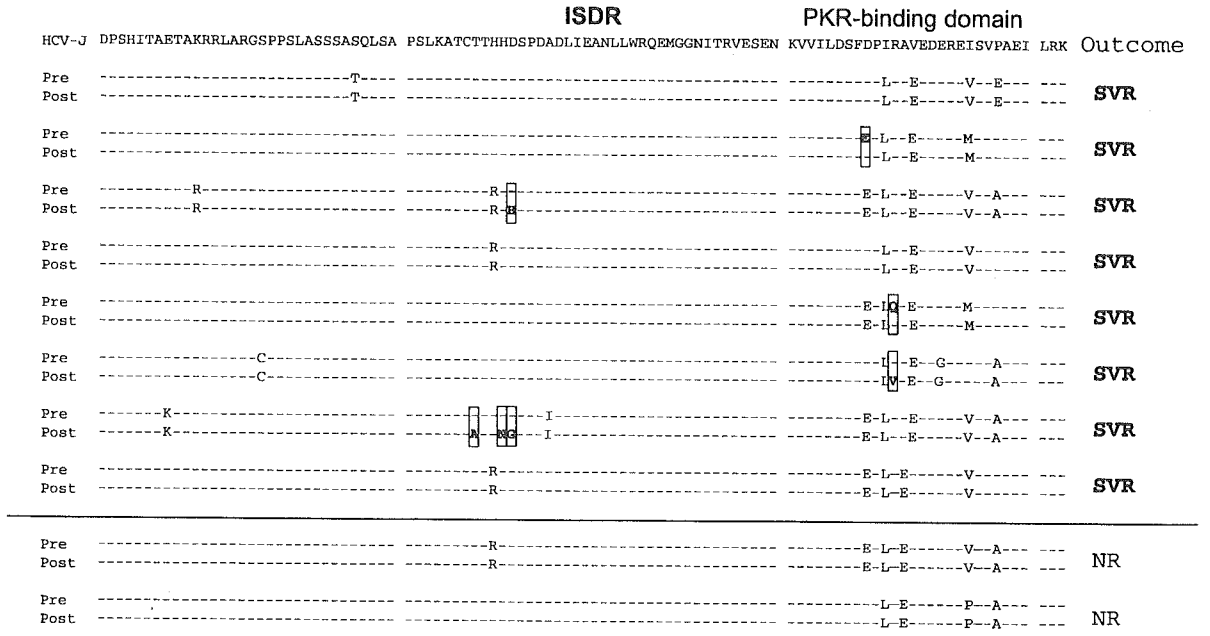

Fig. 2. Amino acid sequence alignments in the NS5A region obtained pre- and post-ribavirin monotherapy. The figure contains data for all 10 patients out of 34 with nucleotide mutations occurring during ribavirin monotherapy. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate identical amino acid residues, with the consensus sequence and HCV-J shown at the top. Outcomes of combination therapy are shown on the right side. In a pairwise comparison between pre- and post-ribavirin monotherapy sera, amino acid alterations were found in 5 of 10 patients. Non-synonymous mutations were exclusively found in SVR patients.

the non-treatment observation period and viral responses to subsequent IFN/ribavirin therapy were evaluated. Interestingly, gene mutation rates in the NS5A region during the non-treatment observation period were significantly higher in SVRs than in NRs: 1.4×10^{-2}/site/year vs. 0.28×10^{-2}/site/year, respectively ($P=0.04$). However, it should be noted that the relative difference in NS5A mutation rates between SVRs and NRs during ribavirin treatment was larger than the relative difference between SVRs and NRs during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$).

Similarly, gene mutation rates in the NS5B region were higher in SVRs (0.46×10^{-2}/site/year) than in NRs (0.14×10^{-2}/site/year), although these differences were not statistically significant. Seven of the nine patients who had mutations occurring during the non-treatment observation period also had mutations during ribavirin monotherapy. However, 53% (8/15) of the patients with mutations during ribavirin monotherapy had no gene mutations during the non-treatment observation period.

4. Discussion

In the present study, we identified HCV gene mutations occurring during ribavirin monotherapy and found that the mutation rate was associated with the virological response to subsequent IFN/ribavirin combination therapy. Since the mutation rate was significantly higher during ribavirin monotherapy than during non-treatment observation periods in the same patients, at least some of the mutations observed during ribavirin treatment were likely an effect of ribavirin administration. Therefore, ribavirin appears to act as a mutagen during clinical treatment, and this mutagenic effect correlates with improvements in the virological response rate resulting from the synergistic use of ribavirin with IFN.

Recently, several in vitro and animal studies [9–11, 18,19] have provided evidence that ribavirin has mutagenic

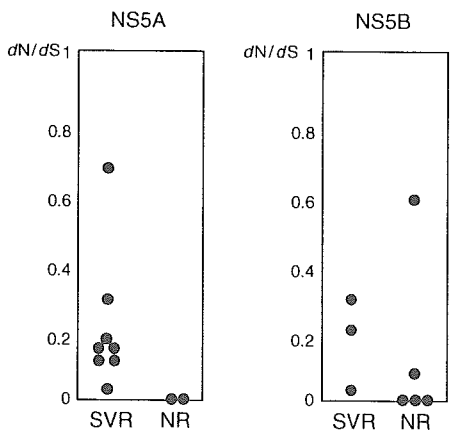

Fig. 3. Ratio of *dN* (nonsynonymous substitution) to *dS* (synonymous substitution) distances for the NS5A and NS5B regions. All 10 patients with nucleotide mutations in the NS5A region and all 8 patients with nucleotide mutations in the NS5B region during ribavirin monotherapy are shown in this figure. All pairwise *dN/dS* ratios were calculated using MEGA ver. 2.1 for each subject. dN/dS in the NS5A region tended to be higher in SVR patients than in NR patients (NS5A: $P=0.25$, NS5B: $P=0.77$).

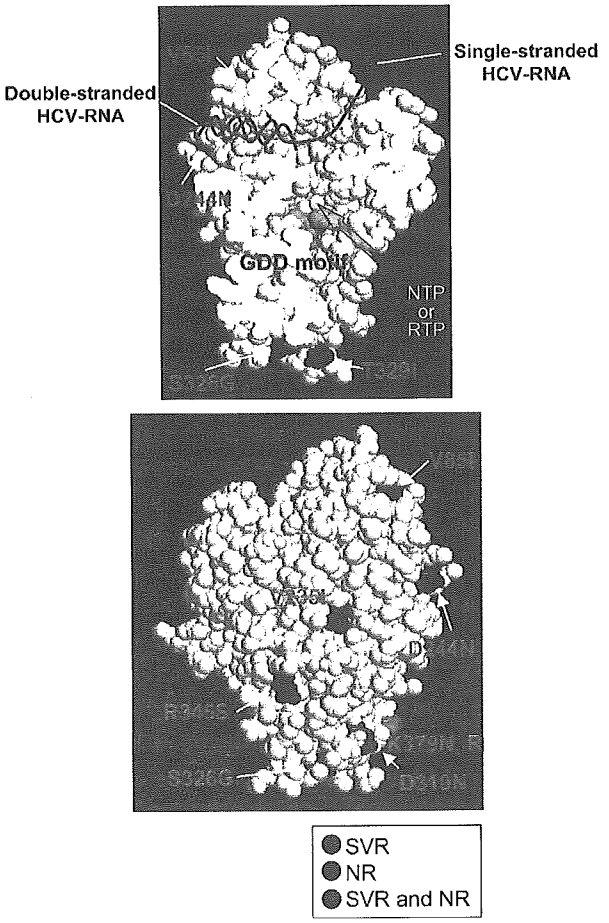

Fig. 4. Crystal structure of HCV NS5B-RNA dependent RNA polymerase. The molecular model of NS5B was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. View of a cross section of the RdRp at level of nucleotide tunnels (A) and the surface (B) are illustrated. Single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and NTP (or ribavirin triphosphate; RTP) enters the enzyme through the right lower NTP tunnel (between the fingers and the thumb). The substitutions observed in SVR patients only, NR patients only, and both sets of patients are shown in red, blue, and magenta, respectively.

activity. Other studies have found that ribavirin increases the mutation rate in a full-length HCV cDNA plasmid [20] and in an HCV replicon [21]. However, none of these studies were human studies; no study to date has documented that ribavirin has mutagenic activity in the clinical setting. In the absence of clinical data, the association between the mutagenic activity of ribavirin and the clinical and virological responses to IFN/ribavirin combination therapy remains unknown. Detecting gene mutations induced by ribavirin and analyzing their association with clinical responses to IFN/ribavirin therapy are extremely difficult because HCV with error mutations may be immediately eliminated by the concurrently administered IFN. Although issues regarding the mutagenic effects of ribavirin remain controversial, the inclusion of four weeks of ribavirin monotherapy immediately before IFN/ribavirin combination therapy in our protocol enabled us to clarify the association between gene mutations induced by ribavirin and the ensuing virological response to the subsequent IFN/ribavirin therapy.

Interestingly, the correlation between mutation rate and virological response to therapy was more evident in the NS5A region, including the ISDR, than in the NS5B region. From our previous analysis of the full-length HCV genome [13,22], NS5A sequences were more variable than NS5B sequences among different clones. Therefore, it seems likely that the stronger relationship observed between mutation rates in the NS5A region and SVR status was due to the relatively greater inherent variability of the NS5A region compared with the NS5B region. Even in the NS5A region, however, serial amino acid mutations rarely occur in the same patients during untreated periods [23]. Hence, our ribavirin monotherapy results suggest that in some patients, ribavirin induces non-synonymous mutations which increase sensitivity to IFN. In turn, this increased susceptibility to IFN could lead to SVR status. Conversely, no functionally important mutations were detected in the NS5B region. Since a ribavirin-induced non-synonymous mutation at a functionally important site in the NS5B region is likely to lead to viral death, even without concurrent IFN administration, the substitutions observed in this critical region are more probably the results of positive or negative selection.

The patients who had gene mutations during the non-treatment observation period were prone to also having mutations during ribavirin monotherapy as well and were more likely to achieve SVR status. These correlations suggest that ribavirin easily induced HCV mutations in such patients. Although mutations could have occurred in the absence of ribavirin, the difference in mutation rates between SVRs and NRs was significantly larger during ribavirin treatment than during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$). The observation that in more than half of the patients, mutations occurred only during ribavirin monotherapy and were not detectable during the non-treatment observation period suggests that mutagenic effects of ribavirin synergistically potentiating the virological response to IFN may play an important role in achieving SVR status.

Gene mutations in the NS5A and/or NS5B regions during ribavirin monotherapy did not occur in all patients in the present study, suggesting that the intensity of the mutagenic effects of ribavirin differed among individual patients. Additionally, some patients who did not have gene mutations in these regions during the non-treatment observation period or during ribavirin monotherapy nonetheless still achieved SVR, suggesting that the synergistic efficacy of ribavirin may not result solely from the mutagenic activity of this agent. Alternatively, ribavirin may have possibly induced mutations during the period of IFN/ribavirin combination therapy in these patients, but as previously discussed, the concurrent IFN might have eliminated the HCV containing

these mutations before the mutations were able to be detected. Additionally, our data did not address the significance of the impact that mutations in regions other than NS5 may have had on viral response to therapy.

HCV populations in vivo consist of a quasispecies nature. Our previous cloning analysis detected small number of minor clones in specimens, which were determined as ISDR-wild type by direct sequencing [24]. Hence, it should be noted that our criteria for mutation could not completely distinguish between de novo mutation and selection of a minor clone.

In the present study, we found that ribavirin also expressed antiviral activity by reducing viral load, presumably because we used a highly quantitative assay for HCV-RNA measurement [17]. However, contradictory results have been reported previously [1–3]. Since the present study identified only a small reduction in viral load, further investigation is needed to confirm our result.

In conclusion, our data demonstrate that clinical administration of ribavirin induces mutations in HCV genes and suggest that, in some patients, mutagenesis may be one of the mechanisms responsible for the synergistic efficacy of ribavirin in IFN/ribavirin combination therapy.

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Polymerase Domain B Mutation Is Associated with Hepatitis Relapse during Long-Term Lamivudine Therapy for Chronic Hepatitis B

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Key Words
Lamivudine · Breakthrough hepatitis · INNO-LiPA · Hepatitis B virus DNA · Domain B

Abstract
Breakthrough hepatitis remains the major issue in long-term lamivudine therapy for chronic hepatitis B. However, the emergence of drug-resistant hepatitis B virus (HBV) is not always accompanied by a relapse of hepatitis. To elucidate factors predictive of breakthrough hepatitis, 53 patients with genotype C of HBV on long-term lamivudine therapy were analyzed. HBV reappeared during therapy in 19 patients with a cumulative incidence of 15% at 1 year, 34% at 2 years, and 60% at 3 years. Within this group, breakthrough hepatitis developed in 12 patients (63%). A polymerase gene domain B mutation (rt180M) emerged in 13 patients, and domain C mutations (rt204I, rt204V) were found in 19 patients. The rt180M mutation was associated with breakthrough hepatitis ($p < 0.05$) with a positive predictive value of 85% and a negative predictive value of 83%. Patients with the rt180M mutation had higher HBV-DNA levels during viral breakthrough compared to patients with rt180wt ($p < 0.05$). The mutational pattern of rt204 was not associated with breakthrough hepatitis. In conclusion, genotypic assays for the rt180M mutation after viral breakthrough may be useful in predicting the risk of breakthrough hepatitis and in deciding when to initiate alternative or additive nucleoside analogue therapy.

Introduction

Lamivudine, a nucleoside analogue, is now widely used as primary therapy for chronic hepatitis B virus (HBV) infection [1]. The initial clinical response is usually favorable with high rates of HBV suppression, normalization of serum alanine transaminase (ALT) levels, loss of detectable serum HBe antigen, as well as histologic improvement [2–7]. However, short-term therapy cannot completely eliminate the HBV pool in the liver [8], and cessation of therapy usually leads to withdrawal hepatitis [9–11]. Consequently, long-term therapy is required in the majority of patients to maintain the suppressive effects of lamivudine.

Unfortunately, long-term therapy has drawbacks as well. Response rates may gradually decrease due to the emergence of drug-resistant HBV. This resistant virus form is characterized by amino acid mutations in the catalytic domains of the polymerase gene. Two mutations are frequently observed in association with lamivudine resistance: a mutation of methionine to isoleucine or va-

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line in codon 204 of the catalytic domain C, and a mutation of leucine to methionine in codon 180 of the catalytic domain B [12]. The emergence of these mutations leads to the reappearance of HBV-DNA (viral breakthrough) and the relapse of hepatitis (breakthrough hepatitis) [13] at cumulative rates of 14–32% at 1 year and 38–49% at 2 years [2, 4, 5, 14, 15].

However, biochemical changes do not always correlate with drug resistance. In some cases of viral breakthrough during long-term lamivudine therapy, patient ALT levels remain unaffected, and these patients continue to exhibit histologic improvement [12, 15]. Conversely, there are also patients who develop a severe flare-up of their hepatitis or even frank decompensation when viral resistance emerges [6, 16–20]. The risk of hepatitis B relapse thus becomes an important issue in patients undergoing long-term treatment with lamivudine. While various factors such as pretreatment HBV-DNA levels, ALT levels, presence of HBe antigen, and certain genotypes, may be associated with the emergence of resistant viral strains, factors predictive of a patient's clinical outcome have not yet been defined. Given the wide variability in patient responses to viral breakthrough and the potential morbidity and mortality associated with the worst outcomes, identifying pretreatment factors predictive of breakthrough hepatitis could be very relevant to clinical practice.

Finally, when analyzing treatment resistance, it is also important to consider the HBV viral genotype. Viral genotype may have some bearing on patient outcomes with long-term lamivudine therapy. For example, patients infected with genotype A who develop viral breakthrough while on lamivudine tend to have higher HBV-DNA levels than patients who harbor other HBV genotypes such as D. Investigators have recently shown that the pattern of polymerase gene mutation leading to lamivudine resistance may be different for genotype A [21]. A double mutation of methionine to valine in codon 204 and leucine to methionine in codon 180 was prevalent in genotype A, while a methionine to isoleucine mutation in codon 204 occurred more frequently in genotype D. These differences in mutational patterns may be linked to an association between genotype and HBV-DNA levels after viral breakthrough.

Currently, the most prevalent HBV genotype in Japan is genotype C [22]. Genotype C is reported to be associated with a more aggressive clinical course and increased resistance to interferon therapy when compared to genotype B [23–25]. Given the pertinence of genotype in lamivudine resistance, differentiating the clinical significance of the various polymerase gene mutations in genotype C becomes critical.

The aim of the present study is to elucidate factors associated with breakthrough hepatitis during long-term lamivudine therapy in HBV genotype C infections. Mutations in domains B and C of the polymerase gene, core promoter gene and precore gene were analyzed to determine if specific mutational patterns might be associated with different clinical outcomes.

Patients and Methods

Therapeutic Protocol

Fifty-three patients with chronic hepatitis B genotype C who were consecutively started on long-term lamivudine monotherapy between August 1999 and November 2003 at Musashino Red Cross Hospital were analyzed retrospectively. There were 32 males and 21 females; mean age was 48.8 ± 11.8 years. At the start of therapy, all patients had detectable levels of HBV-DNA in their blood by polymerase chain reaction (PCR), as well as elevations in serum ALT levels. All patients were also found not to have either hepatitis C or human immunodeficiency virus antibodies in their blood. No patient received interferon or any other antiviral agents during the study or within 6 months of initiating lamivudine therapy. Patients were treated with a single oral dose of 100 mg of lamivudine every day; median duration of lamivudine therapy was 689 (range 207–1,736) days. All patients remained on lamivudine therapy throughout the course of study except those who developed breakthrough hepatitis. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Quantification of HBV-DNA

Blood samples were obtained at the start of therapy and then once every month during therapy. The serum level of HBV-DNA was determined using transcription-mediated amplification and hybridization protection assays (Fujirebio Inc., Tokyo, Japan) that have a detection range of 3.7–8.7 log genome equivalents (LGE)/ml [26].

Definitions

Viral breakthrough was defined as an elevation of more than 1 LGE/ml of HBV-DNA accompanied by mutations in the polymerase gene on 3 consecutive determinations during monthly testing after a period of HBV-DNA suppression. No case that met this definition experienced a spontaneous decline in HBV-DNA thereafter. Breakthrough hepatitis was defined as a sustained elevation in serum ALT levels on 2 consecutive determinations 2 weeks apart in concert with viral breakthrough.

Analysis of Precore and Core Promoter Mutations

Mutations in the precore and core promoter regions were analyzed at baseline. The A1762T and G1764A mutations in the basic core promoter [27] were detected by a commercially available en-

zyme-linked specific probe assay (Smitest HBV core promoter mutation detection kit, Genome Science Laboratory, Tokyo). The G1896A stop codon mutation in the precore region was detected by an enzyme-linked mini sequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo). Use of both of these assays has been described previously [28].

Analysis of Lamivudine-Resistant HBV

Blood samples at the time of viral breakthrough were analyzed for mutations in the HBV polymerase associated with lamivudine resistance using INNO-LiPA HBV DR analysis (Innogenetics, Inc., Ghent, Belgium) [29, 30]. Briefly, DNA isolated from the serum was amplified by nested PCR and used for hybridization to the LiPA strips. The probes on the INNO-LiPA HBV DR strip cover the amino acids of codon 180 (wild-type leucine (L) and mutant methionine (M)) and codon 204 (wild-type methionine and mutants valine (V) and isoleucine (I)). The amino acid positions on the HBV polymerase gene are numbered for consistency with the newly established standardization of nomenclature for lamivudine-resistance mutations rt180M and rt204V/I (originally designated as L528M or L526M and M552V/I or M550V/I) [31].

Statistical Analysis

For statistical analysis the STAT View software package was used. Categorical data were analyzed using Fisher's exact test. Continuous variables were compared with Student's t test. A Kaplan-Meier estimate and the log-rank test were used to calculate the median time to and the significance of viral breakthrough, as well as the median time to and the significance of breakthrough hepatitis. Cox's proportional hazard model and stepwise logistic regression analysis were used for multivariate analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics, Pretreatment Variables and Clinical Course

Prior to initiation of lamivudine therapy, the mean HBV-DNA level was 7.1 ± 1.1 LGE/ml, and the mean ALT was 215 ± 285 U/l. Of a total of 53 patients infected with genotype C of HBV, 30 patients had detectable HBeAg in their serum (56.6%). A precore stop codon mutation was detected in 21 patients (42%), and core promoter mutations were detected in 44 patients (88%). The median treatment period was 689 (range 207–1,736) days.

During therapy, detectable levels of HBV-DNA fell below 4 LGE/ml in 42 (79.3%) patients. Viral breakthrough occurred in a total of 19 patients; in these patients, the median time to viral breakthrough was 473 (range 224–1,128) days. The cumulative incidence of viral breakthrough was 15% at 1 year, 34% at 2 years, and 60% at 3 years (fig. 1).

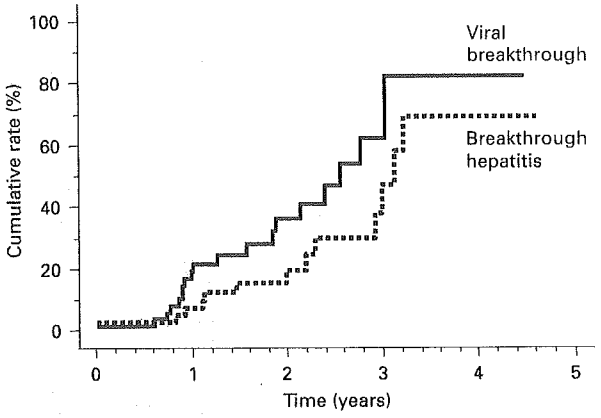

Fig. 1. The cumulative rate of viral breakthrough and breakthrough hepatitis. Kaplan-Meier plot of time to viral breakthrough and time to breakthrough hepatitis in 53 patients treated with lamivudine.

Among those 19 patients who developed viral breakthrough, 12 patients (63%) also developed breakthrough hepatitis. The median time to hepatitis after viral breakthrough was 111 days. The other 7 patients remained in biochemical remission. The cumulative incidence of breakthrough hepatitis was 4% at 1 year, 17% at 2 years, and 45% at 3 years (fig. 1).

Pretreatment variables including age, gender, presence of HBe antigen, HBV-DNA levels, ALT levels, and precore and core promoter mutations were analyzed. These variables were not found to be associated with viral breakthrough or with breakthrough hepatitis (table 1).

Variables at 24 Weeks of Treatment

HBV DNA levels after 24 weeks of lamivudine therapy correlated significantly with eventual viral breakthrough. At week 24 of therapy, HBV-DNA levels were above 4 LGE/ml in 23 patients. Moreover, there was a significant difference in time to viral breakthrough between those whose HBV-DNA levels were above and those whose levels were below 4 LGE/ml after 24 weeks of therapy ($p = 0.007$, Kaplan-Meier log-rank test; fig. 2a). Patients with HBV-DNA levels above 4 LGE/ml had a 3.5-fold higher probability of viral breakthrough compared to the other patients (Cox's proportional hazard model, 95% CI 1.33–9.34, $p = 0.012$). In contrast, the HBV-DNA levels above 4 LGE/ml at week 24 were not associated with breakthrough hepatitis (fig. 2b).

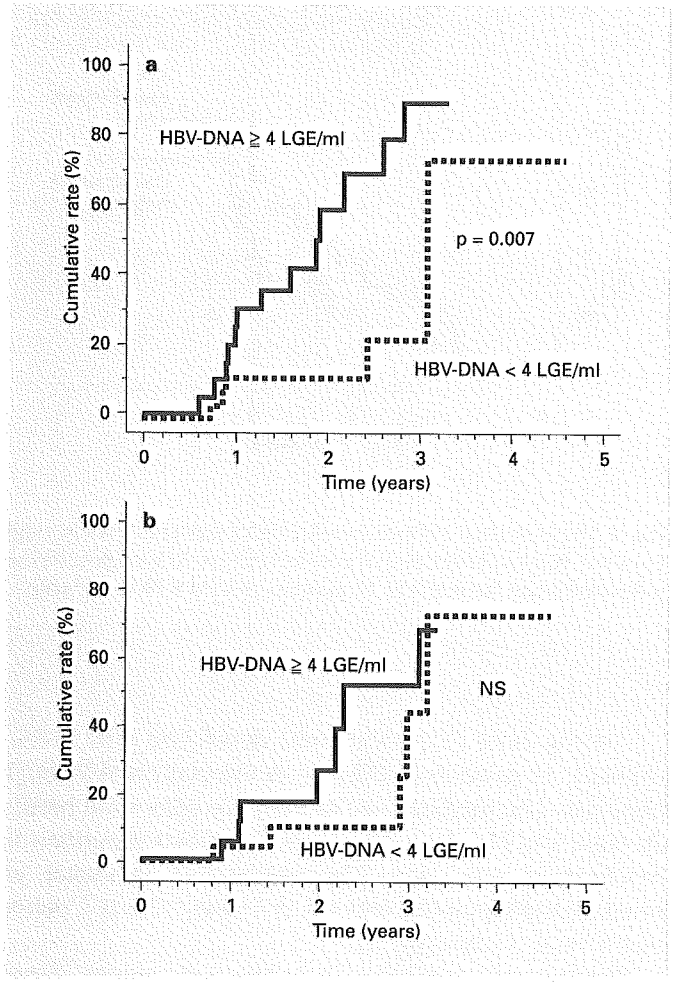

Fig. 2. The cumulative rate of viral breakthrough and breakthrough hepatitis according to HBV-DNA levels after 24 weeks of lamivudine therapy. Patients were divided into 2 groups according to HBV-DNA levels after 24 weeks of lamivudine therapy. Kaplan-Meier plots of time to viral breakthrough (**a**) and time to breakthrough hepatitis (**b**) are shown. Log-rank tests show that higher HBV-DNA levels after 24 weeks of lamivudine therapy are associated with the more rapid development of viral breakthrough (p = 0.007) but not with breakthrough hepatitis.

Resistance-Associated Mutations, HBV-DNA Levels after Viral Breakthrough

At the time of viral breakthrough, a polymerase gene mutation in domain B (rt180M) was detected in 13 patients, and a mutation in domain C was detected in 19 patients (rt204I in 10, rt204V in 6 patients and a mixture of rt204I and rt204V in 3 patients). Six patients had a single rt204I mutation; 4 patients had rt180M/rt204I double mutations; 6 patients had rt180M/rt204V double mutations; and 3 patients had rt180M/rt204I/V (mixture) double mutations. The emergence of the rt180M mutation was significantly associated with breakthrough hepatitis: breakthrough hepatitis occurred in 11 of 13 patients with the rt180M mutation, while only 1 of 6 patients with rt180wt (baseline wild-type) developed significant biochemical changes (p = 0.01, Fisher's exact test; table 2). The positive predictive value of the presence of rt180M was 85%, and the negative predictive value was

Table 1. Pretreatment variables in association with viral breakthrough and breakthrough hepatitis

	Viral breakthrough		Breakthrough hepatitis	
	no (n = 34)	yes (n = 19)	no (n = 41)	yes (n = 12)
Gender (male/female)	22/12	10/9	16/25	5/7
Age, years	49.8 ± 11.9	47.1 ± 11.8	49.2 ± 11.5	47 ± 13.3
ALT, U/l	255 ± 338	141 ± 128	230 ± 314	159 ± 148
HBeAg positive/negative	17/17	6/13	22/19	8/4
HBV-DNA, LGE/ml	7.0 ± 1.1	7.3 ± 1.1	7.1 ± 1.1	7.2 ± 1.2
CP mutations (M/W)	25/6	19/0	32/6	12/0
PC mutation (M/W)	14/17	7/12	15/23	6/6

HBeAg = Hepatitis Be antigen; CP = core promoter; PC = precore; M = mutant type; W = wild type. There were no variables that had a statistically significant association with viral breakthrough or with breakthrough hepatitis.

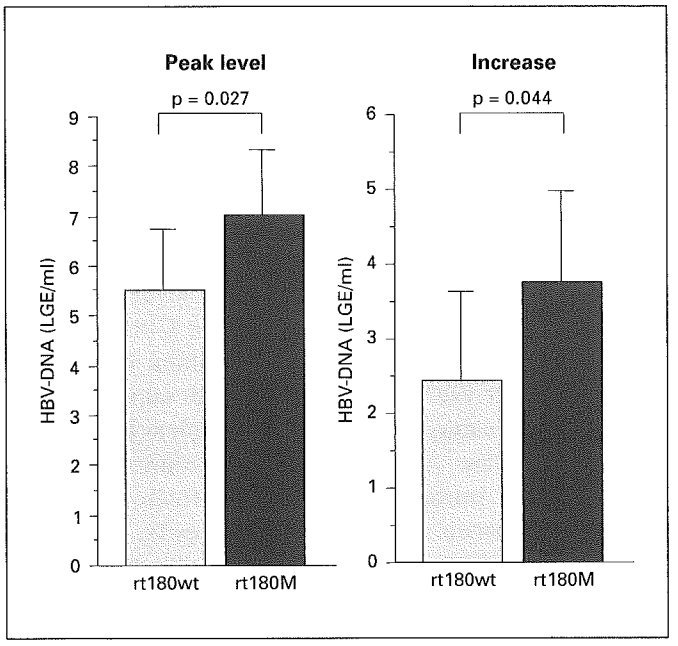

Fig. 3. The association between domain B mutations and HBV-DNA levels after viral breakthrough. After viral breakthrough, patients with the rt180M mutation had higher peak levels of HBV-DNA (p = 0.027) and larger increases of HBV-DNA (p = 0.044) compared to patients with rt180wt.

Table 2. Mutational patterns of the polymerase gene in association with breakthrough hepatitis

Mutational pattern	Breakthrough hepatitis		
	no (n = 7)	yes (n = 12)	p value
rt180M/rt180wt	2/5	11/1	0.01
rt204I/rt204V/rt204V+I	5/2/0	5/4/3	NS
rt204V (yes/no)	2/5	7/5	NS
rt204I (yes/no)	5/2	8/4	NS

NS = Not significant.

83%. Patients with the rt180M mutation had a 27.5-fold higher probability of breakthrough hepatitis compared to those patients with rt180wt (logistic regression analysis, 95% CI 2.00–378.93, p = 0.013). In contrast, mutational patterns of rt204 were not associated with breakthrough hepatitis: only 8 of 13 patients with rt240V mutation and 5 of 10 patients with rt240I developed breakthrough hepatitis (p = 0.35, Fisher's exact test; table 2).

During viral breakthrough, patients with the rt180M mutation had a larger increase in HBV-DNA levels (3.7 ± 1.2 vs. 2.4 ± 1.2 LGE/ml, p = 0.044) and higher peak values of HBV-DNA (7.0 ± 1.3 vs. 5.5 ± 1.2 LGE/ml, p = 0.027) compared to those patients with rt180wt (fig. 3).

Discussion

In this study, we found that the rt180M mutation in domain B of the HBV polymerase gene was significantly associated with breakthrough hepatitis during long-term lamivudine therapy in patients with chronic hepatitis B from genotype C. The positive predictive value of this mutation for breakthrough hepatitis was 85%, and the negative predictive value was 83%. The rt180M mutation was always detected as a double mutation with an rt204 mutation. Moreover, it was linked to higher subsequent levels of HBV-DNA during viral breakthrough compared to similar episodes in patients with a single rt204 mutation. Conversely, the mutational patterns of rt204, namely rt204I or rt204V, were not significantly associated with breakthrough hepatitis. Thus, the development of an rt180M mutation in patients on long-term lamivudine therapy might be a useful predictor for breakthrough hepatitis. As such, the presence of this mutation may also be helpful in deciding whether or not to proceed with alternative or additive nucleoside analogues as salvage therapy.

Lamivudine plays an important role in the treatment of patients with chronic hepatitis B. Short-term treatment is insufficient for clearing the virus [8], while long-term treatment is associated with the development of drug-resistant HBV. These strains of drug-resistant HBV do not always precipitate a relapse of hepatitis [12, 15], but the mechanism and predictive factors for the differing clinical outcomes have not been identified.

Previous studies examined factors such as genotype [21], ALT levels [32–34], HBV-DNA levels prior to therapy [21, 33–35], the degree of decline in HBV-DNA levels during therapy [17, 36, 37], the presence of HBeAg [32, 35, 38], and the presence of core promoter mutations [39]. While all of these factors have been found to be related to the appearance of resistant virus strains, the data are inconsistent, and the ultimate role of these factors remains controversial [36]. Moreover, how these and other factors are related to different clinical outcomes after the reappearance of HBV has not been elucidated. This study attempts to identify those predictive factors for break-

through hepatitis during long-term lamivudine therapy that may have an important impact on clinical outcomes.

We found that pretreatment variables, such as mutations in the core promoter and precore genes, HBV DNA levels, ALT levels, and the presence of HBe antigen, were not associated with viral breakthrough or with breakthrough hepatitis. This finding leads us to believe that identifying high-risk patients prior to initiating lamivudine therapy may not be possible. All cases with breakthrough had mutation in the core promoter, but the difference did not reach statistical significance possibly due to the small number of patients with the wild-type sequence in the core promoter. Further study including a larger number of patients with the wild-type sequence in core promoter may be necessary to elucidate the significance of the mutation in the core promoter in breakthrough.

However, when patients were analyzed after 24 weeks of lamivudine therapy, we found that HBV-DNA levels were significantly associated with the development of viral breakthrough, a finding consistent with previous reports [17, 36, 37]. Additionally, higher rates of spontaneous mutations in the viral genome are likely to be associated with higher replication levels [40], leading to the emergence of resistant HBV. Given these findings, monitoring for HBV-DNA levels at 24 weeks may be useful in targeting patients at higher risk for viral breakthrough but not necessarily for breakthrough hepatitis.

When parameters at the time of viral breakthrough were analyzed, the rt180M mutation was significantly associated with the occurrence of breakthrough hepatitis. Among those we studied the rt180M was the sole maker that was associated with breakthrough hepatitis, therefore close monitoring and detection of this mutation may be useful clinically in the prediction of breakthrough hepatitis.

The mechanism underlying the association of rt180M mutation with breakthrough hepatitis is not well understood. Usually, the rt204V/I mutant virus is less efficient at replication than the rt204wt virus. However, in tissue culture, the mutants with both B and C domain mutations (rt180M and rt204I/V) have higher reverse transcriptase activity and replication capacity than those single C-domain mutants (rt180wt and rt204I/V). Domain C rt204 is located in the conserved YMDD motif of the RNA-dependent RNA polymerase and is involved in nucleotide binding [41], while domain B is involved in template positioning [42]. Since these two domains interact in the molecular model of HBV reverse transcriptase [43], it might be speculated that the rt180M mutation interacts with the rt204 mutations. In essence, in double mutants, the B-domain mutation rt180M may compensate for the replication defective C-domain mutants [44], thus accounting for the higher reverse transcriptase activity and replication capacity compared to single C-domain mutants. These in vitro findings are consistent with our findings that patients with both B and C domain mutants were associated with higher HBV-DNA levels during viral breakthrough than those with single C-domain mutants. It has also been suggested that cellular immune responsiveness to HBV may increase after suppression of viremia when using antiviral therapy such as lamivudine [45]. Thus, when viral breakthrough occurs in patients whose cellular immunity has been relatively restored, a relapse of hepatitis may occur. Additionally, the amino acid position 180 may be a more vulnerable target to the restored immune system, a possibility that needs to be examined by further study.

In conclusion, the results of this study offer potentially important clinical ramifications for patients infected with genotype C hepatitis B who are on long-term lamivudine therapy. The quantification of HBV viremia after 24 weeks of lamivudine therapy may predict patients who are at a higher risk for viral breakthrough. Closer monitoring may thus be warranted in those patients with HBV-DNA levels of >4 LGE/ml. When viral breakthrough does occur, genotypic assays for the rt180M mutation should be performed since this mutation may be predictive of hepatitis relapse.

It is our belief that using both a quantitative assay for HBV viremia at 24 weeks and genotypic assays for polymerase mutant detection after viral breakthrough may serve as an effective means of monitoring long-term lamivudine therapy in chronic hepatitis B genotype C patients. Together, the results from these assays can provide useful information that may influence the decision to initiate early induction of salvage 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).

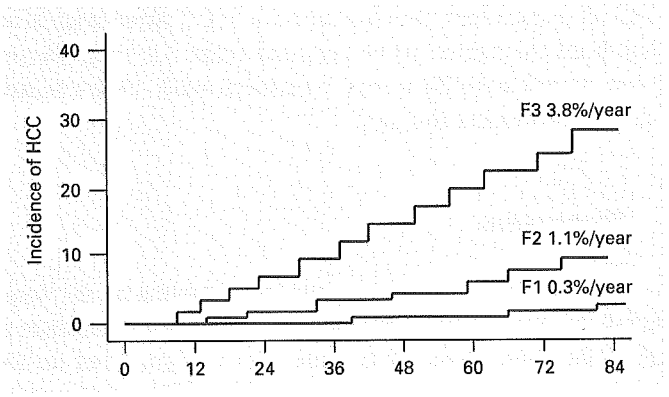

Fig. 1. Incidence of HCC was 0.1% in patients with fibrosis score F1, 1.1% in F2 and 3.8% in F3 (Kaplan-Meier method).

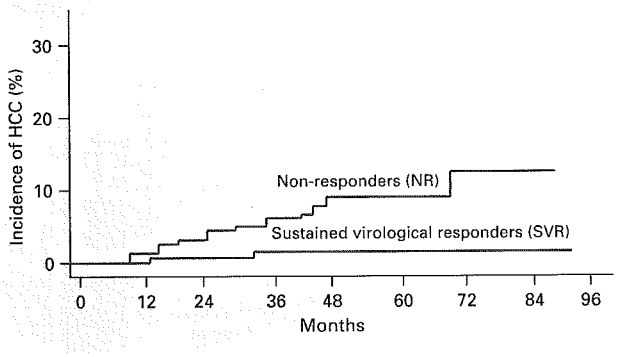

Fig. 2. Incidence of HCC was compared between sustained virological responder and non-responder patients. HCC development was significantly higher in the non-responders than in the sustained virological responders after IFN monotherapy.

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

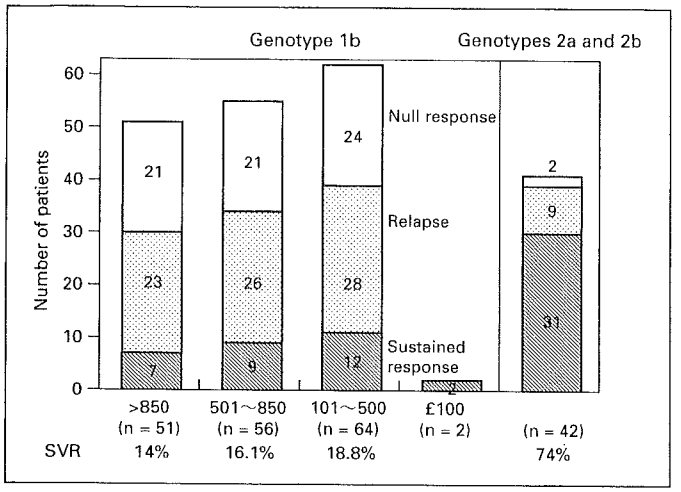

Fig. 3. Outcome of ribavirin and IFN combination therapy. ▨ = Sustained response; ▩ = relapse; □ = null response.

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.

Acknowledgement

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G to A Hypermutation of Hepatitis B Virus

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G to A hypermutation of the human immunodeficiency virus type 1 (HIV-1) is induced by a deaminase APOBEC3G and is related to host antiviral defense. APOBEC3G has also been found to reduce the replication of HIV-1 by an unknown mechanism. This enzyme also reduces the production of hepatitis B virus, although the mechanism for this action has not been clearly elucidated. The hypermutated hepatitis B virus (HBV) is rarely found in usual sequencing analyses. Using peptide nucleic acid mediated by polymerase chain reaction clamping, we detected the hypermutated HBV DNA in 1 of 8 patients with acute HBV infection and 4 of 10 with chronic HBV infection. In the latter group, hypermutated genomes were found only in eAb-positive patients. As much as 72.5% of G residues were mutated in the hypermutated clones. G to A substitutions were predominant in almost all clones sequenced compared with other substitutions. G to A mutated viral genomes also were found in HepG2–derived cell lines that continuously produced HBV into the supernatant. Both alpha and gamma interferon reduced virus production in these cell lines, but they did not alter the frequency of the hypermutation. Transcripts of APOBEC3G, as well as some other deaminases, were found in these cell lines. In conclusion, our results show that part of the minus strand DNA of HBV is hypermutated both *in vitro* (HepG2 cell lines) and *in vivo*. The role and mechanism of hypermutation in reducing HBV replication should be further investigated to understand the anti-HBV defense system. (HEPATOLOGY 2005;41:626-633.)

Hepatitis B virus (HBV) is a small enveloped DNA virus that replicates in hepatocytes in a noncytolytic manner. Chronic infection with the virus often leads to chronic hepatitis and liver cirrhosis. Hepatocellular carcinoma arises in chronic carriers at a higher frequency than noninfected individuals.[1-4]

The replication cycle of the HBV includes pregenome RNA synthesis and reverse transcription, resulting in the production of the minus strand DNA, which serves as a template of the plus strand DNA.[5] The life cycle of this virus resembles that of the human immunodeficiency virus 1 (HIV-1), which also replicates through reverse transcription.[6]

Recent reports showed that a cytosine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which is packaged in HIV-1 virions, induces G to A hypermutation to a nascent reverse transcript of HIV-1, which contributes in part to the innate antiviral activity.[7-10] The antiviral activity of APOBEC3G is species specific[11,12] and may represent the different actions of the protein.[13,14] The virion infectivity factor encoded by lentivirus genomes associates with APOBEC3G to prevent the enzyme from being packaged into virions and triggers its proteasomal degradation.[15-18] The negative strand DNA of the HBV might be a target of such antiviral deaminase activity. In fact, naturally occurring HBV genomes bearing the hallmarks of retroviral G to A hypermutation have been reported in clones obtained from 2 HBV carriers.[19] Both of these clones represented subgenomes arising from reverse transcrip-

Abbreviations: HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B early antigen; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

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tion of packaged spliced mRNA. However, such hypermutated genomes have otherwise never been reported, nor deposited in DNA databases. Moreover, whether such hypermutated sequences are generated in liver cells or in leukocytes is unknown.

Inhibition of HBV replication by APOBEC3G was observed recently in a transient transfection system.[20] However, no induction of hypermutations to the HBV genome was observed. Instead, prevention of pre-genome RNA packaging was observed.

The aims of the current study were to determine the frequency of viral genomes with G to A substitutions in HBV carriers and patients with acute HBV infection, and to determine whether the hypermutated sequences are generated in hepatic cell lines. We identified such hypermutated viral genomes in 5 of 18 HBV carriers and patients with acute HBV infection and the expression of known deaminases that are potentially responsible for the hypermutation in cultured hepatoma cell lines.

Materials and Methods

Serum Samples. Serum samples from 18 adult Japanese patients with HBV infection were studied. At the time of the study, 8 of these patients had acute HBV infection and tested positive for immunoglobulin M anti–hepatitis B core antibody. The remaining 10 patients were chronic carriers. All serum samples were stored at −80°C until examined. All patients were negative for serum markers of both hepatitis C virus and HIV-1 infection, and none was on antiviral treatment.

Serological Markers of HBV Infection. Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and hepatitis B early antigen (HBeAg) as well as anti-HBe were detected by radioimmunoassay (Abbott Diagnostics, Abbott Park, IL). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents per milliliter. The lower detection limit of this assay is 3.7 log genome equivalents/mL (equivalent to 5,000 copies/mL). The antibody against hepatitis C virus was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Analysis of HBV DNA in Cell Lines That Stably Produce HBV. Two cell lines known to produce wild-type HBV and one cell line known to produce lamivudine-resistant HBV (with mutations of L528M and M552V) were created by transfecting 1.4 genome length sequences of HBV to HepG2 cell lines. These cell lines produced HBV that showed a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers (M. Tsuge et al., manuscript in preparation) and could infect human hepatocyte chimeric mice (manuscript in preparation). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and then treated with media containing interferon alpha or gamma. After 3 days of interferon treatment, the cells were harvested and lysed with 250 μL lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.5% [vol/vol] NP-40) followed by centrifugation for 2 minutes at 15,000*g*. Replicative intermediate of the HBV was immunoprecipitated and subjected to Southern blot analysis and quantitative analysis by light cycler. The effect of lamivudine was analyzed similarly, except that cells were harvested after 5 days of treatment.

Detection of Hypermutated Clones by Polymerase Chain Reaction With PNA Clamping, Cloning, and Sequencing. HBV DNA was extracted from 100 μL serum or culture supernatant by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μL H_2O. The first round of polymerase chain reaction (PCR) was performed with an outer primer set (PLF1 and BR112 [Table 1]) and a second-round PCR with an inner primer set (PLF2 and PLR2 [Table 1]). The peptic nucleic acid (PNA) oligonucleotide, initially designed to detect lamivudine-resistant variant genome,[21] was an 18-mer (PNA 552 [Table 1]) that exactly matched the 18-nucleotide sequence of the original YMDD sequence of DNA polymerase/reverse transcriptase, which contained GG and TG sequences (AGT TAT ATG GAT GAT GTG). The PCR with PNA clamping was performed in a total volume of 25 μL, consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L $MgCl_2$), 0.2 mmol/L each of dNTPs, 1 μL of the DNA solution, 12.5 pmol each primer, 150 pmol PNA 552, and 1 unit of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) together with 0.2 μg anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denaturation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, annealing and extension of primer at 63°C for 50 seconds), followed by a final extension at 63°C for 7 minutes. Part of the X gene was amplified with an outer primer pair (HBV1 and HBV2) and an inner primer (PLF2 and HBV2) (Table 1) for the first- and second-round amplifications, respectively. The amplification for the first-round PCR included initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denatur-

Table 1. Oligonucleotides and PNAs Used in the Current Study

Primer	Sequence
	HBV amplification
PLF1	5′-GGT ATG TTG CCC GTT TGT CC-3′
BR112	5′-TTC CGT CGA CAT ATC CCA T-3′
PLF2	5′-CCT ATG GGA GTG GGC CTC AG-3′
PLR2	5′-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3′
HBV1	5′-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3′
HBV2	5′-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3′
BR109	5′-AAG GGA GTA GCC CCA ACG TT-3′
	PNA
PNA552	H2N-CAC ATC ATC CAT ATA ACT-CON2H
PNA552V	H2N-CAC ATC ATC CAC ATA ACT-CON2H
	Amplification of mRNAs of deaminases
APO1a	5′-CAG AGC ACC ATG ACT TCT-3′
APO1d	5′-ATT GTG GCC AGT GAG CTT CA-3′
APO2a	5′-AGA AGG AAG AGG CTG CTG TG-3′
APO2b	5′-AGA ACG GCT GCC TGC CAA CT-3′
APO2c	5′-GAA GGC TGG CAG GAT GGT GT-3′
APO2d	5′-CAG GTG ACA TTG TAC CGC AG-3′
APO3Aa	5′-TCT TAA CAC CAC GCC TTG AG-3′
APO3Ad	5′-GAA GAT GCG CAG TCT CAC GT-3′
APO3Ba	5′-AGA GCG GGA CAG GGA CAA GC-3′
APO3Bb	5′-GCG TAT CTA AGA GGC TGA AC-3′
APO3Bd	5′-CGA AGG ACC AAA GGG TCA TT-3′
APO3Be	5′-ACA AGT AGG TCT GGC GCC GT-3′
APO3Ca	5′-AGG ACG CTG TAA GCA GGA AG-3′
APO3Cb	5′-CCG ATG AAG GCA ATG TAT GG-3′
APO3Cc	5′-GTC GTC GCA GAA CCA AGA GA-3′
APO3Cd	5′-GAT GTG TAC CAG GTG ACC TG-3′
APO3Da	5′-CTG GGA CAA GCG TAT CTA AG-3′
APO3Dd	5′-AGT CTG AGA TGA AGA GGT GG-3′
APO3Fa	5′-CTT GGG TCC TGC CGC ACA GA-3′
APO3Fd	5′-TCA TCC TTG GCC GGC TAG TC-3′
APO3Ga	5′-GAC TAG CCG GCC AAG GAT GA-3′
APO3Gb	5′-CAC AGT GGA GCG AAT GTA TC-3′
APO3Gc	5′-GTT CGG AAT ACA CCT GGC CT-3′
APO3Gd	5′-ACT CCT GGT CAC GAT GCA GC-3′

ation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, primer annealing at 60°C for 1 minute, and extension of primer at 63°C for 4 minutes), followed by the final extension at 63°C for 7 minutes. The second-round amplification was performed under the same conditions without a primer extension for 3 minutes. The estimated error rate of the Taq DNA polymerase was 1.76×10^{-5} per site in amplifying approximately 10^2 copies of plasmid under the same conditions as described previously and cloning and sequencing.[21] Products (1 μL each) of the second-round of PNA PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°C, 1 minute; 58°C, 1 minute; 72°C, 1.5 minutes) after initial denaturation at 94°C for 4 minutes and followed by the final extension at 72°C for 7 minutes. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, WI) with the standard method, and then transformed into *Escherichia coli* JM 109 (Takara Shuzo Co., Otsu, Japan). Sequencing was performed in the ABI PLISMTM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems). Ten independent clones from each serum sample of patients or supernatant of cell cultures were sequenced for analysis and compared for nucleotide sequences obtained by direct sequencing of PCR products. Hypermutation was defined as clones with a statistically significant number of G to A substitutions.

Sequence Analysis. Nucleotide sequences were aligned and parameters of hypermutation were evaluated with Hypermut Program Package[22] (http://www.hiv.lanl.gov/HYPERMUT/hypermut.html). We used nucleotide sequences obtained by direct sequencing as reference sequences and tentatively labeled clones with a statistically significant ($P < .05$ by Fisher's exact test) number of G to A substitutions as "hypermutated."

Detection of mRNA of Known Deaminases by Reverse Transcription and PCR. Total RNA was extracted from HepG2 cell lines by using cell-to-cDNAII kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed with random primer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan) at 42°C for 60 minutes according to the instructions provided by the manufacturer. Synthesized cDNAs were used to detect mRNAs of known deaminases using primers listed in Table 1. Each of these primers was carefully designed to amplify only the target member of the APOBEC families. Amplification of specific deaminases was confirmed by amplifying each deaminase cDNA by using cDNAs obtained from organs reported to be positive for the expression of each deaminase. The amplicons were analyzed in 2% agarose gel, and the nucleotide sequences were confirmed by direct sequencing.

Results

Frequent Detection of G to A Substituted HBV Genomes by PCR With PNA Clamping in Patients With Acute or Chronic Hepatitis B Virus Infection. Using PCR with PNA clamping, clones with multiple G to A substitutions were found (Table 2). In contrast, only small numbers of other substitutions were identified in these clones. A hypermutated genome of HBV was found in 1 of 8 patients with acute HBV infection and 4 of 10 patients with chronic HBV infection (Table 2). We cloned and sequenced more than 20 clones without PNA and found no hypermutated clones. Among patients with chronic HBV infection, hypermutated clones were identified only in eAb-positive patients (Table 2). Figure 1