# Association of Lamivudine-Resistant Mutational Patterns with the Antiviral Effect of Adefovir in Patients with Chronic Hepatitis B

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- ABSTRACT -

# Association of Lamivudine-Resistant Mutational Patterns with the Antiviral Effect of Adefovir in Patients with Chronic Hepatitis B

Adefovir has a potent antiviral activity as a rescue treatment against lamivudine-resistant strains. The aim of this study was to assess the patterns of lamivudine-resistant mutations and their influence on the virologic response to adefovir rescue therapy in patients with lamivudine-resistant chronic hepatitis B. Sixty-seven patients with lamivudine-resistant chronic hepatitis B were treated with adefovir monotherapy. Baseline blood samples were analyzed for lamivudine-resistant mutations via restriction fragment mass polymorphism. Virologic responses, ALT normalization and loss of HBeAg were assessed. Serum HBV DNA levels were measured using real-time PCR at baseline and 24 weeks of adefovir therapy. Of the 67 patients with chronic hepatitis B, 65 patients (97%) had lamivudine-resistant mutations in the YMDD motif [27 (41%) rtM204I, 22 (34%) rtM204V, and 16 (25%) rtM204I/V]. In addition to the YMDD mutations, the rtL180M, rtL80I, and rtV173L mutations were also present in 78, 43, and 11% of patients, respectively. The rtM204V mutation always accompanied rtL180M, and rtL80I was always observed in conjunction with rtM204I. Decrease in mean serum HBV did not differ between patients carrying the rtM204I versus rtM204V mutant at week 24 (-3.3 versus -3.3 $\log_{10}$ copies/mL, respectively; P = 0.303). The presence of the rtL180M, rtL80I, and rtV173L did not significantly affect viral load reduction during adefovir administration. These results demonstrate that the rtL80I mutant is co-selected with rtM204I as a compensatory mutation in the same manner as rtL180M with rtM204V, and that adefovir shows similar antiviral efficacy against all of the evaluated patterns of lamivudine-resistant HBV mutations.

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**Key words:** hepatitis B virus, mutation, reverse transcriptase, antiviral resistance

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# ABBREVIATIONS

ALT, alanine aminotransferase; HBV, hepatitis B virus; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PCR, polymerase chain reaction; RFMP, restriction fragment mass polymorphism

# I. INTRODUCTION

Chronic hepatitis B is an important cause of morbidity and mortality worldwide (Lok, 2002; Chen et al., 2006; Iloeje et al., 2006). The goals of therapy in patients with chronic hepatitis B are sustained hepatitis B virus(HBV) DNA suppression, normalization of serum aminotransferase(ALT) levels, and improvement in liver necroinflammation. The long-term objectives include the prevention of cirrhosis, end-stage liver disease, and hepatocellular carcinoma and subsequent prolongation of survival (European Association for the Study of the Liver., 2003; Hadziyannis et al., 2005). These goals are achieved through the suppression of serum HBV DNA and hepatitis B e antigen (HBeAg) loss and/or seroconversion to the antibody to HBeAg (anti-HBe) in HBeAg-positive patients (Conjeevaram and Lok, 2003).

Treatment of chronic HBV infection with the nucleoside analog and reverse transcriptase (rt) inhibitor lamivudine effectively suppresses HBV replication without major side effects (Lok et al., 2003). Unfortunately, the clinical benefit is rarely sustained under long-term treatment due to the selection of lamivudine-resistant mutants, which occur at an annual rate of 14-32% (Lai et al., 1998; Dienstag et al., 1999; Lai et al., 2003; Lok et al., 2003; Shaw et al., 2004). HBV has a high mutation rate because its reverse transcriptase lacks a proofreading function. The most commonly described mutations are the substitution of valine or isoleucine for methionine at residue 204 (rtM204I/V). These YMDD motif mutations are necessary and sufficient to confer high-level lamivudine resistance. A second mutation, leucine 180 to methionine (rtL180M) in the upstream B subdomain of HBV polymerase accompanies rtM204 mutations. Other HBV rt mutations, such as rtV173L and rtL80V/I, have also been implicated in treatment failure (Ogata et al., 1999; Delaney et al., 2003).

Adefovir dipivoxil (Adefovir) is an orally bioavailable prodrug of adefovir, a nucleotide analog of adenosine monophosphate that inhibits both HBV rt and DNA polymerase activity. Adefovir effectively suppresses not only wild-type HBV, but also lamivudine-resistant HBV mutants (Xiong et al., 1998; Benhamou et al., 2001). Adefovir has an antiviral profile with potent activity against lamivudine-resistant strains, making it useful as a rescue treatment for lamivudine resistance, and adefovir shows infrequent selection of drug-resistant mutants at up to 2 years of therapy. However, suboptimal responses have also been reported as a low

initial virologic response after adefovir rescue therapy (Fung et al., 2006). The influence of various lamivudine- resistant mutations on the antiviral effect of adefovir is not well understood. Westland *et al.* (Westland et al., 2005) reported that adefovir therapy displayed a similar antiviral efficacy in patients with lamivudine-resistant virus regardless of the mutational pattern. On the other hand, Suzuki et al. (Suzuki et al., 2006) reported that changes in rtM204I viral loads were greater than changes in rtM204V viral loads during adefovir therapy. The present study investigated patterns of lamivudine-resistant mutations in 67 patients with lamivudine-resistance and evaluated the effect of these mutations on the antiviral response to adefovir therapy.

# II. MATERIALS AND METHODS

## A. Patients

Sixty-seven consecutive patients were included in this analysis. Fifty-eight (87%) patients were men, and the mean age was 42 (range, 26-61) years. Fifty-five (82%) patients were HBeAg-positive, and the mean ALT was 255 IU/L (range, 34-1814). The mean HBV DNA was 7.09 ± 1.00 $\log_{10}$ copies/mL. The baseline characteristics and demographics of the patients are listed in Table 1. Before enrollment, all patients had shown viral breakthrough, which is defined as an increase in serum HBV DNA by ≥1 $\log_{10}$ copies/mL above nadir on two consecutive occasions after an initial virologic response or an initial decline in HBV DNA by >2 $\log_{10}$ copies/mL (Fung et al., 2006). The patients had not received any antiviral drug before lamivudine administration. All patients were switched to adefovir monotherapy from lamivudine without an overlapping period; however, 1 month of overlapping therapy was needed in one patient due to the development of hepatitis flare after 1 month of switching. Adefovir was administered orally at a dosage of 10mg/d for more than 24 weeks.

## B. Blood tests

Routine biochemical tests were determined using standard procedures before and every 3 months during adefovir therapy. Serial blood samples were taken before and during therapy and stored at -70°C until use for HBV molecular analysis. HBeAg was tested with commercial radioimmunoassay kits (Abbott Laboratories, North Chicago, IL, USA). Serum HBV DNA levels were quantified at baseline and then every 12 weeks during adefovir therapy using an HBV polymerase chain reaction(PCR) kit (Abbott Laboratories, Wiesbaden, Germany), which has a detection limit of 28 copies/mL. Serum HBV DNA reduction was analyzed based on baseline and week 24 HBV DNA data.

Table 1. Patient Characteristics at Baseline

| Lamivudine-resistance pattern groups | n | Age, mean (range) | Sex, male, n(%) | Serum HBV DNA level ($log_{10}$ copies/mL), mean ± SD | Serum ALT (IU/L), mean ± SD(n†) | HBeAg positive, n(%) |
|---|---|---|---|---|---|---|
| rtM204I | 27 | 43 | 24(89) | 7.16±0.79 | 307±379 | 24(89) |
| rtM204V | 22 | 43 | 19(86) | 7.36±0.96 | 168±167 | 16(73) |
| RtM204I/V | 16 | 41 | 13(81) | 6.79±1.12 | 296±216 | 14(88) |
| *P*- Value | | 0.792 | 0.782 | 0.183 | 0.199 | 0.280 |
| + rtL180M | 51 | 43 | 43(84) | 7.09±1.00 | 260±305(48) | 42(82) |
| - rtL180M | 16 | 42 | 15(94) | 7.12±0.87 | 278±230 | 13(81) |
| *P*- Value | | 0.889 | 0.334 | 0.906 | 0.833 | 0.920 |
| rtM204I + rtL180M | 13 | 43 | 11(85) | 7.00±0.83 | 372±528(11) | 12(92) |
| rtM204I - rtL180M | 14 | 42 | 13(93) | 7.31±0.75 | 295±241 | 12(86) |
| *P*- Value | | 0.773 | 0.496 | 0.316 | 0.628 | 0.586 |
| + rtL80I | 28 | 43 | 27(96) | 7.16±0.89 | 327±240(26) | 25(89) |
| - rtL80I | 39 | 42 | 31(80) | 7.05±1.02 | 222±310(38) | 30(77) |
| *P*- Value | | 0.624 | 0.045 | 0.660 | 0.151 | 0.193 |
| rtM204I + rtL80I | 19 | 44 | 18(95) | 7.16±0.86 | 308±257(17) | 17(90) |
| rtM204I - rtL80I | 8 | 40 | 6(75) | 7.15±0.65 | 373±599 | 7(88) |
| *P*- Value | | 0.290 | 0.136 | 0.974 | 0.703 | 0.882 |
| + rtV173L | 7 | 39 | 6(86) | 7.23±1.06 | 147±126 | 6(86) |
| - rtV173L | 60 | 43 | 52(87) | 7.08±0.96 | 279±298(57) | 49(82) |
| *P*- Value | | 0.243 | 0.944 | 0.688 | 0.250 | 0.792 |
| rtM204V + rtL180M + rtV173L | 6 | 39 | 5(83) | 7.27±1.15 | 163±129 | 5(83) |
| rtM204V + rtL180M - rtV173L | 32 | 43 | 27(84) | 7.09±1.05 | 240±207(31) | 25(78) |
| *P*- Value | | 0.283 | 0.949 | 0.698 | 0.390 | 0.774 |
| Total | 67 | 42(26-61) | 58(87) | 7.09±1.00 | 255±284 | 55(82) |

Note. rtM204I, methionine to isoleucine substitution at rt204; rtM204V, methionine to valine substitution at rt204; rtM204I/V, methionine to isoleucine or valine substitution at rt204; rtL180M, leucine to methionine substitution at rt180; rtL80I, lysine to isoleucine substitution at rt80; rtV173L, valine to leucine substitution at rt173.

†Numbers excepting two patients with normal ALT at baseline who were excluded from the analysis of the normalization of serum ALT after 24 weeks of adefovir therapy.

**C. Detection of mutations by restriction fragment mass polymorphism (RFMP) analysis**

Mutations in the YMDD motif of the HBV DNA polymerase gene (rt204), rt180, rt80 and rt173 were identified at baseline using RFMP analysis as previously described (Kim et al., 2005). HBV DNA was extracted from 200 μL of serum using a QIAamp blood kit (Qiagen, Chatworth, CA, USA), according to the manufacturer's instructions; 2 μL of the viral DNA was used for the PCR reaction. For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)-based genotyping, PCR was performed in an 18 μL reaction mixture containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, 10 pmol of each primer, and 0.4 units of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplification conditions included initial denaturation at 94 °C for 2 min, 10 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 15 s, and extension at 72 °C for 30 s, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s. The sequences of the forward and reverse primers are shown in Table 2.

Restriction enzyme digestion of PCR products was performed by mixing the PCR reaction mixture with 10 μL of buffer containing 50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, and 1 unit of *Fok*I. The reaction mixture was incubated at 37 °C for 2 h and further incubated at 45 °C for 2 h with *Bst*F5I. The resulting digest was desalted by vacuum filtration through a 384-well sample preparation plate containing 5 mg of polymeric sorbent (Waters, Miliford, MA, USA) per well. The desalted reaction mixtures were re-suspended with matrix solution containing 50 mg/mL 3-hydroxy picolinic acid (Sigma, Saint Louis, MO, USA), 0.05 M ammonium citrate (Sigma), and 30% acetonitrile(Sigma) and were spotted in 3 μL volumes on a polished AnchorChip plate. Mass spectra were acquired on a linear MALDI-TOF MS (Bruker Daltonics Biflex IV, Billerica, MA, USA) workstation in the positive ion, delayed extraction mode. Typically, TOF data from 20-50 individual laser pulses were recorded and averaged on a transient digitizer, after which the averaged spectra were automatically converted to mass by data processing software (Bruker Data Analysis for TOF 1.6m). The genotypic analysis obtained by RFMP was confirmed by sequencing analysis.

Table 2. Primers used for amplification in RFMP assays of the rtM204I/V, rtL180M, rtL80I, and rtV173L mutations

| Primer | Sequences (5'-3') | Position | Polarity |
|---|---|---|---|
| rfmp204f | TTCCCCCACTGTTTGGCTggatgTCAGTTAT | 712-738 | Sense |
| rfmp204r | TACAGACTTGGCCCCCAATACCACATGA | 771-744 | Antisense |
| Rfmp180f | ATTCCTATGGGAGTGGGCCTCAGTggatgCGTTTCTC | 634-666 | Sense |
| Rfmp180r | ACGAACCACTGAACAAATGGCACTAGTAAACTG | 705-673 | Antisense |
| Rfmp80f | CTCACCAACCTCTTggatgTCCTCCAATTTGTCCTGG | 334-366 | Sense |
| Rfmp80r | TAAAACGCCGCAGAggatgACAACCAGCGA | 395-370 | Antisense |
| Rfmp173f | TCATCCTGGGCTTTCggatgCAAGATTCCTATGGGA | 614-645 | Sense |
| Rfmp173r | CTGAGCCAGGAGAAggatgCGGACTGAGGCC | 675-649 | Antisense |

Primers were designed based on the consensus sequences extracted from the multiple alignment of HBV sequences retrieved from the Entrez Nucleotide database of the National Center for Biotechnology Information, Bethesda, MD, USA. A five-nucleotide sequence(ggatg) embedded in the forward primer to introduce a FokI site in amplicon is indicated by lower case letters. A single base mismatch (underlined G and A) was introduced to erase the naturally occurring FokI site (nucleotides 741-745). Nucleotide sequence positions were numbered according to Ono *et al*.(1983).

**D. Sequencing analysis**

A sample of 5 μL of the viral DNA was used for the PCR reaction. The HBV polymerase domain was amplified using each 10 pmol of primers with the sequences 5′–TCC TAG GAC CCC TGC TCG TGT TAC-3′ (nucleotides 177-200) and 5′- CTG TAA ATA GAC CTA TTG ATT GGA-3′ (nucleotides 959-982) in 25 μL of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, and 0.4 units of Platinum® *Taq* DNA polymerase (Invitrogen). The amplification conditions included initial denaturation at 94°C for 2 min, 10 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and extension at 72°C for 30 s, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. Amplification products were purified using a QIAquick PCR purification kit (QIAGEN), according to the manufacturer's instructions. Purified PCR products were sequenced using the BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) and ABI prism model 3100 DNA sequencer (Applied Biosystems) using the above forward and reverse primers in both directions.

**E. Definition**

An initial virologic response was defined as HBV DNA < 4 $\log_{10}$ copies/mL at 24 weeks of

treatment.

**F. Statistical analysis**

Statistical testing was performed using SPSS version 11.5(SPSS Inc., Chicago, IL). The results are reported as the mean ± standard deviation(SD). HBV DNA levels were logarithmically transformed for analysis. Continuous variables were compared using the independent samples *t*-test and analysis of variance(ANOVA). Categorical data were compared using the Pearson $\chi^2$ test or Fisher's exact test. *P*-values < 0.05 were considered statistically significant.

# III. RESULTS

## A. Virologic and biochemical response to adefovir

At the start of adefovir treatment, all patients had HBV DNA >4 $\log_{10}$ copies/mL and 60 (90%) had HBV DNA >6 $\log_{10}$copies/mL. At 24 weeks of treatment, the mean reduction in HBV DNA was 3.1 ± 1.4 $\log_{10}$copies/mL. An initial virologic response was achieved in 37 (55%) patients and ALT normalization was observed in 43 (64%) patients. HBeAg loss was observed in 9 of 55 HBeAg-positive patients(16%) after 24 weeks of adefovir therapy.

## B. Antiviral efficacy of Adefovir between the HBeAg-positive and -negative groups

Both HBeAg-positive and -negative patients were enrolled in this study; 55 patients were HBeAg-positive, and 12 were HBeAg-negative. The average age, sex, mean serum ALT, and mean serum HBV DNA levels were similar in the HBeAg-positive and -negative groups. The virologic response did not differ between the HBeAg-positive and –negative groups; the mean changes in HBV DNA from baseline were -3.11 and -3.22 $\log_{10}$ copies/mL, respectively ($P$ = 0.808).

## C. Baseline hepatitis B virus polymerase mutations

Genotypic analyses of baseline samples were performed on all patients(n = 67) to identify lamivudine-resistant mutations (Table 3). Of the 67 patients with genotyping data, 65 (97%) had lamivudine-resistant mutations in the YMDD motif [27 (41%) rtM204I, 22 (34%) rtM204V, and 16 (25%) rtM204I/V] (Table 4). In addition to the YMDD mutations, the rtL180M, rtL80I, and rtV173L mutations were also present in 51(78%), 28(43%), and 7(11%) patients, respectively.

The rtM204V mutation was only found in conjunction with the rtL180M mutation (22 of 22, 100%), whereas the rtM204I mutation occurred with the rtL180M mutation in 48% (13 of 27) of patients. The rtL80I mutation was exclusively observed in patients with the rtM204I mutation. No patient with the rtM204V mutation also had the rtL80I mutation (0 of

22, 0%). Patients with the rtM204I/V mutation showed 100% (16 of 16) and 56% (9 of 16) co-existence with rt180M and rtL80I, respectively. The rtV173L mutation was mostly associated with the rtM204V(or rtM204I/V)-rtL180M double mutation(6 of 7, 86%) with only one exception(rtM204I + rtV173L).

Table 3. HBV DNA Changes and Biochemical Responses in All Mutational Patterns at 24 Weeks of Adefovir Therapy

| Mutational Patterns | n(%) 67(100.0) | HBV DNA* (Mean ± SD, $log_{10}$ copies/mL) | | | ALT (Mean ± SD, IU/L) | | |
|---|---|---|---|---|---|---|---|
| | | Week 0 | Week 24 | Change | Week 0 | Week 24 | Normalization ratio (%) |
| rtM204I | 4 (6.0) | 7.61±0.51 | 3.18±0.86 | -4.43±1.19 | 214±190 | 28±6 | 4/4(100) |
| rtM204I+rtL180M | 3 (4.5) | 6.59±0.45 | 3.35±1.29 | -3.24±1.34 | 693±972 | 34±28 | 2/3(66.7) |
| rtM204I/V+rtL180M | 7 (10.4) | 6.32±1.15 | 3.83±0.80 | -2.50±1.28 | 211±200 | 36±16 | 5/7(71.4) |
| rtM204V+rtL180M | 18 (26.9) | 7.39±0.86 | 4.05±1.51 | -3.34±1.56 | 187±179 | 38±18 | 10/17†(58.8) |
| rtM204I+rtL80I | 9 (13.4) | 7.21±0.87 | 3.99±1.55 | -3.21±1.48 | 358±257 | 49±36 | 6/9(66.7) |
| rtM204I+rtL180M+rtL80I | 10 (14.9) | 7.12±0.89 | 4.17±0.96 | -2.94±1.02 | 209±248 | 44±17 | 2/8†(25) |
| rtM204I/V+rtL180M+rtL80I | 7 (10.4) | 7.08±1.16 | 4.23±1.02 | -2.84±0.84 | 374±246 | 29±12 | 6/7(85.7) |
| rtM204V+rtL180M+rtV173L | 4 (6.0) | 7.22±1.48 | 3.89±1.81 | -3.33±0.74 | 81±32 | 33±19 | 3/4(75) |
| rtM204I/V+rtL180M+rtL80I+rtV173L | 2 (3.0) | 7.38±0.08 | 3.98±0.39 | -3.40±0.31 | 326±33 | 37±1 | 2/2(100) |
| rtM204I+rtV173L | 1 (1.5) | 7.00 | 3.05 | -3.95 | 50 | 27 | 1/1(100) |
| No mutation | 2 (3.0) | 5.80±0.31 | 4.60±3.06 | -1.20±3.38 | 162±52 | 29±12 | 2/2(100) |
| *P* -Value | | 0.272 | | 0.398 | 0.189 | | 0.244 |

*HBV DNA level as measured by quantitative real-time polymerase chain reaction(PCR).
†Patients with normal ALT at baseline were excluded from the analysis.

Table 4. Incidence of Co-selection Among the Lamivudine-Resistant Mutations at Baseline

| YMDD Mutants | | Co-selected mutations | | | | | |
|---|---|---|---|---|---|---|---|
| Subgroups | n | rtL180M (n=51) | | rtL80I (n=28) | | rtV173L (n=7) | |
| | | +/- | n(%) | +/- | n(%) | +/- | n(%) |
| rtM204I | 27 | + | 13(48) | + | 19(70) | + | 1(4) |
| | | - | 14(52) | - | 8(30) | - | 26(96) |
| rtM204V | 22 | + | 22(100) | + | 0(0) | + | 4(18) |
| | | - | 0(0) | - | 22(100) | - | 18(82) |
| rtM204I/V | 16 | + | 16(100) | + | 9(56) | + | 2(13) |
| | | - | 0(0) | - | 7(44) | - | 14(87) |
| Total | 65 | | 65 | | 65 | | 65 |

**D. Effect of lamivudine-resistant mutational pattern on the virologic and biochemical response to adefovir**

The virologic and biochemical responses for all mutational patterns after 24 weeks of adefovir therapy are shown in Table 3, and no significant differences were observed before or after 24 weeks of adefovir therapy among the mutant groups. Following 24 weeks of adefovir therapy, the mean reduction in serum HBV DNA was 3.1 $\log_{10}$ copies/mL ($P <$ 0.001 compared to baseline).

To determine the effect of lamivudine-resistant mutations on the antiviral effect of adefovir, changes in serum HBV DNA were compared between groups with major mutational patterns. At the same time, ALT normalization, initial virologic response, and HBeAg loss were also analyzed. The characteristics of each lamivudine-resistant pattern group at baseline were similar except for the male predominance in the patients with rtL80I compared to those without rtL80I (P= 0.045, Table 1). As shown in Table 5, no significant difference in antiviral effects was observed in response to the YMDD mutants, rtM204I, rtM204V, or rtM204I/V. Analysis only considering the presence of rtL180M did not reveal any significant differences in antiviral responses (Table 6). To assess the effect of the rtL180M mutation on the antiviral response to adefovir, changes in serum HBV DNA were compared between patients carrying rtM204I + rtL180M versus those carrying rtM204I mutant HBV alone. The results showed that the presence of the rtL180M mutation in HBV does not have a statistically significant effect on viral load reduction by adefovir; patients with and without rtL180M showed mean reductions of 3.01 and 3.61 $\log_{10}$ copies/mL, respectively ($P$ = 0.224). However, the patients with the rtM204I + rtL180M mutations showed less frequent serum ALT normalization than the patients with the rtM204I mutation(4/11 versus 11/14, $P$ = 0.049 with Pearson $\chi^2$ test). When analyses were done with the same method as the rtL180 mutation, the individual contributions of the rtL80I and rtV173L mutations on the suppression of serum HBV DNA by adefovir were not statistically significant (Table 7 and 8). Additionally, the analyses of serum ALT normalization, initial virologic response, and HBeAg loss showed no significant results.

Table 5. Comparisons of Antiviral Responses at 24 Weeks of Adefovir Therapy Among Patients with Different YMDD Mutant Subgroups

| Pattern of mutation | n | Change in HBV DNA ($log_{10}$ copies/mL), mean ± SD | Normalization of serum ALT (%) | Initial virologic response *, n (%) | Loss of HBeAg (%) |
|---|---|---|---|---|---|
| rtM204I | 27 | -3.33±1.27 | 15/25† (60) | 16 (59) | 4/24‡ (17) |
| rtM204V | 22 | -3.34±1.44 | 13/21† (62) | 12 (55) | 3/16‡ (19) |
| rtM204I/V | 16 | -2.76±1.02 | 13/16 (81) | 8 (50) | 2/14‡ (14) |
| *P* -Value | | 0.303 | 0.330 | 0.836 | 0.948 |

*Initial virologic response was defined as HBV DNA < 4 $log_{10}$copies/mL after 24 weeks of treatment.
†Patients with normal ALT at baseline were excluded from the analysis.
‡Patients without HBeAg at baseline were excluded from the analysis.

Table 6. Comparisons of Antiviral Responses at 24 Weeks of Adefovir Therapy Among Patients with or without rtL180M Mutation

| Pattern of mutation | n | Change in HBV DNA($log_{10}$ copies/mL), mean ± SD | Normalization of serum ALT (%) | Initial virologic response *, n (%) | Loss of HBeAg (%) |
|---|---|---|---|---|---|
| + rtL180M | 51 | -3.07±1.23 | 30/48† (63) | 25 (49) | 7/42‡ (17) |
| - rtL180M | 16 | -3.31±1.78 | 13/16 (81) | 12 (75) | 2/13‡ (15) |
| *P*- Value | | 0.547 | 0.225 | 0.088 | 1.000 |
| rtM204I + rtL180M | 13 | -3.01±1.05 | 4/11† (36) | 5 (39) | 2/12‡ (17) |
| rtM204I - rtL180M | 14 | -3.61±1.41 | 11/14 (79) | 11 (79) | 2/12‡ (17) |
| *P*- Value | | 0.224 | 0.049 | 0.054 | 1.000 |

*Initial virologic response was defined as HBV DNA < 4 $log_{10}$copies/mL after 24 weeks of treatment.
†Patients with normal ALT at baseline were excluded from the analysis.
‡Patients without HBeAg at baseline were excluded from the analysis.

Table 7. Comparisons of Antiviral Responses at 24 Weeks of Adefovir Therapy Among Patients with or without rtL80I Mutation

| Pattern of mutation | n | Change in HBV DNA($log_{10}$ copies/mL), mean ± SD | Normalization of serum ALT (%) | Initial virologic response *, n (%) | Loss of HBeAg (%) |
|---|---|---|---|---|---|
| + rtL80I | 28 | -3.04±1.09 | 16/26† (62) | 13 (46) | 2/25‡ (8) |
| - rtL80I | 39 | -3.20±1.55 | 27/38† (71) | 24 (62) | 7/30‡ (23) |
| *P*- Value | | 0.638 | 0.426 | 0.220 | 0.160 |
| rtM204I + rtL80I | 19 | -3.07±1.23 | 8/17† (47) | 9 (47) | 1/17‡ (6) |
| rtM204I - rtL80I | 8 | -3.93±1.21 | 7/8 (88) | 7 (88) | 3/7‡ (43) |
| *P*- Value | | 0.110 | 0.088 | 0.090 | 0.059 |

*Initial virologic response was defined as HBV DNA < 4 $log_{10}$copies/mL after 24 weeks of treatment.
†Patients with normal ALT at baseline were excluded from the analysis.
‡Patients without HBeAg at baseline were excluded from the analysis.

Table 8. Comparisons of Antiviral Responses at 24 Weeks of Adefovir Therapy Among Patients with or without rtV173L Mutation

| Pattern of mutation | n | Change in HBV DNA($log_{10}$ copies/mL), mean ± SD | Normalization of serum ALT (%) | Initial virologic response *, n (%) | Loss of HBeAg (%) |
|---|---|---|---|---|---|
| + rtV173L | 7 | -3.44±0.58 | 6/7 (86) | 4 (57) | 1/6‡ (17) |
| - rtV173L | 60 | -3.10±1.43 | 37/57† (65) | 33 (55) | 8/49‡ (16) |
| *P*- Value | | 0.537 | 0.410 | 1.000 | 1.000 |
| rtM204V + rtL180M + rtV173L | 6 | -3.35±0.59 | 5/6 (83) | 3 (50) | 1/5‡ (20) |
| rtM204V + rtL180M - rtV173L | 32 | -3.05±1.39 | 21/31† (68) | 17 (53) | 4/25‡ (16) |
| *P*- Value | | 0.388 | 0.646 | 1.000 | 1.000 |

*Initial virologic response was defined as HBV DNA < 4 $log_{10}$copies/mL after 24 weeks of treatment.
†Patients with normal ALT at baseline were excluded from the analysis.
‡Patients without HBeAg at baseline were excluded from the analysis.

# IV. DISCUSSION

The selection of lamivudine–resistant mutations is the main concern with lamivudine treatment. Overlapping therapy with add-on adefovir is recommended for lamivudine-resistant patients by most practice guidelines to decrease the risk of hepatitis flares during the transition period (Liaw, 2007; Lok and McMahon, 2007). The guidelines established by the American Association for the Study of Liver Diseases (AASLD), which were published when the present study was initiated, suggest an overlap period of 2 to 3 months to minimize the risk of a hepatitis flare during transition in patients with lamivudine resistance (Lok and McMahon, 2004). However, several reports suggested that the discontinuation of lamivudine in patients with lamivudine resistance is not associated with an increased frequency of hepatitis flares (Liaw et al., 2004; Wong et al., 2004), and the ministry of Health, Welfare, and Family Affairs does not reimburse for the add-on therapy in Korea. Thus, the patients were switched from lamivudine to adefovir therapy. Only 1 of 67 patients developed a hepatitis flare, which occurred 1 month after switching, but it was well controlled by overlapping 1 month of lamivudine therapy.

Mutations that result in the replacement of the methionine at position 204 of the deoxynucleoside triphosphate-binding site of the HBV reverse transcriptase with isoleucine or valine confer high-level resistance to lamivudine, but reduce the replication efficiency (Warner et al., 2007). However, the rtM204V mutation alone is associated with a much lower resistance to lamivudine than rtM204I alone, rtM204I + rtL180M, or rtM204V + rtL180M (Enomoto et al., 2007). The present study confirms previous findings that the rtM204I substitution occurs in isolation, whereas rtM204V is found only in association with other changes, notably rtL180M in the B motif (Allen et al., 1998; Stuyver et al., 2001; Bartholomeusz and Schaefer, 2004). Several other mutations, including rtL80V/I, rtL82M, rtF166L, rtV173L, and rtA200V, have also been reported in patients receiving lamivudine therapy (Delaney et al., 2001).

Delaney *et al*. (Delaney et al., 2003) reported the incidence of the rtV173L mutation in lamivudine-resistant patients with chronic hepatitis B as 17% (36/216). They also found that rtV173L was associated only with the rtL180M-rtM204V double mutation (36/36, 100%), suggesting that rtV173L specifically emerges in viruses with this pattern of lamivudine-

resistant mutation. Westland *et al*. (Westland et al., 2005) also identified the same pattern of lamivudine-resistant mutation (rtV173L + rtL180M + rtM204V) at a high frequency in patients with lamivudine-resistance (23 of 122, 19%). In the present study, the rtV173L mutation was found in three patterns of mutations as follows; 4 (5.8%) patients with rtV173L + rtL180M + rtM204V mutations, 2 (2.9%) patients with rtV173L + rtL180M + rtM204V + rtL80I, and 1 (1.5%) patient with rtM204I + rtV173L. Interestingly, the last pattern shows that rtV173L can also develop in the absence of the rtL180M-rtM204V double mutation.

The analysis of a large sequence database revealed that co-selection of rtL80V/I occurred in 46% of isolates, in which lamivudine resistance was attributable to rtM204I, but in only 9% of those in which resistance was attributable to rtM204V (Warner et al., 2007). *In vitro* phenotyping showed that although the rtL80I mutant alone replicated less efficiently and was hypersensitive to lamivudine compared to the replication efficiency and sensitivity of its wild-type parent, the presence of rtL80I enhanced the replication efficiency of rtM204I/V mutants without significantly affecting lamivudine resistance, particularly in the case of rtM204I (Warner et al., 2007). In the present study, the rtL80I mutation was associated with 70% of patients with the rtM204I mutation and half of the patients with the rtM204I/V mutation but was not associated with any of the patients with the rtM204V mutation.

The present study investigated the effect of the lamivudine-resistant mutational pattern on the virologic response to adefovir. The results suggest that mutations in HBV polymerase associated with resistance to lamivudine, such as rtM204I, rtM204V, rtL180M, rtL80I, and rtV173L, do not influence the antiviral effect of adefovir as a rescue therapy. However, the co-selection of the rtL180M mutation in patients with the rtM204I mutation decreased serum ALT normalization significantly (36 versus 79%, $P = 0.049$) after adefovir therapy. Compared to patients with the rtM204I mutation alone, the initial virologic response tended to be lower in the group with rtL180M (39 versus 79%, $P = 0.054$).

Westland *et al*. (Westland et al., 2005) also reported similar antiviral efficacy of adefovir in patients with lamivudine-resistant virus from all four patterns (rtL180M + rtM204V, rtV173L + rtL180M + rtM204V, rtM204I, and rtL180M + rtM204I) and stated that the individual contributions of the rtV173L and rtL180M mutations on the suppression of serum HBV DNA by adefovir were not significant. On the other hand, Suzuki *et al*. (Suzuki et al., 2006) measured sequential viral loads of mutants during co-administration of adefovir in addition

to lamivudine in patients with chronic hepatitis B with lamivudine resistance and showed that the viral loads of rtM204I decreased at the most rapid rate among rtM204I, rtM204V, and rt180M, although the difference was not statistically significant. Moreover, when viral loads of both mutants (rtM204I and rtM204V) were similar at the commencement of adefovir therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. Considering these findings, Suzuki *et al*. suggested the rtM204I may be more sensitive to adefovir. Thus, the result of the present study are not consistent with those of Suzuki *et al*., but differences between the two study groups, such as the coexistence of additional mutations and co-administration of lamivudine, may have affected the outcomes.

Lada *et al*. (Lada et al., 2004) studied the *in vitro* susceptibility of lamivudine-resistant HBV to adefovir. In their clinical study of adefovir, 7 of the 35 patients studied carried HBV strains with the triple lamivudine resistance-associated amino-acid changes rtV173L/L180M/M204V at baseline. Serum HBV reduction was lower in seven patients with the triple mutation compared to the patients who had only the rtL180M/M204V mutations at week 48 of adefovir therapy. In the *in vitro* system of Lada *et al*., the presence of the V173L mutation reduced viral replication from 45 to 32% ($P < 0.01$). However, they suggested that the relative lower efficacy of adefovir on the triple mutant may not have any relevance in patients.

# V. CONCLUSION

In conclusion, the present study suggests that adefovir monotherapy in patients with lamivudine resistance has a similar antiviral efficacy against all of the evaluated patterns of lamivudine-resistant HBV mutations. These results need to be validated in further studies with large numbers of patients.

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# 만성 B형 간염 환자에서 라미부딘 내성 돌연변이의 유형과 아데포비어의 항바이러스 효과와의 상관관계

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아데포비어는 라미부딘 내성 균주에 대한 구조요법으로서 강력한 항바이러스 효과를 가지고 있다. 본 연구는 라미부딘 내성 돌연변이의 유형을 분석하고 이들 돌연변이가 라미부딘 내성 B형 간염 환자에서 아데포비어 치료시 항바이러스 효과에 미치는 영향을 알아보고자 하였다. 라미부딘 내성 B형 간염 환자 67명을 아데포비어 단독요법으로 치료하였다. 치료전 채혈한 환자의 혈청에서 제한효소분절질량다형성(restriction fragment mass polymorphism, RFMP)을 이용하여 라미부딘 내성 돌연변이를 분석하였다. 아데포비어 치료에 따른 바이러스 반응, ALT 정상화 그리고 HBeAg 소실을 분석하였다. 아데포비어 투여 전과 투여 24주 후의 혈청 HBV DNA를 real-time PCR을 이용하여 정량분석하였다. 67명의 만성 B형 간염 환자들 중 65명(97%)에서 YMDD motif의 라미부딘 내성 돌연변이[27 (41%) rtM204I, 22 (34%) rtM204V, 그리고 16 (25%) rtM204I/V]가 관찰되었다. YMDD 돌연변이에 부가적으로 rtL180M, rtL80I, 그리고 rtV173L 돌연변이가 각각 78%, 43%, 그리고 11%의 환자들에서 동반되었다. rtL180M 돌연변이는 rtM204V 돌연변이와 그리고 rtL80I 돌연변이는 rtM204I 돌연변이와 항상 동반되었다. 아데포비어 투여 24주 후 rtM204I 돌연변이를 동반한 환자군과 rtM204V를 동반한 환자군들 사이에 평균 혈청 HBV DNA의 감소는 유의한 차이를 보이지 않았다(-3.3 대 -3.3 $\log_{10}$copies/mL; P = 0.303). rtL180M, rtL80I, 그리고 rtV173L 돌연변이의 존재는 아데포비어 투여 중 HBV DNA의 감소에 유의한 영향을 주지

않았다. 결론적으로 rtL180M 돌연변이가 rtM204V와 항상 동반되듯이 rtL80I 돌연변이 또한 rtM204I의 보상적 돌연변이로 발생됨을 확인할 수 있었으며 아데포비어는 본 연구에서 관찰된 모든 라미부딘 내성 돌연변이 유형에 대해 유사한 항바이러스 효과를 보여주었다.

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**핵심어:** B형 간염 바이러스, 돌연변이, 역전사효소, 항바이러스제 내성