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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.