drug. No deaths were noted during the trial. ALT flare (ALT levels >2-fold of baseline levels and >10× ULN) occurred in three patients, all in the 1 mg group (Table 3). In each of these patients, the ALT flare was transient and was associated with declining HBV-DNA.

Discussion

In multinational clinical trials, entecavir has demonstrated potent inhibition of viral replication in lamivudine-refractory patients.[14,16] The results of the present study confirm the efficacy of entecavir in Japanese patients with more than 90% of patients in both treatment groups achieving the primary end-point (a reduction in HBV-DNA from baseline of ≥ 2 $\log_{10}$ copies/mL or to <400 copies/mL by PCR assay at week 48). Although there was no significant difference between the two groups at Week 48, the time course for reduction in HBV-DNA was more rapid with the 1 mg dose than with the 0.5 mg dose, and more than 90% of subjects in the 1 mg group had achieved the primary end-point by Week 8, whereas this proportion of responders achieved the primary end-point in the 0.5 mg group by Week 24. As entecavir's genetic barrier to resistance is reduced in the presence of lamivudine resistance-associated mutations, these results stress the importance of rapidly suppressing viral replication to avoid development of an entecavir-resistant virus.

The reduction in mean HBV-DNA seen in these entecavir-treated patients is consistent with that observed in a phase III clinical trial of entecavir in lamivudine-refractory patients (ETV-026)[16] and a phase II clinical trial of entecavir in Chinese lamivudine-refractory patients (ETV-056).[17] These two studies recruited patients with higher baseline viral load than our study of Japanese patients and therefore the observed change in viral load following treatment is more pronounced.

Although the cut-offs for HBV-DNA undetectability and ALT normalization were slightly higher in the present study (<400 copies/mL and <1.25× ULN) compared with ETV-026 and ETV-056 (<300 copies/mL and ≤1× ULN), the proportions of patients achieving the secondary end-points were consistent among treatment groups in this study and those observed in the global and Japanese programs. At the time this study was designed, the WHO toxicity scale was used to define ALT normalization.

Emergence of entecavir-resistant HBV in nucleoside-naive patients is rare due to entecavir's ability to suppress viral load to undetectable levels and its high genetic barrier.[21] Amino acid substitutions rtL180M and rtM204I (associated with resistance to lamivudine) reduce *in vitro* viral susceptibility to entecavir by approximately eightfold.[21] For resistance to entecavir to occur, additional amino acid substitutions are required at either rtT184, rtS202, or rtM250.[16,21] In the present study, virological breakthrough (HBV-DNA increase of ≥ 1 $\log_{10}$ copies/mL from nadir) was observed in one patient in the 1 mg group (at week 44). DNA sequencing of the polymerase gene in this patient revealed the presence of amino acid substitutions rtL180M and rtM204V at baseline and at week 52; there was no change in the pattern of these amino acid substitutions and none of the additional amino acid substitutions which are required for entecavir resistance (at positions rtT184, rtS202, or rtM250) were seen to emerge. ALT levels were within the normal range by the end of treatment (week 52) in this patient. In study ETV-026, rates of entecavir resistance associated with virological breakthrough were 1% and 9% after 1 and 2 years, respectively.[22] Furthermore, cumulative 4-year resistance rates in lamivudine-refractory patients of approximately 40% have been reported[23] and 29% (12/42) of Japanese patients who were treated with entecavir 1 mg through 3 years had evidence of substitutions associated with entecavir resistance[24] indicating that longer term follow up in the patients described here is necessary.

Previous reports indicate that entecavir treatment is well tolerated and associated with a low incidence of on-treatment ALT flare. The most frequent adverse events in these studies were upper respiratory tract infection, headache, fatigue, cough, nausea, and nasopharyngitis.[16,25,26] We observed a similar pattern of adverse events in our clinical trial. The incidences of serious adverse events, grade 3–4 elevations of ALT, and ALT flares were also comparable to rates previously reported in multinational clinical studies.[16,25,26] Moreover, there were no discontinuations of entecavir therapy due to adverse events and no deaths. These results confirm the safety and tolerability of entecavir in lamivudine-refractory Japanese patients.

ALT flares represent a particular safety problem in patients with hepatitis B, potentially leading to decompensated hepatic disease. In this clinical trial, there was no washout period between the end of lamivudine treatment and the initiation of entecavir therapy, and no overlap. Consistent with that seen in the previous clinical studies,[14,25,26] the incidence of ALT flare was low in this clinical trial. The three cases of ALT flare that did occur (all in the 1 mg group) were not associated with hepatic decompensation. All three ALT flares occurred within 8 weeks of initiating entecavir treatment, were associated with at least a 2 $\log_{10}$ reduction in HBV-DNA levels, and resolved on continued entecavir treatment. There was no re-elevation of ALT observed. This may be attributable to the transient reconstitution of the host's immune response as a result of the inhibition of viral replication.[14,25] Our results confirm that it is not necessary to overlap lamivudine therapy with entecavir during the transition from lamivudine to entecavir therapy.[14,25] It should be noted that this study did not include patients with decompensated liver disease and that entecavir's safety profile in this group of patients has yet to be established.

The results of this clinical trial demonstrate the antiviral efficacy of entecavir, at 0.5 mg and 1 mg, in Japanese patients with lamivudine-refractory chronic hepatitis B. Entecavir was generally well tolerated in this population, indicating that there are no specific safety concerns for the treatment of Japanese patients with this agent. The efficacy and resistance profiles of 0.5 mg and 1 mg doses at 48 weeks were similar; however, the rapid suppression of viral load seen with the 1 mg dose is likely to contribute to a more robust long-term resistance profile than with the 0.5 mg dose. For this reason, a daily dose of 1 mg entecavir is recommended for treatment of lamivudine-refractory Japanese patients.

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Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: Two-year follow-up☆

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***Background/Aims*: We studied the long-term efficacy (median follow-up of 28 months) of adefovir (ADV) in combination with lamivudine (LAM) in 132 LAM-resistant Japanese patients with chronic genotype C-dominant hepatitis B virus (HBV) infection.**

***Methods*: The viral response (undetectable HBV-DNA by PCR assay) and the predictor of viral response were evaluated. The emergence of ADV-resistant mutants was investigated during the combination therapy.**

***Results*: The cumulative probability of viral response was 69% at 12 months, and 81% at 24 months. Multivariate analysis identified baseline HBe antigen status ($P = 0.0001$), aspartate aminotransferase level (AST) ($P = 0.001$) and HBV-DNA level ($P = 0.002$) as determinants of viral response to treatment. At the beginning of ADV therapy, substitutions at rtA181 (rtA181T and rtA181S) were identified in 3 patients (2.3%). In the remaining 129 patients, the rtM204 mutants were identified at baseline, and two (1.6%) of the 129 patients developed new ADV-resistant mutants; one was rtA181S and another was rtA181T plus rtN236T mutation.**

***Conclusions*: Adefovir and lamivudine combination therapy effectively suppressed viral replication and maintained the efficacy well in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants is rare, at least over a period of 2 years, in patients with combination therapy.**

***Keywords*: Adefovir dipivoxil; Lamivudine-resistant mutant; Hepatitis B virus; rtA181T; rtN236T; Combination therapy**

1. Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma [1,2]. To date, interferon and three nucleoside and nucleotide analogues (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection in Japan, while telbivudine is licensed in Europe and North America [3,4]. Nucleoside and nucleotide analogues

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Abbreviations: LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

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suppress HBV replication in most patients and improve transaminase levels and liver histology [5–7]. However, prolonged therapy results in the emergence of drug-resistant mutants.

The rate of emergence of drug-resistant mutants is higher in patients treated with LAM than ADV and ETV, and the emergence of such mutants is followed by increases in viral load and re-elevation of transaminase levels [8–10]. Most LAM-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [11,12]. Both experimental and clinical studies have shown that ADV and ETV could suppress not only wild-type but also LAM-resistant strains and have been confirmed as salvage therapy for LAM-refractory patients [13,14]. However, a few studies have already reported the emergence of resistant mutants to these drugs. ADV-resistant mutations are more common in LAM-resistant patients than in treatment-naïve patients during ADV monotherapy, and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [15,16]. However, a recent study reported that LAM-resistant HBeAg-negative patients treated with combination therapy of ADV with LAM did not develop resistance to ADV over a period of 3 years and the rate of undetectable HBV-DNA in combination therapy was higher than in the ADV monotherapy [14].

Recently, we reported the efficacy of ADV plus LAM combination therapy in patients with LAM-resistant chronic HBV infection [17]. However, the number of patients was limited and the virological analysis was inadequate in that study. In the present study, we analyzed the efficacy of ADV plus LAM combination therapy in 132 LAM-resistant patients with chronic hepatitis B over a period of 2 years. We also investigated the emergence of ADV-resistant mutants before and during the combination therapy.

2. Patients and methods

2.1. Patients

A total of 132 consecutive adult Japanese patients with chronic HBV infection were treated with adefovir dipivoxil at Toranomon Hospital, Tokyo, Japan, in addition to ongoing LAM treatment for more than 52 weeks starting in 2002. Enrolment in this study and the start of ADV treatment were determined by the following criteria: (1) Increase in serum HBV-DNA levels of $\geqslant$1 log copies/ml during LAM treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy. (2) Detection of mutations of the YMDD motif and/or other mutations related to LAM resistance before the start of ADV treatment, as diagnosed by the PCR-based method described later and/or direct sequence analysis. (3) No history of treatment with other nucleoside analogues such as famciclovir and entecavir. The exclusion criteria were as follows: (1) Serum creatinine levels $\geqslant$1.5 mg/dl. (2) Patients coinfected with hepatitis C, delta viruses, or HIV. (3) History of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

2.2. Methods

Patients received a 10-mg once-daily dose of oral ADV, in addition to ongoing LAM treatment (100 mg/day). Blood samples were obtained once every month during the ADV + LAM combination therapy, and analyzed for virological markers, biochemical markers, together with liver function tests, renal function tests, and complete blood cell counts. The primary efficacy measures were undetectable HBV-DNA level by PCR assay (<2.6 log copies/ml) and normalization of ALT level (<50 IU/ml); the secondary efficacy measure was HBeAg seroconversion. The rate of each measure was evaluated 6, 12, 18 and 24 months after the start of ADV + LAM treatment.

2.3. Analysis of virological markers

HBsAg, HBeAg and antibody against HBeAg (anti-HBe) were determined by commercially available radioimmunoassay systems (Abbott Japan, Tokyo, Japan). HBV-DNA serum level was determined by using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). The HBV genotype was determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Tokyo) based on the method of Usuda et al. [18].

2.4. Detection of antiviral-resistant mutations

Substitution at rtM204 of the YMDD motif was identified at baseline by using the Enzyme-Linked Mini-sequence Assay with a commercial assay kit (PCR-ELMA; Genome Science). HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo) and dissolved in 20 μl H_2O. Detection of substitutions at rtA181 and rtN236 was achieved by PCR with restriction fragment length polymorphism (RFLP). For this purpose, HBV-DNA extracted from serum samples was amplified by PCR using primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCACTG AACAAATGGCACTAGTAAGCTGA -3′ for rtA181, and 5′-CCA CTTTTCTTTTGTCTTTGGGTATACATTTAA-3′ and 5′-GATCG GCAGAGGAGCCACAA -3′ for rtN236. The PCR products were digested with five units of restriction enzyme *Esp*I for rtA181, DraI for rtN236 and subjected to electrophoresis in 3.5% agarose gel. With regard to the sensitivity of the RFLP assay, when the mutant was mixed with 10-fold the amount of wild-type, the mutant ($\geqslant 10^2$ copies/ml) could be detected. The nucleotide and amino acid substitutions of the detected mutant samples were confirmed by direct sequence analysis.

2.5. Statistical analysis

All data were analyzed using the statistical package SPSS II (version 10.0, SPSS Inc, Chicago, IL). Non-parametric tests including the chi-squared test, Fisher's exact probability test, and the Mann–Whitney *U*-test were used to compare the background characteristics and efficacy. The cumulative rate of undetectability of HBV-DNA and HBeAg loss was calculated using the Kaplan–Meier method and differences between the curves were tested using the log-rank test. Univariate analyses were conducted using logistic regression analysis. All factors found to be at least marginally associated ($P < 0.15$) were entered into multivariate analysis using a stepwise Cox regression analysis. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Study population

The clinical and virological profiles of the 132 patients at the start of ADV + LAM treatment are shown in Table 1. At the commencement of ADV + LAM treatment, 41 patients (31.1%) had cirrhosis, and 79 patients (59.8%) were positive for HBeAg. Six of the 132 patients were treated with ADV at the time of virological breakthrough and the remaining 126 patients were treated at the time of breakthrough hepatitis.

3.2. Virological and biochemical response

The cumulative rates of undetectable serum HBV-DNA levels (<2.6 log copies/ml) were 56% at the end of 6 months, 69% at 12 months, 81% at 24 months and 87% at 36 months. The cumulative rates of normalized serum ALT levels were 73% at the end of 6 months, 85% at 12 months and 99% at 24 months. Of the 79 HBeAg-positive patients, the cumulative rates of HBeAg loss were 10% at 6 months, 16% at 12 months, 34% at 24 months and 39% at 36 months. The cumulative rates of HBeAg seroconversion were 7.5% at 6 months, 13% at 12 months, 24% at 24 months and 32% at 36 months.

3.3. Baseline parameters associated with virological response as determined by univariate and multivariate analyses

Univariate analysis identified six baseline parameters that influenced the undetectability of serum HBV-DNA during therapy: HBeAg status (negative; $P < 0.00001$), HBV-DNA (<7 log copies/ml; $P < 0.00001$), AST (>150 IU/L; $P < 0.00001$), ALT (>200 IU/L; $P = 0.0074$), fibrosis (liver cirrhosis; $P = 0.0057$) and T-Bil (>1 mg/dl; $P = 0.0535$). No association with other factors was noted: patient age, sex, serum albumin, serum creatinine, platelet count, YMDD mutant status and HBV genotype.

Multivariate analysis that included the above variables identified four parameters that independently influenced the virologic response: HBeAg status ($P = 0.0001$), AST ($P = 0.001$), HBV-DNA ($P = 0.002$), and fibrosis ($P = 0.015$) (Table 2). These results confirmed that HBeAg status is the most influential factor of undetectability of HBV-DNA. The time to undetectable HBV-DNA was significantly shorter in HBeAg-negative than in-positive patients ($P = 0.00001$). The time to normalization of ALT level was also shorter in HBeAg-negative than in-positive patients (Fig. 1a and b). The rates of undetectable HBV-DNA in the HBeAg-negative group were 94% at the end of 12 months and 100% at 24 months. On the other hand, the undetectability rates of HBV-DNA in the HBeAg-positive group were 47% at the end of 12 months, 68% at 24 months and 78% at 36 months (Fig. 1, Table 3). Therefore, we thought that it was important to investigate the predictive factor(s) of virologic response in HBeAg-positive patients. There were 21 non-responders (HBV-DNA ≥4.5 log copies/ml at 6 months of ADV + LAM), whose HBV-DNA level were all over 7 log copies/ml. Therefore we selected the responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) with high levels HBV-DNA (≥7 log copies/ml) at baseline and we found 15 patients who fulfilled the criteria. The 36 HBeAg-positive patients with high levels HBV-DNA underwent sequence analysis of the RT lesion in the polymerase gene. However, there were no differences in the RT lesion; i.e., rtH55, rtL80, rtV173, rtM180, rtI233, and rtN337, between responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) and non-responders (HBV-DNA >4.5 log copies/ml at 6 months of ADV + LAM).

Table 1
Baseline characteristics at commencement of adefovir dipivoxil ($n = 132$)

Age (years)*	47 (26–73)
Gender (Male:Female)	105:27
Prior LAM therapy (month)*	31 (8–110)
ADV treatment duration (month)*	28 (12–50)
Presence of cirrhosis (%)	41/132 (31.1)
HBV genotype (A:B:C:D)	7:5:119:1
HBeAg-positive (%)	79/132 (59.8)
HBV-DNA (log copies/ml)*	7.3 (3.3–>7.6)
rtM204 mutant (%)	130/132 (98.4)
I:V:I + V#	69:28:33
AST (IU/L)*	132 (31–1413)
ALT (IU/L)*	132 (24–1563)
T-Bil (mg/dl)*	0.8 (0.6–6.0)
Albumin (g/dl)*	3.9 (2.8–4.7)
Serum creatinine (mg/dl)*	0.8 (0.4–1.3)

* Data are median values (range).
# I → YIDD, V → YVDD, I + V → YIDD + YVDD mix.

3.4. Genotypic analysis of ADV- and LAM-resistant mutants

Genotypic resistance to ADV was looked for in PCR positive (HBV-DNA ≥ 2.6 log copies/ml) samples. Number of samples tested at baseline, 1 year and 2 years were 131 of 132 samples, 45 of 45 samples, 16 of 16 samples, respectively. The substitutions at rtA181 and rtN236 were assessed annually by RFLP method and direct sequence. At baseline, substitutions at rtA181 were identified in 3 patients (2.3%), whose genotypes were rtA181T without substitution at rt204, rtA181S without substitution at rt204 and rtA181T plus rtM204I double mutation (Fig. 2). On the other hand, substitution at rt236 was not identified at the start of ADV. In

Table 2
Multivariate analysis of baseline factors associated with virological response

Factors	Category	Hazard ratio	95% CI	P
HBeAg status	1: negative	1		
	2: positive	0.380	0.242–0.595	0.0001
AST (IU/L)	1: <150	1		
	2: ⩾150	2.115	1.357–3.296	0.001
HBV-DNA (log copies/ml)	1: <7	1		
	2: ⩾7	0.532	0.353–0.797	0.002
Cirrhosis	1: no cirrhosis	1		
	2: cirrhosis	1.683	1.107–2.559	0.015

Note. Virological response: undetectable serum HBV-DNA by amplicor monitor assay (<2.6 log copies/ml).

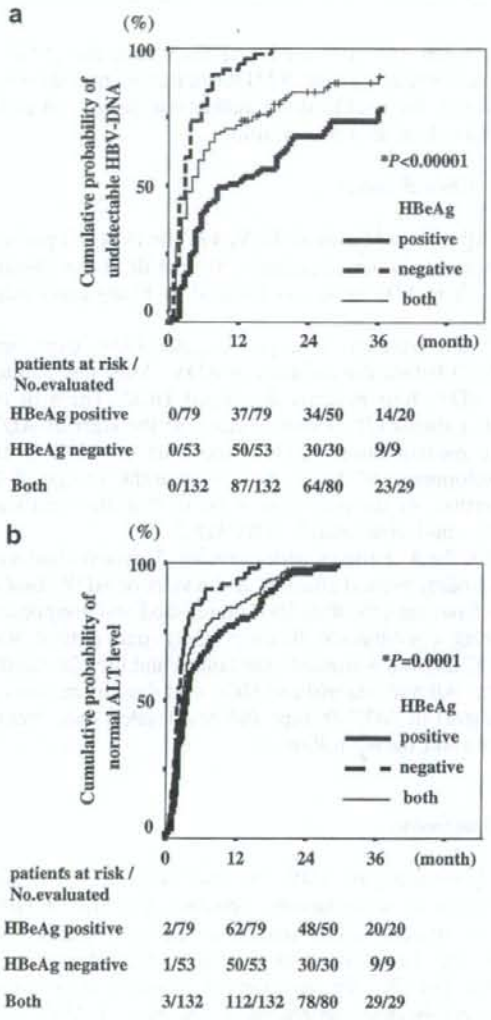

Fig. 1. (a) Cumulative probability of undetectable HBV-DNA during ADV + LAM combination therapy in patients with HBeAg-positive, -negative and both. (b) Cumulative probability of normal ALT during ADV + LAM combination therapy in patients HBeAg-positive, -negative and both. *P values between HBeAg-positive and -negative groups.

the remaining 129 patients, rtM204 mutations without substitutions at rt181 and rt236 were identified. Following ADV + LAM combination therapy, new ADV-resistant strains were identified in two patients (1.6%); one had rtA181S and the other had rtA181T plus rtN236T double mutation; they were the only two patients (among the 129 patients) who showed virological rebound during ADV + LAM therapy (Fig. 3). The cumulative rate of ADV-R was calculated every year; 1% of the first year, 1% of the second year, 1% of the third year and 8% of the fourth year. However, long follow-up studies of larger population samples are needed for a more accurate evaluation of the cumulative rate.

During combination therapy, 105 patients achieved virological response. Ninety-eight of 105 (93.3 %) patients maintained virological response. Only one patient was included according to our definition of virological breakthrough that was defined as increase in serum HBV-DNA levels of ⩾1 log copies/ml (3.6 log copies/ml) during combination therapy and also developed rtA181S mutation (Fig. 3b). However, the remaining 6 patients showed fluctuated HBV-DNA level of between <2.6 and 3.1 log copies/ml transiently, whose genotypes were wild-type at rtA181 and rtN236 during treatment.

3.5. Clinical course of patients who had developed rtA181 mutations at the start of ADV + LAM combination therapy

Three patients developed substitutions at rtA181 associated with LAM resistance. All patients were HBeAg-positive at the start of LAM. As shown in Fig. 2, two of the three patients developed rtA181T and rtA181S without YMDD mutation and the viral load did not respond sufficiently to ADV therapy. The patient with rtA181S continued to show HBV-DNA >7 log copies/ml after 2 years of ADV + LAM treatment (Fig. 2a). Subsequently, the patient was changed to 0.5 mg of ETV, which resulted in 2 log copies/ml reduction in viral load and improvement of ALT. The other patient developed rtA181T mutation mixed with wild strain (Fig. 2b). At the end of 6-month

Table 3
Undetectable rate of HBV-DNA by Amplicor monitor assay in HBeAg-negative and -positive patients

HBV-DNA (log copies/ml)	Baseline	6 months	12 months	18 months	24 months
HBeAg-negative					
<2.6	0 (0%)	40 (75%)	50 (94%)	47 (100%)	30 (100%)
2.6–<4.5	3 (6%)	13 (25%)	3 (6%)	0 (0%)	0 (0%)
≥4.5	50 (94%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total, *n* (%)	53 (100%)	53 (100%)	53 (100%)	47 (100%)	30 (100%)
HBeAg-positive					
<2.6	0 (0%)	32 (40%)	37 (47%)	34 (54%)	34 (68%)
2.6–<4.5	3 (4%)	26 (33%)	29 (37%)	21 (35%)	15 (30%)
≥4.5	76 (96%)	21 (27%)	13 (16%)	8 (13%)	1 (2%)
Total, *n* (%)	79 (100%)	79 (100%)	79 (100%)	63 (100%)	50 (100%)

ADV + LAM therapy, the HBV-DNA level diminished by 1.5 log copies/ml and ALT level improved to the normal range. At that time, only the mutant strain (rtA181T) was detected, suggesting that the viral reduction was due to the suppression of wild-type HBV strain. The HBV-DNA level was persistently above 5 log copies/ml even at the end of 1 year of ADV + LAM therapy. On the other hand, in the patients with rtA181T + rtM204I mutation, viral load rapidly decreased to the undetectable HBV-DNA level at the end of 6 months of ADV + LAM combination therapy (Fig. 2c).

3.6. Clinical course and clonal analysis in patients who developed ADV-related mutation during combination therapy

Fig. 3 shows the clinical course of patients with ADV-resistant mutants. The first ADV-resistant HBV strain was isolated from a 32-year-old Japanese man with genotype C (Fig. 3a). At 15 months after the start of LAM, viral and biochemical breakthroughs were observed. To suppress the viral HBV-DNA, ADV was added to LAM therapy. The mutant strain with rtA181T associated with ADV resistance appeared at 6 months of ADV + LAM therapy, while another rtN236T mutation appeared at 3 years of ADV therapy. Moreover, breakthrough hepatitis was observed after 3.5 years of ADV + LAM therapy (Table 4). Interestingly, the rtA181T at the end of 6 months of ADV therapy, due to single nucleotide substitution (TGG to TGA), resulted in early termination of overlapping HBs gene by creating a stop codon. On the other hand, at the end of 3 years of ADV therapy, all rtA181T mutant strains changed to double nucleotide substitutions (TGG to TTA), which induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L) developed.

Another mutant strain was detected in a 38-year-old Japanese man with genotype C-HBV infection (Fig. 3b). Following 46 months of ADV + LAM therapy when the viral load was increased, the rtA181S mutant strain without YMDD mutation was detected; however, the viral load diminished naturally to an undetectable level in a few months.

3.7. Clinical events

After the addition of ADV, 4 of the 132 (3%) patients elevated in serum creatinine >0.5 mg/dl above baseline and their ADV dose was reduced to 10 mg every other day.

Eight patients developed hepatocellular carcinoma (HCC) before the addition of ADV. After the addition of ADV, four patients developed HCC. Three of the four patients (75%) had cirrhosis at the start of ADV. The median duration from the start of ADV to the development of HCC was 14 months (range, 6–26 months). At the diagnosis of HCC, 3 of the 4 patients (75%) had undetectable HBV-DNA.

Of the 41 patients with cirrhosis, 5 patients had ascites and/or pleural effusion at the start of ADV. In 4 of the 5 patients, the fluid level diminished and disappeared during combination therapy. Only one patient with HCC showed worsened liver failure and died 22 months later. All patients without HCC and decompensation at the start of ADV therapy did not develop liver decompensation during follow-up.

4. Discussion

The efficacy of ADV combined with LAM has been reported in some studies; however, the rate of HBV-DNA undetectability under combination therapy was found to be the same as in patients treated with ADV alone [14,19]. We investigated whether combination therapy is characterized by a low risk of ADV resistance. In this study, we studied the long-term efficacy of ADV when added to LAM in 132 patients with chronic hepatitis B who developed LAM resistance. The results demonstrated that combination therapy

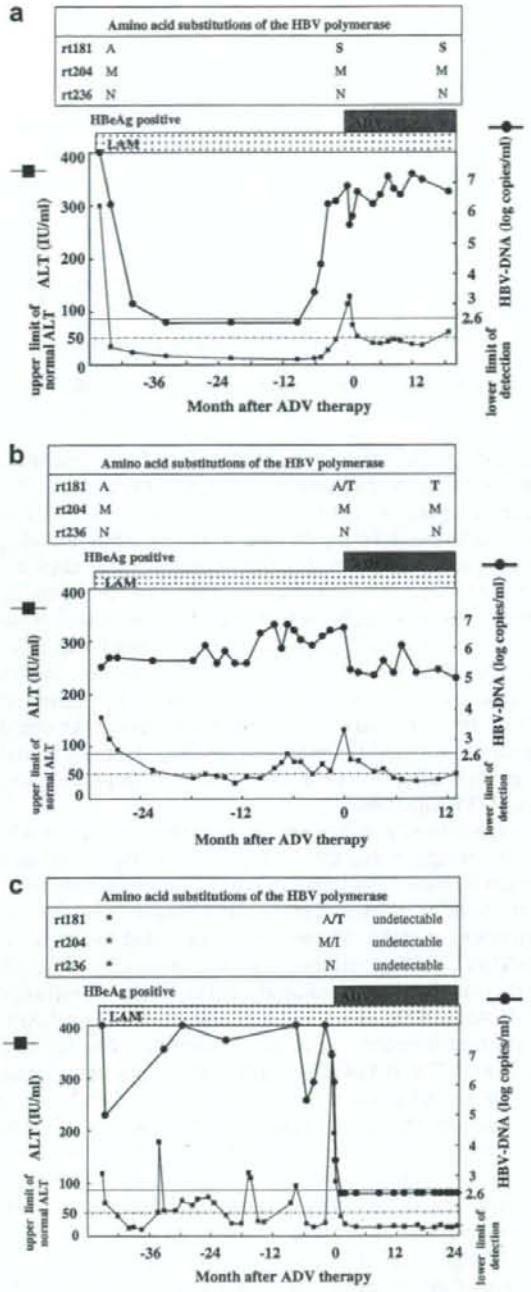

Fig. 2. Clinical course of three patients who showed emergence of ADV-resistant mutants at the commencement of ADV therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above each graph. (a) Clinical course of a patient who developed the rtA181S mutant. (b) Clinical course of a patient who developed the rtA181T mutant. (c) Clinical course of a patient who developed the rtA181T with rtm204I mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase; *no data.

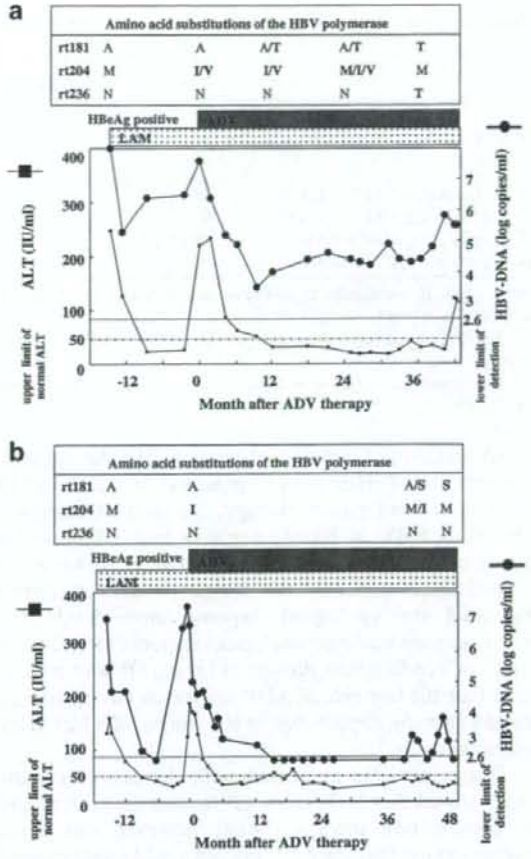

Fig. 3. Clinical course of two patients with LAM-resistant HBV who showed the emergence of an ADV-resistant mutant during combination therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above the graph. (a) Clinical course of a patient who developed the rtA181T + rtN236T mutant. (b) Clinical course of a patient who developed the rtA181S mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

rapidly and consistently suppressed the HBV-DNA. Moreover, we demonstrated that the emergence of ADV-resistant mutants was rare during the combination therapy for up to 3 years. However, our virological analysis showed that substitutions at rt181, which were associated with both LAM and ADV resistance, need to be evaluated during combination therapy.

Multivariate analysis in this study revealed that the baseline HBeAg status, AST level and HBV-DNA level influenced the cumulative probability of undetectability of serum HBV-DNA. A number of previous studies also identified almost the same predictors of virological response during ADV alone or combination therapy [20,21]. In particular, the undetectable rate of HBV-

Table 4
Clonal analysis of samples from the patient who developed resistance to ADV + LAM combination therapy

	Relative rate (%) of clones (No. of clones/total)				
	Wild	rtM204I/V	rtA181T(1)	rtA181T(2)	rtA181T + rtN236T
rtA181	–	–	T(HBsAgstop)*	T(sW172L)#	T(sW172L)#
rtM204	–	I/ V	–	–	–
rtN236	–	–	–	–	N
(1) At the start of ADV + LAM	40 (4/10)	60 (6/10)	0	0	0
(2) 6 months after ADV + LAM	0	59 (13/22)	41 (9/22)	0	0
(3) 2 years after ADV + LAM	16 (4/25)	36 (9/25)	36 (9/25)	12 (3/25)	0
(4) 3 years after ADV + LAM	0	0	0	0	100 (20/20)

Note. rtM204I, methionine to isoleucine substitution at rt204; rtM204V, methionine to valine substitution at rt204; rtA181T, alanine to threonine substitution at rt181.

* The single nucleotide substitution (TGG to TGA) resulted in rtA181T mutation and early termination of overlapping HBs gene by creating a stop codon.

The double nucleotide substitution (TGG to TTA) resulted in amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L).

DNA was more frequent and faster in HBeAg-negative patients than in HBe-positive patients. At the end of 12 months of combination therapy, the rates of undetectability of HBV in HBeAg-negative and HBe-positive patients were 94% and 47%, respectively. However, in HBe-positive patients, the longer treatment course increased the virological response more frequently. Some patients achieved virological response after 2 more years of combination therapy (Fig. 1a). It was considered that the low risk of ADV resistance during combination therapy contributed to the longer effect of HBV suppression.

Our result is in agreement with the previous study that showed that ADV-resistant mutants are infrequent in combination therapy [14,19]; however, our study demonstrated that the ADV mutant could have emerged during combination therapy. We identified the emergence of rtA181T/S and/or rtN236T mutation in two of the 129 patients with YMDD mutant as an ADV-resistant strain during the ADV + LAM combination therapy. To our knowledge, this is the first report of emergence of ADV-resistant mutant followed by breakthrough hepatitis during combination therapy as shown in Fig. 3a. A previous open-label study in HBeAg-negative LAM-resistant patients demonstrated that combination therapy did not result in the development of resistance to ADV over a period of 3 years, in contrast to ADV monotherapy that was associated with the development of such resistance in 21% of the patients after the first year [14]. Although another recent study reported the appearance of ADV-resistant mutants in three patients during combination therapy, they were all initially switched to ADV monotherapy and later changed to the combination therapy after several months [20]. Another recent study reported an emergence rate of ADV resistance during ADV + LAM of 1% at 1 year and 4% at 3 years; however, no virological rebound was noted [21]. The patients in our study continued to show a viral load of up to 5.8 log copies/ml and developed breakthrough hepatitis. Some studies of ADV monotherapy reported that the rise in ALT after the emergence of rtA181 and/or rtN236 mutant is mild to moderate [9,14,24]. Several *in vitro* studies including our previous study [22–25] demonstrated that the rtA181T and rtN236T mutant leads to a minor reduction in the susceptibility to both LAM and ADV. However, one study of 998 naïve patients treated with ADV showed that the rtA181V + rtN236T mutation was significantly associated with virological breakthrough [26]. In our study, a similar phenomenon emerged; patients with ADV resistance developed breakthrough hepatitis after rtN236T mutation that appeared after rtA181T mutation.

Interestingly, clonal analyses of HBV in patients with ADV-resistant mutants in this study showed that such mutants were mixed with rtA181T mutants without substitutions at rt204, and rtM204I/V mutants without substitution at rt181. Moreover, we identified two types of rtA181T mutant strains; one was a single nucleotide substitution that induced prematurely terminated HBsAg and the other was a double nucleotide substitution that induced amino acid substitutions in the HBs antigen. The rtA181T mutant with prematurely terminated HBsAg cannot replicate and spread by itself because of the lack of HBs antigen. This type of strain is thought to replicate *in vivo* supplied HBs antigen from wild-type strains as helpers. Thus, the mutants changed themselves to the HBV with mature HBsAg by additional nucleotide substitution. Our previous study identified the rtA181T with mature HBsAg first; however, the mutant emerged during LAM therapy and it did not show a stepwise process [25].

We also demonstrated that the substitution at rt181 was associated with not only ADV resistance but also LAM resistance. At the commencement of ADV + LAM combination therapy, the substitutions at rtA181 as LAM resistance were identified in three patients (2.3%), who exhibited poor viral reduction

during the combination therapy. Of note, the rtA181S mutation is a novel LAM-resistant strain that has never been reported. There are a few reports of the rtA181 mutation associated with LAM resistance. A recent study reported the presence of rtA181T mutants in 3 of 57 (5.3%) LAM-resistant patients [15] and another study showed that 6 of 145 (4%) LAM-resistant patients developed rtA181T/V mutation [21]. If ADV therapy produces insufficient reduction of LAM-resistant HBV, it is important to suspect the emergence of ADV-related mutant at the commencement of ADV therapy and plan a new treatment strategy. However, there is no consensus at present on the management of patients with ADV + LAM-resistant mutant. Entecavir was the only agent reported to be effective both *in vitro* and *in vivo*. In our study, the patient with rtA181S mutation was switched to entecavir therapy; however, this did not produce a sufficient reduction in the viral load. On the other hand, recent studies reported the efficacy of tenofovir for patients with LAM-resistant mutants [27,28]. Further studies are needed to clear this issue.

In conclusion, ADV in combination with LAM effectively suppressed viral replication and was efficacious in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants was rare in patients on ADV + LAM combination therapy at least for 2 years. However, virological analysis showed that the substitution at rt181, which was associated with both LAM and ADV resistance, was needed for careful monitoring before and during combination therapy.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Hepatocarcinogenesis Following HCV RNA Eradication by Interferon in Chronic Hepatitis Patients

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Abstract

Objective Interferon (IFN) therapy reduces the incidence of hepatocarcinogenesis in patients with hepatitis C viral (HCV) infection who achieve a sustained virological response (SVR). The aim of the present study was to determine the rate of hepatocarcinogenesis and the risk factor in sustained virological responders.

Patients and Method The study subjects were 1,193 patients with HCV-related chronic liver disease and IFN- or IFN plus ribavirin-induced SVR. The age, male/female ratio, and liver fibrosis stage [(F0-F3)/LC] were 15-83 years, 808/385, and 1106/41, respectively. Patients were followed-up for 8.3 years (range, 0 to 19.0 years) and the incidence of hepatocellular carcinoma was recorded.

Results Hepatocellular carcinogenesis was detected in 23 patients during the follow-up. The crude rates of hepatocarcinogenesis at 5, 10, and 15 years were 1.5%, 2.4% and 4.1%, respectively. Multivariate analysis identified cirrhosis, male sex and age older than 50 years as determinants of hepatocarcinogenesis with hazard ratios of 12.9 ($p<0.001$), 6.45 ($p=0.012$), and 20.2 ($p=0.004$), respectively.

Conclusion Long-term follow-up of patients with chronic HCV infection is necessary even in those who show SVR, especially in male elderly patients with cirrhosis.

Key words: hepatitis C virus, hepatocellular carcinoma, chronic hepatitis, sustained virological response, cox proportional hazard model

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Introduction

Interferon (IFN) is effective in eliminating HCV and reducing serum alanine aminotransferase (ALT) in some patients with chronic hepatitis C viral (HCV) infection (1-3). A reduction in the incidence of hepatocellular carcinoma (HCC) in patients with HCV-associated hepatitis and cirrhosis treated with IFN has been reported by many investigators (4-14). The previous study (14) suggested that fluctuations and persistently high levels of ALT in patients with chronic HCV infection enhances the carcinogenic process. From the viewpoint of liver carcinogenesis, IFN plays a suppressive action on the development of HCC through reduction or complete remission of inflammatory activity. Multivariate analysis has indicated that IFN lowers the carcinogenesis rate in those patients who show IFN-induced reduction in ALT levels (15). In patients with IFN-induced normalization of ALT levels, the groups at high risk for carcinogenesis were older, male, and a more advanced histologic stage (16). Patients who show elimination of HCV RNA are considered to exhibit normalization of ALT levels. Therefore, the incidence of carcinogenesis is assumed to be lower in patients with sustained virological response (SVR) than in those who show biochemical response (BR) and no response (NR) to IFN therapy. SVR was defined as persistent disappearance of HCV RNA after therapy, BR as normal ALT values without elimination of HCV RNA for at least 6 months after therapy, and NR as persistently abnormal or only transient normalization of ALT for less than 6 months. However, he-

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patocarcinogenesis still occurs in patients with SVR (17-31). In the studies of Toyoda et al (17) and Ikeda et al (18), the risk factors for carcinogenesis were not discussed due to few sustained virological responders with carcinogenesis. Tokita et al (19) and Kobayashi et al (20) indicated that the risk factors of hepatocarcinogenesis after elimination of HCV RNA are severe fibrosis, male sex, and regular consumption of moderate amounts of alcohol, and old age at the start of IFN treatment. Their hazard ratios could not be estimated because of the relatively small number of patients with SVR. Ikeda et al (21) indicated the hazard ratios of risk factors; older age, increased aspartate aminotransferase (AST), and decreased platelet count. However, the study population was restricted to patients who received IFN monotherapy and it did not include patients who received either pegylated interferon (PEG IFN) or combination therapy of IFN and ribavirin.

The aims of this study were to estimate the rate of hepatocellular carcinogenesis in patients with chronic HCV infection who show SVR to IFN monotherapy or combination therapy of IFN and ribavirin and to determine the risk factors that affect carcinogenesis rate in such patients using multivariate analysis.

Patients and Methods

Study population

In this retrospective cohort study, all patients with chronic HCV infection who started IFN therapy between February 1987 and July 2006 in the Department of Hepatology, Toranomon Hospital were analyzed in the database. Prior to IFN therapy, they were positive for anti-HCV (second- or third generation anti-HCV, enzyme-linked immunosorbent assay, Dainabot, Osaka, Japan) and HCV RNA. Anti-HCV was assayed using stored frozen sera at -80°C. HCV RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc., Belleville, NJ) or the branched DNA probe assay (b DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan). The medical records of 1,193 patients with HCV infection, who had achieved HCV RNA elimination after IFN therapy or the combination therapy of IFN and ribavirin were obtained. The sera of all patients were negative for hepatitis B surface antigen (HBsAg; radioimmunoassay, Austria, Abbott Laboratories, Detroit, MI). The study protocol was approved by the Human Ethics Review Committee of Toranomon hospital.

Clinical background and laboratory data

The background of 1,193 patients who achieved SVR is shown in Table 1. They included 809 men and 384 women, who were 15 to 83 years old with a median age of 50 years at the commencement of therapy. HCV genotype was analyzed by the immunoserological typing method using a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan).

Table 1. Patients' Profiles, Virological, Histological Characteristics of the Patients Prior to Their Interferon (IFN) Therapy and Protocol of IFN Therapy

Number of patients	1193
Sex (M / F)	809 / 384
Age (years) *	50 (15-83)
Observation period (year) *	8.3 (0.0-19.0)
HCV genotype	
Genotype 1a, 1b	494 (41.4%)
Genotype 2a, 2b	670 (56.2%)
Genotype 1+2	5 (0.4%)
Genotype 3	1 (0.1%)
Undetermined	23 (1.9%)
Histological stage of hepatitis	
F0 (no fibrosis)	7 (0.6%)
F1 (slight fibrosis)	738 (61.9%)
F2 (moderate fibrosis)	289(24.2%)
F3 (severe fibrosis)	72 (6.0%)
F4 (cirrhosis)	41 (3.4%)
Not examined	46 (3.9%)
IFN therapy	
Monotherapy	1032 (86.5%)
Combination therapy with ribavirin	161 (13.5%)
Type of IFN	
IFN-α	850 (71.2%)
PEG IFN-α	46 (3.9%)
IFN-β	251 (21.0%)
IFN-α/PEG IFN-α+IFN-β	47 (3.9%)

IFN: interferon, PEG IFN: pegylated interferon

*Data are median (minimum, maximum) values.

The HCV genotype was 1 (genotype 1a and 1b) in 494 patients, 2 (genotype 2a and 2b) in 670 patients, 1 plus 2 in 5 patients, 3 in 1 patient. Before treatment, 1,131 patients underwent liver biopsy with or without peritoneoscopy to assess the staging of liver fibrosis and the grade of inflammatory activity based on the classification of Desmet (32). Staging of liver fibrosis was defined as F0 (no fibrosis), F1 (fibrosis portal expansion), F2 (bridging fibrosis), F3 (bridging fibrosis with architectural distortion) and F4 (cirrhosis). Additionally, 16 patients were diagnosed as cirrhosis by peritoneoscopy without biopsy, laboratory values or clinical features: 1147 patients were diagnosed with chronic hepatitis (n=1,106) and cirrhosis (n=41) (F0/F1/F2/F3/F4=7/738/289/72/41).

Treatment protocol

IFN was performed once in 973 patients and more times of therapy in 220 patients (twice/three times/four times/five times/six times=166/38/13/2/1). IFN and ribavirin combination therapy was used to eliminate HCV RNA for 161 patients, while IFN monotherapy eliminated HCV RNA for the other 1,032 patients. The type of IFN was IFN-α (natural or recombinant)/PEG IFN-α in 896 patients (75.1%); IFN-α in 850 patients (71.2%), PEG IFN-α in 46 patients (3.9%), IFN-β (natural) in 251 patients (21.0%) and IFN-α or PEG IFN-α and IFN-β in 47 patients (3.9%).

A total of 613 patients (51.4%) received 3 to 9 million units of IFN everyday for 8 weeks followed by twice or three times a week for 1 to 305 weeks (for 16 to 22 weeks in 75% of patients), 304 patients (25.5%) received 3 to 9 million units of IFN everyday for 1-5 weeks followed by three times a week, 5 patients (0.4%) for 12 weeks and one patient (0.1%) for 24 weeks followed by intermittent administration. A total of 124 patients (10.4%) underwent short therapy with IFN everyday for 4-8 weeks, 2 patients (0.2%) for 10-12 weeks, 18 patients (1.5%) for 18-24 weeks. 2 patients (0.2%) had a prolonged administration of IFN for 11 and 13 months. And 63 patients (5.3%) underwent intermittent administration of three times a week for 4weeks to 70 months. This protocol is one of the low-dose intermittent IFN therapies. A total of 48 patients (4.0%) underwent 50-180 μg of PEG IFN once a week: 8 patients for 24 weeks and 40 patients for 48 weeks.

Follow-up and diagnosis of hepatocellular carcinoma

Almost all patients were followed-up every week or biweekly during IFN monotherapy. This included hematological, biochemical, and virological tests. Patients treated with pegylated IFN were also checked every week or biweekly. After the completion of treatment, monthly follow-up was continued until the virological response could be determined. When SVR was confirmed, imaging studies were conducted once or twice per year in the majority of patients; these included computed tomography (CT) or ultrasonography (US), except those patients who were lost to follow-up. Angiography was performed only when HCC was highly suspected on CT or US. The presence of a characteristic hypervascular nodule on angiography was considered a specific finding for HCC, and histological confirmation was usually not required in the majority of such cases. The clinical trends of tumor markers were also taken into account. When angiography could not be performed, the hepatic mass was considered HCC when CT showed a hypervascular mass and the tumor marker level was elevated. No fine needle biopsy or histopathological examination was performed before treatment.

The date of the last follow-up in this study was March 1, 2007. The median observation period of the entire group was 8.3 years with a range of 0.0 to 19.0 years. As for patients of the combination therapy, the median follow-up period was 3.2 years with a range of 0.0 to 7.5 years.

Statistical analysis

Non-parametric procedures were employed for the analysis of background clinicopathological parameters, including Mann-Whitney U-test. The rate of hepatocarcinogenesis was calculated for the period between the end of IFN therapy and appearance of HCC, using Kaplan-Meier technique (33). Differences in carcinogenesis curves were tested using the log-rank test. Independent factors associated with the appearance of HCC were studied using stepwise Cox regression analysis (34). The following seven variables were analyzed for potential covariates for liver carcinogenesis; age, sex, fibrotic stage of hepatitis at the initiation of the IFN therapy, HCV genotype, use of ribavirin (monotherapy or combination therapy), type of IFN (α or β, and number of treatments. Factors found significant were entered into a multivariate Cox proportional hazard model. A P-value less than 0.05 was considered significant. Data were analyzed using the SPSS software ver. 11.0.1J (SPSS Inc., Chicago, IL).

Crude rates of hepatocarcinogenesis

During a median observation period of 8.3 years with a range of 0.0 to 19.0 years, HCC was diagnosed in 23 (1.9%) of the 1,193 patients. The median interval between the end of therapy and detection of HCC was 3.1 years (range, 0-12.9 years).

The characteristics of HCC patients are shown in Table 2. Patients who developed HCC before the initiation of IFN therapy were excluded. Four patients (Nos. 2, 5, 15 and 22) developed HCC before the diagnosis of SVR but after the elimination of HCV RNA. The surgically resected liver tissue was also examined by the PCR method in 4 cases (Nos. 1, 6, 13 and 14), which showed no HCV RNA.

HCC patients included 21 men and 2 women; the median age at the start of IFN therapy and at diagnosis of HCC was 58 (range, 50-70) and 62 (51-76), respectively. The HCV genotype was 1 in 9 patients and 2 in 14 patients. Chronic hepatitis was diagnosed in 14 patients (F1/F2/F3=2/12/0) and cirrhosis in 9, at the time of initiation of IFN therapy. The type of therapy for hepatitis was IFN monotherapy in 22 patients and the combination therapy in 1. The number of HCC tumors was one in 18 patients, two in 3 patients and more than two in 2 patients. A typical hypervascular mass on angiography or perfusion defect on CT during arterial portography (CT-AP) was noted in 20 patients. Angiography could not be performed in the other three patients; they had a hypo-enhanced, iso enhanced, and hyper-enhanced tumor on CT, respectively. Treatment was radical in 21 patients; including hepatectomy in 17 and percutaneous locoregional therapy in 4 patients. At the time of surgical resection, the fibrosis staging was histopathologically examined in 16 patients. Twelve patients was diagnosed as hepatitis (F1/F1-2/F2/F3=2/1/6/3) and 4 as cirrhosis. In

Table 2. Carcinogenesis after HCV RNA Elimination

No	Gender	Age at the start of IFN	Age at the carcinogenesis	Genotype	Type of IFN	Fibrosis staging before IFN Tx	Interval between the end of IFN Tx and carcinogenesis, yr	Number of Tumor	Tumor size, mm	Treatment for HCC	Fibrosis staging at the time of carcinogenesis	Differentiation of HCC
1	M	50	51	1b	α	F2	1.0	1	15	Hepatectomy	F3	Moderate
2	M	52	54	2a	α	F1	0.6	1	18	Hepatectomy	F2	Well
3	F	54	59	2a	α	F2	3.5	1	17	Hepatectomy	F1-2	Moderate
4	M	55	60	1b	α	F2	3.7	1	16	Hepatectomy	F2	Moderate
5	M	55	56	1b	Peg α+Rib	F2	0.0	1	21	RFA	-	-
6	M	55	57	2a	α2a	F4	1.9	1	19	Hepatectomy	F4	Moderate
7	M	57	67	2	α	F2	8.9	1	47	Hepatectomy	F1	Moderate
8	M	55	59	2a	α2a	F4	3.1	1	18	Hepatectomy	F4	Moderate
9	M	55	62	1b	β	F4	6.5	1	16	Hepatectomy	F4	Poor
10	M	57	58	1b	α	F2	0.9	1	16	Hepatectomy	F2	Moderate
11	M	57	59	1b	α	F2	1.2	1	20	Hepatectomy	F2	Moderate
12	M	58	66	2a	α2b	F1	8.7	1	26	Hepatectomy	F1	Well
13	M	58	62	1b	β	F2	3.9	1	30	Hepatectomy	F2	poor>moderate
14	M	59	69	2b	α	F2	9.1	1	21	Hepatectomy	F2	Moderate
15	M	59	61	1b	α	F4	0.1	1	30	Hepatectomy	F3	poor>moderate
16	M	61	63	2	α	F2	1.8	4+LN meta	23	Hepatectomy+MCT	F3	moderate>well
17	M	62	65	2a	β	F4	2.4	2	20,20	RFA	-	-
18	M	62	75	2a	α2a	F4	12.9	1	23	Hepatectomy	F4	Moderate
19	M	63	66	2a	β	F2	3.6	Uncountable	Diffuse	No treatment	-	
20	M	65	71	2a	α	F2	5.0	2	12, 8	Hepatectomy	-	Necrosis
21	F	66	68	2a	α	F4	0.9	1	13	RFA	-	-
22	M	69	72	1b	β	F4	0.4	2	13, 13	Hepatectomy	-	moderate, poor
23	M	70	76	2a	β	F4	5.4	1	10	RFA+PMCT	-	-

IFN: interferon, Tx: therapy, Peg: pegylated interferon, Rib: ribavirin, LN meta: lymph node metastasis,
RFA: radiofrequency ablation, MCT: microwave coagulation therapy, PMCT: percutaneous microwave coagulation therapy.

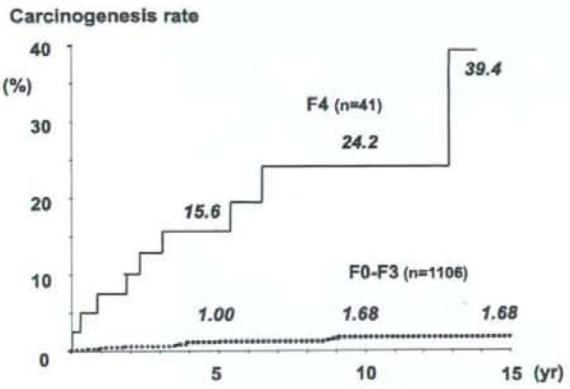

Figure 1. Rates of hepatocarcinogenesis in 41 patients with cirrhosis (F4) and 1,106 patients with liver fibrosis stage F0-F3.

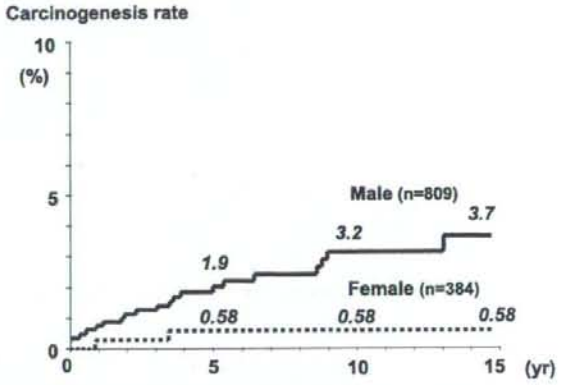

Figure 2. Rates of hepatocarcinogenesis in 809 male patients and 384 female patients.

comparison with the staging at the initiation of IFN therapy, 3 cases showed improvement in the fibrosis, 10 showed no change, and 3 showed progression.

The crude rates of hepatocarcinogenesis in the SVR patients were 1.5%, 2.4% and 2.7% at the end of the 5th year, 10th year and 15th year, respectively.

Determinants of hepatocarcinogenesis

The rate of carcinogenesis was significantly higher in 41 patients with cirrhosis (F4) than in 1,106 patients with liver fibrosis of F0-F3 ($p<0.0001$, Fig. 1). The respective cumulative HCC development rates in patients with cirrhosis at 5, 10, and 15 years after SVR were 15.6%, 24.2% and 39.4%. On the other hand, the respective rates for patients with F0-F3 were 1.00%, 1.68% and 1.68% at 5, 10, and 15 years after SVR. When patients were divided into two groups with F2-4 and with F0-1, rates of the former group were 4.16%, 6.52% and 7.58%, while those of latter group were 0.13%, 0.40% and 0.40%. The incidence rates of HCC increases with the fibrotic stage; those with F1, F2 and F4 were 0.14%, 3.43% and 15.6% at 5 years, 0.40%, 5.22% and 24.2% at 10 years, and 0.40%, 5.22% and 39.4% at 15 years, respectively.

The rate of hepatocarcinogenesis among 809 male patients was significantly higher than among 384 female patients ($p=0.018$, Fig. 2); the respective rates at 5, 10 and 15 years were 1.87%, 3.18% and 3.67% for males and 0.58%, 0.58% and 0.58% for females.

The rate of hepatocarcinogenesis among 570 patients aged >50 years was greater than among 623 patients aged <51 years at the start of IFN therapy ($p<0.0001$, Fig. 3); the respective rates at 5, 10 and 15 years for the former group were 2.92%, 4.93% and 5.81%, compared with 0.16%, 0.16% and 0.16% for the later.

Multivariate analysis identified three factors to be associated with the rate of development of HCC: sex, age at start IFN treatment, and fibrotic stage in the liver tissue. Multi-

variate analysis was performed using non-time dependent proportional hazard analysis. Fibrotic stage, sex, and age were identified as significant independent factors that influenced the rate of future hepatocarcinogenesis (Table 3). Cirrhosis (F4) was associated with a higher risk of hepatocarcinogenesis with a hazard ratio of 12.9 (95% confidence interval, 5.5-30.6, p<0.001) compared with F1-3 stage. Similarly, male sex (6.45, p=0.012) and older age than 50 years (20.2, p=0.004) were associated with a higher risk. Serological grouping of HCV, type of therapy (monotherapy or combination therapy), type of IFN of the final therapy, and number of therapies did not significantly influence the rate of hepatocarcinogenesis. When the patients were divided into two groups with F0-1 and with F2-4, hazard ratios of F2-4, male and older age were 13.4 (3.1-57.8, p<0.0001), 7.00 (1.63-29.99, p=0.0009) and 17.6 (2.3-131.6, p=0.005). When the patients were divided into two groups with F0-2 and F3-4, hazard ratios of F3-4, male and older age were 5.8 (2.5-13.8, p<0.0001), 6.78 (1.58-29.07, p=0.01) and 22.9 (3.0-172.2, p=0.002).

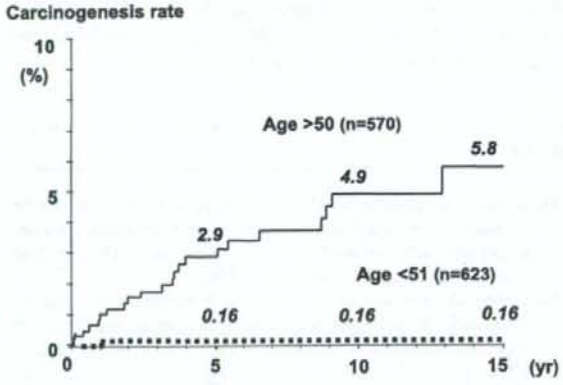

Figure 3. Rates of hepatocarcinogenesis in 570 patients older than 50 and 623 patients younger than 51 years.

Discussion

Epidemiological data on the rate of development of HCC in patients with chronic hepatitis (35) and those with cirrhosis (36) indicate that the life expectancy of patients with HCV-related chronic liver disease is significantly influenced by the development of HCC. Up to 75% of patients with HCV infection and cirrhosis eventually develop HCC (15). IFN can be considered to have anti-carcinogenic properties through its anti-inflammatory action, since several studies have already described that the cancer suppressive activity of IFN in those patients who show HCV RNA eradication was similar to that of patients with ALT normalization without HCV RNA elimination (BR) (15, 37-40). After excluding patients with cirrhosis, the previous report (40) showed that the rate of carcinogenesis was lower in patients with SVR than in those with BR because HCV-elimination does not result in re-elevation and exacerbation of ALT. As a follow-up to the above studies, the rate of hepatocarcinogenesis in SVR patients with either chronic hepatitis or cirrhosis was estimated in the present study.

In spite of the anti-carcinogenic effect of SVR, 23 cases developed HCC following elimination of HCV RNA among 1,193 patients. The median interval between the end of IFN therapy and carcinogenesis is 3.1 years with a range of 0.0 to 12.9 years. Among 23 cases, 22 patients had regular examinations of at least once a year, and 21 of them received radical treatment such as hepatectomy or radiofrequency ablation. The high rate of radical treatment was probably due to the preserved liver function after HCV RNA elimination.

HCCs in six cases that were detected in the year after the end of the interferon therapy could have been already present before elimination of HCV RNA. Even when we exclude these cases, multivariate analysis identified the same factors such as higher histological stage, male sex and age older age as determinants of hepatocarcinogenesis. The haz-

Table 3. Factors Associated with Hepatocarcinogenesis in Sustained Virological Responders with Chronic HCV Infection

Factors	Category	Hazard ratio	95% confidence interval	p
Fibrotic stage	1: F0-3	1		
	2:F4	12.9	(5.5-30.6)	<0.001
Gender	1: women	1		
	2: men	6.45	(1.51-27.64)	0.012
Age (years)	1: <51	1		
	2: >50	20.2	(2.7-152.9)	0.004

ard ratios of cirrhosis, male sex and age older than 50 years were 10.9 (4.0-29.8, p<0.001), 10.4 (1.4-78.2, p=0.024) and 17.0 (2.2-130.7, p=0.006), respectively.

The rates of hepatocarcinogenesis in patients with histological stage F0-F3 were 1.00%, 1.68%, 1.68% at 5, 10, and 15 years after SVR, respectively. These rates were about 20% less than the rates reported previously for patients with chronic hepatitis; 4.8%, 13.6%, and 26.0%, respectively (35). The rates of hepatocarcinogenesis in patients with cirrhosis were 15.6%, 24.2%, and 39.4% at 5, 10, and 15 years, respectively, which were about 65% less than the rates reported previously for patients with cirrhosis; 21.5%, 53.2% and 75.2%, respectively (36). These results indicate that IFN has a more marked effect in reducing the rate of hepatocarcinogenesis in patients with F0-F3 than in those with cirrhosis. Furthermore, the difference in the rate of hepatocarcinogenesis in patients who show SVR and those with chronic HCV-infected patients increases with time, since the likelihood of development of HCC before elimination of HCV RNA decreases as time passes after IFN therapy.

Although some studies have reported that elderly male patients with severe fibrotic stage could be at a high risk for hepatocarcinogenesis even when they show SVR, the hazard ratios in such patients have not been reported probably because of shorter follow-up period and the relatively small number of patients. In this study, the follow-up period was longer than that of previous studies, allowing meaningful multivariate analysis (e.g., Cox hazards model). The results of such analysis showed that the risk of carcinogenesis increases with the histologic stage of the liver, age and male sex. This finding was similar to that reported in a study of untreated patients (41, 42) or IFN-treated hepatitis patients with the histological stage of F0-F3 (15).

Treatment of patients with chronic HCV infection using PEG IFN-α and ribavirin resulted in persistently negative tests for serum HCV RNA in 40-50% of patients with HCV genotype 1 and 75-80% with HCV genotype 2 or 3. The present study also showed that neither type of IFN (α or β) nor the use of ribavirin altered the rate of carcinogenesis. Further studies are needed with a longer follow-up period since the follow-up period of patients treated with the combination therapy ranged from only 0 to 7.5 years (median 3.2years) and was shorter than that of patients who received IFN monotherapy.

In conclusion, the results emphasize the importance of long-term follow-up of patients with chronic HCV infection, even those who show SVR to IFN therapy, especially male elderly patients with severe fibrosis of the liver.

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Repeated administration of recombinant human serum albumin caused no serious allergic reactions in patients with liver cirrhosis: a multicenter clinical study

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Background. We carried out a multicenter study to evaluate the safety of recombinant human serum albumin (rHSA), developed using the methylotrophic yeast *Pichia pastoris*, during and after repeated administration in patients with liver cirrhosis. ***Methods.*** rHSA was administered to 423 cirrhosis patients with ascites or edema. rHSA was administered three times over 3 days, and each 3-day treatment course was repeated at least three times with an interval of at least 2 weeks between courses. Adverse drug reactions (ADRs) were monitored during and after repeated rHSA administration. Specific antibody titers against *Pichia* yeast components were measured before and after treatment. Efficacy was evaluated on the basis of changes in serum albumin level, colloid osmotic pressure, and body weight. ***Results.*** ADRs were observed in 96 of 423 patients (22.7%), with no serious allergy or difference in the incidence of ADRs observed among the first, second, and third administrations. Specific IgE and IgG antibodies were detected before treatment in 19 and 422 patients, respectively. However, allergic ADRs were observed in 14 patients in whom specific IgE antibodies were not detected. No obvious relationship between allergic ADRs and specific IgE or IgG titers was identified. Serum albumin levels and colloid osmotic pressure increased significantly ($P < 0.0001$), and body weight decreased significantly ($P < 0.0001$) after rHSA administration. ***Conclusions.*** rHSA caused no serious allergic reactions even when three treatment courses were administered at intervals of at least 2 weeks.

Key words: recombinant human serum albumin (rHSA), *Pichia pastoris*, liver cirrhosis, allergic reactions, antibody testing

Introduction

Plasma-derived human serum albumin (pHSA) is used to correct circulating plasma volume and improve colloid osmotic pressure. As pHSA is manufactured from human blood, the supply is limited and the risk of infection by unknown viruses or prions cannot be completely ruled out. To alleviate these problems, recombinant human serum albumin (rHSA) has been developed from *Pichia pastoris*[1] without using any animal-derived materials.

rHSA and pHSA have been shown to be identical in structure and physicochemical and immunochemical properties.[2–4] A phase III controlled clinical study of patients in liver cirrhosis, with increased serum albumin as the primary end point, showed that the efficacy of rHSA is comparable to that of pHSA.[5,6] A similar efficacy has been demonstrated in patients with hemorrhagic shock, thermal burns, or nephrotic syndrome.[5,6] No serious allergic symptoms were observed in these studies.[5,6]

At present, many recombinant pharmaceuticals with established efficacy and safety are being used, and among blood products, recombinants of blood coagulation factor VIII[7,8] are increasingly replacing plasma-derived preparations. Despite the high purity of rHSA, the potential onset of allergic reactions triggered by *Pichia* yeast components must be investigated, because the administered doses of rHSA are higher than those of other recombinants, and because administration might be repeated. This multicenter study was therefore conducted in cirrhosis patients to evaluate the safety and efficacy of rHSA during and after repeated administration of rHSA.

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Methods

Patients

A total of 423 patients with liver cirrhosis [etiology: hepatitis B virus (HBV) in 59 patients, hepatitis C virus (HCV) in 264, alcohol consumption in 47, primary biliary cirrhosis (PBC) in three, autoimmune hepatitis in three, non-B, non-C hepatitis in ten, HBV plus HCV in four, HCV plus alcohol consumption in seven, and other in 26] were enrolled in this study from July 2002 to January 2005. Written informed consent was obtained from all patients.

All patients meeting the following criteria were included in this study: serum albumin level <3.0 g/dl; presence of cirrhotic ascites or edema (legs or back); and age, 20–75 years. Beginning with the second treatment course, patients with serum albumin level <3.0 g/dl or cirrhotic ascites or edema (legs or back) were eligible.

Patients who met one or more of the following criteria were excluded from each course: prothrombin time <30% or 5 s≥ reference or control time; total bilirubin ≥5.0 mg/dl; serum creatinine ≥4.0 mg/dl; presence of hepatocellular carcinoma (HCC) with tumor embolism in the portal vein (main trunk or primary/secondary branch), main trunk of the hepatic vein, or postcaval vein; New York Heart Association class III/IV; history of shock in response to any pHSA preparation component; hepatic encephalopathy of grade II or higher severity at the time of consent; and pregnancy or lactation in women. Also excluded were any patients the investigator considered inappropriate for the study.

Protocol amendment

During the course of this study, a phase I study of rHSA was conducted in the United States in healthy volunteers with high specific IgE antibodies against *Pichia* yeast components (≥0.7 U_A/ml). In the American study, serious allergic adverse drug reactions (ADRs) were observed in two of four patients (bronchospasms in one patient and bronchospasms and generalized urticaria in one patient), leading to discontinuation of the study. Consequently, new enrollment in the present study was temporarily suspended in May 2003 while the cause was investigated. A passive cutaneous anaphylaxis (PCA) reaction test in rats showed that the products used in the American study were more antigenic than those used in the present study. Afterward, purification techniques were improved and the problem was resolved.

In November 2003, the present study was restarted with an amended protocol that included the following additional inclusion criteria: specific IgE antibody titers against *Pichia* yeast components were required to be <0.35 U_A/ml before each treatment, and hospital admission is indispensable from the time of the first administration (day 1) to the day after the final administration (day 4).

Treatment

After confirmation of a negative skin prick test against Pichia yeast components, 25 g/day of rHSA (25% rHSA, Bipha, Chitose, Japan) was intravenously administered over 2 h each day for 3 days. This course of treatment was repeated for three courses, and optionally for five courses. Each course was started after an interval of at least 2 weeks after the previous course.

Concomitant medication

During the study period, concomitant use of other investigational products was not allowed. Administration of plasma protein products, blood transfusion, reinfusion of concentrated ascites, paracentesis, and invasive treatment of complications associated with the underlying disease (e.g., endoscopic variceal ligation or sclerotherapy for esophageal varices, percutaneous ethanol injection therapy, or radiofrequency ablation for HCC) were prohibited from the day the inclusion/exclusion criteria were examined, except for specific IgE testing, until day 10. Continued use of already prescribed diuretics was allowed; however, the start of diuretics was prohibited during days 1–4. Branched-chain amino acid formulae and other pharmaceuticals could be used concomitantly.

Measurements and observation

Inclusion/exclusion criteria were examined within 3 days prior to day 1 of each treatment course, and specific IgE screening, which was added as an inclusion criterion by the protocol amendment, was performed within 14 days prior to day 1.

ADRs were monitored during the study period. Hematological tests and urinalysis were performed at baseline (within 3 days prior to day 1) and on days 4 and 10, and antibody tests were performed on day 1 (pretreatment) and days 4 and 10 in each course to evaluate safety.

Serum albumin level, colloid osmotic pressure, and body weight were measured on days 1 (pretreatment) and 4 during each course to evaluate efficacy. Serum albumin level and colloid osmotic pressure were measured at Mitsubishi Chemical Medience Corporation (Tokyo, Japan)