Adefovir and Lamivudine Combination Therapy in Patients with Entecavir-Resistant Chronic Hepatitis B: Antiviral Responses and Evolution of Mutations

Hyung Joon Yim^a Hyun Jung Lee^a Sang Jun Suh^a Yeon Seok Seo^b
Chang Wook Kim^c Chang Don Lee^c Sang Hoon Park^d Myung Seok Lee^d
Choong Kee Park^e Hee Bok Chae^f Moon Young Kim^g Soon Koo Baik^g
Yun Soo Kim^h Ju Hyun Kim^h Jung Il Leeⁱ Jin Woo Leeⁱ Sun Pyo Hong^j
Soon Ho Um^b

^aDepartment of Internal Medicine, Korea University Ansan Hospital, Ansan, ^bDepartment of Internal Medicine, Korea University Anam Hospital, Seoul, ^cDepartment of Internal Medicine, The Catholic University of Korea Uijeongbu St. Mary's Hospital, Uijeongbu, ^dDepartment of Internal Medicine, Hallym University Kangnam Sacred Heart Hospital, Seoul, ^eDepartment of Internal Medicine, Hallym University Sacred Heart Hospital, Anyang, ^fDepartment of Internal Medicine, Chungbuk National University Hospital, Cheongju, ^gDepartment of Internal Medicine, Yonsei University Wonju Severance Christian Hospital, Wonju, ^hDepartment of Internal Medicine, Gil Hospital, Gachon University of Medicine and Science, ⁱDepartment of Internal Medicine, Inha University Hospital, Incheon, and ^jGeneMarix Inc., Yongin, Republic of Korea

Key Words

Adefovir · Chronic hepatitis B · Entecavir · Lamivudine · Resistance

Abstract

Objective: This study was designed to prospectively evaluate the antiviral responses and evolution of resistance mutations during adefovir (ADV) plus lamivudine (LMV) therapy in patients with entecavir (ETV)-resistant hepatitis B virus (HBV) infection. **Methods:** Twenty chronic hepatitis B (CHB) patients who had been receiving ETV for more than 6 months and developed virologic breakthrough due to ETV resistance were consecutively enrolled. **Results:** Patients received ADV plus LMV therapy for 12 months. The baseline mean serum HBV DNA level was $5.59 \pm 1.28 \log_{10}$ IU/ml. The rtT184L/I/A/F (50%), rtS202G (25%) and mixed ETV-resistant mutations (25%) were detected at enrollment. The mean reduction in

serum HBV DNA levels from baseline to 12 months was $-2.3 \pm 1.06 \log_{10}$ IU/ml ($p < 0.001$). Seventeen patients were followed up for the full 12 months, and complete virologic response (HBV DNA < 20 IU/ml) was observed in 4 patients (23.5%). Among the remaining 13 patients who still had detectable HBV DNA, 7 patients showed disappearance of ETV-resistant mutations or reduction of the proportion of ETV-resistant mutants. An ADV- and LMV-resistant mutant (rtA181T) emerged in 2 patients (11.7%). **Conclusions:** ADV plus LMV combination therapy suppresses ETV-resistant mutants in the viral population and significantly reduces serum HBV DNA levels in ETV-resistant CHB patients.

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Y.H.J. received research funding from Glaxo-Smith-Kline Korea and a consultancy fee from Bristol-Meyers Squibb Korea and Gilead Science Korea.

Introduction

Chronic hepatitis B (CHB) is a worldwide health burden, especially in Asia [1, 2]. Several new oral antiviral agents have emerged as promising therapies for CHB during the last decade. Entecavir (ETV) is a potent antiviral agent which has a high genetic barrier for development of resistance [3, 4]. ETV resistance is rare in treatment-naïve CHB patients [5], and long-term administration of ETV results in regression of fibrosis or even reversal of cirrhosis [6]. In this regard, recent guidelines recommend ETV as one of the preferred first-line therapies [7, 8]. However, resistance to ETV has been reported in treatment-experienced patients; the incidence of ETV resistance increased over 50% at treatment year 5 in lamivudine (LMV)-refractory patients [5]. Therefore, optimal treatment of ETV resistance need to be investigated, but clinical data are lacking.

Considering the cross-resistance profile, adefovir (ADV) or tenofovir (TDF) are good options for managing ETV resistance [9]. However, TDF is still not available in many Asian countries. Although ADV is also effective for ETV resistance, ADV monotherapy can predispose to development of ADV resistance because ETV-resistant hepatitis B virus (HBV) retains LMV resistance as background mutations [10, 11]. As the ADV resistance rate is reportedly as high as 18% during the first year for LMV-resistant CHB when administered alone [12], it would be more reasonable to incorporate a drug without cross-resistance to reduce the risk of ADV resistance.

A case report has described successful treatment with a combination of ADV and LMV in a patient with ETV resistance [12]. No additional data on this combination therapy are available in a larger number of patients. It is thought that ADV and LMV combination therapy would have the potential to reduce the risk of ADV resistance and achieve lasting suppression of HBV DNA in patients with ETV resistance.

During rescue therapy due to antiviral resistance, drug-resistant mutations evolve [13–15]. The proportion of preexisting mutations may be decreased, or newly developed mutations may emerge. The changes in the mutation profile may affect the outcome of the rescue therapy. Therefore, in addition to HBV DNA quantification, serial monitoring and searching for resistance-associated mutations are needed to provide more information in terms of the assessment of antiviral responses.

The aim of this study was to evaluate the antiviral responses of ADV and LMV combination therapy in pa-

tients with ETV resistance. Also, we investigated the evolution of ETV-resistant mutations during the combination therapy.

Patients and Methods

Study Design

This was an investigator-initiated, multicenter, prospective, open-label, single-arm study designed to prospectively evaluate the antiviral responses of ADV 10 mg and LMV 100 mg combination therapy for 12 months in CHB patients resistant to ETV. Twenty patients were consecutively recruited from nine hospitals affiliated with seven universities between February 2010 and February 2011. Previously, we conducted a randomized controlled trial comparing the efficacy of ETV monotherapy versus ADV plus LMV combination therapy in 219 LMV-resistant CHB patients [16]. One hundred and nine patients received ETV 1 mg for 2 years. Eighteen patients developed ETV resistance and subsequent virologic breakthrough. Sixteen of the 18 patients agreed to participate in this study (fig. 1). Additionally, 4 patients who were referred from their primary care physician for ETV resistance and met the inclusion criteria agreed to participate. Written informed consent was obtained from the 20 patients. ETV was maintained until the start of ADV and LMV combination therapy. Biochemistry, HBV DNA quantification, serology for hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) and testing for resistance-associated mutations were performed every 3 months. Prothrombin time and hematology were assessed every 6 months. Compliance to medication was monitored by a careful interview with the physicians at each visit. Any untoward medical events were recorded for safety issues. The study protocol was approved by the institutional review board at all study sites (ClinicalTrials.gov identifier: NCT 01546116). This study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Inclusion and Exclusion Criteria

Inclusion criteria were as follows: HBeAg-positive or -negative CHB, positive hepatitis B surface antigen (HBsAg) for more than 6 months, age over 18 years, history of treatment with ETV for more than 6 months, proven ETV-resistant mutations determined by restriction fragment mass polymorphism (RFMP) assay and an HBV DNA level over 2,000 IU/ml. All patients needed to have compensated liver disease determined by a Child-Pugh-Turcotte score ≤ 7 , prolongation of prothrombin time ≤ 3 s, serum albumin > 3 g/dl, total bilirubin < 2.5 mg/dl and no history of variceal bleeding, ascites or hepatic encephalopathy. Only patients who were willing to give informed consent and met all the above criteria were included.

Exclusion criteria were as follows: one or more predefined laboratory abnormalities including serum phosphorous level < 2.4 mg/dl, serum creatinine level > 1.5 mg/dl, creatinine clearance < 50 ml/min, absolute neutrophil count $< 1,000$ cells/ml, hemoglobin level < 10 g/dl in males and < 9 g/dl in females or serum α -fetoprotein > 100 ng/ml, and history of treatment with interferon- α , thymosin $\alpha 1$ or nucleos(t)ide analogues other than ETV within 6 months of screening; history or evidence of ADV resistance; history of organ transplantation; positive antibody test to human immunodeficiency virus, hepatitis C virus or hepatitis D virus; pregnant or breast-feeding condition; hepatocellular car-

Fig. 1. Flowchart of study participants. RCT = Randomized controlled trial; Cr = creatinine; CBC = complete blood count; INR = international normalized ratio.

cinoma or uncontrolled malignant diseases, or habitual alcohol consumption of more than 140 g per week for men and 70 g per week for women.

Endpoints of the Study

The primary efficacy endpoint was the degree of HBV DNA reduction from baseline during the 12-month period of ADV and LMV combination therapy. The secondary efficacy endpoint was the rate of complete virologic response, which was defined as the decrease of HBV DNA to an undetectable level (<20 IU/ml) by real-time PCR assay, normalization of alanine transaminase (ALT; ≤ 45 IU/l), HBeAg to anti-HBe seroconversion, development of an ADV-resistant mutation and virologic breakthrough through 12 months of treatment. The safety endpoint was the development of any adverse events. In addition, the evolution of antiviral-resistant mutations was evaluated.

Assays

Assays were performed at central laboratories (HBV DNA quantification, HBsAg, HBeAg and anti-HBe tests at Seoul Clinical Laboratory, Seoul, Korea; RFMP at Green Cross Reference Laboratory, Yongin, Korea), except for biochemical, hematologic and coagulation tests, which were performed at the local laboratories of each study site using standard methods. HBV DNA was measured by the Cobas TaqManTM assay (Roche Diagnostics, Branchburg, N.J., USA). The lower detection limit was 20 IU/ml. HBsAg, HBeAg and anti-HBe were measured by electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland). Ge-

notypic resistance was evaluated by an RFMP assay based on amplification and mass detection of oligonucleotides excised by restriction enzyme digestion using matrix-assisted laser desorption/ionization time of flight mass spectrometry as previously described [10, 17, 18]. Briefly, viral DNA was extracted using a QIAamp blood kit (Qiagen, Chatsworth, Calif., USA) according to the manufacturer's instructions and amplified using primers for detection of mutations resistant to ETV [5'-GGATGCCTGGGCTTTTCG CAAG-3' (nucleotides 618–633), 5'-GGATGCACTCCCA TAGG-3' (nucleotides 648–637) for the rt169 codon; 5'-CCTCAGTCC GTTCTCCTCTGAAGCCGGCTCAGTTT-3' (nucleotides 651–676), 5'-TTGGCCCCCAATACCACACTGAAGTCCGGTCCAT ATA-3' (nucleotides 764–736) for the rt184 and rt202 codons; 5'-TTCCCCCACTGTTTGGCTGAAGTTCGGAGTTAT-3' (nucleotides 711–738), 5'-AAAGTACCCCAACTTCCACTGAA GTTTCCGGATATCC-3' (nucleotides 908–880) for the rt204 and rt250 codons], ADV [5'-CCTATGGGAGTGGGTCCAACCTCAG CGTTTCTC-3' (nucleotides 637–666), 5'-GAAAGCCAAACA GTGGGGGAAAGC-3' (nucleotides 732–709) for the rt181 co- don; 5'-TTACCAATTTTCTTTTGTCTCCAACCTGGGTAAATA TTT-3' (nucleotides 800–833), 5'-TAGCCCCAACGTTTGGTT TTATT-3' (nucleotides 863–841) for the rt236 codon] and LMV [5'-ATTCCTATGGGAGTGGGCCTCAGTG GATGC GTTCTC-3' (nucleotides 634–666), 5'-ACGAACCACTGAA CAAATGGCACTAGTAAACTG-3' (nucleotides 705–673) for the rt180 codon; 5'-TTCCCCCACTGTTTGGCTGGATGTCA GTTAT-3' (nucleotides 712–738), 5'-TACAGACTTGGCCCCC AATACCACATGA-3' (nucleotides 771–744) for the rt204 co-

don]. Nucleotide sequence positions were numbered as previously detailed [19]. The presence of mutations was assessed using matrix-assisted laser desorption/ionization time of flight mass spectrometry as described previously [10, 17, 18]. The lower detection limit of the RFMP was estimated to be 100 IU/ml.

Definitions

Complete virologic response was defined as above (HBV DNA <20 IU/ml). Early virologic response (EVR) was defined as achievement of undetectable HBV DNA at month 3. Biochemical response was defined as ALT $\leq$ 45 IU/l in both sexes. Serologic responses included HBeAg loss or seroconversion of HBeAg to anti-HBe. According to the previous guidelines [7], virologic breakthrough was defined as a confirmed increase in HBV DNA level of more than 1 log₁₀ IU/ml (10-fold) compared to the nadir HBV DNA level on treatment, biochemical breakthrough as an increase in ALT above the upper limit of normal after achieving normalization during continued treatment, and genotypic resistance as detection of mutations that have been shown in *in vitro* studies to confer resistance to the nucleos(t)ide analogues being administered. Primary nonresponse was defined as <1 log₁₀ IU/ml decrease in HBV DNA level from the baseline at 3 months of treatment [7].

Statistical Analyses

Statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, Ill., USA). For the evaluation of the antiviral efficacy through the 12 months of the treatment period, repeated-measurement analysis of variance (ANOVA) was performed, and post hoc analysis was done with the Bonferroni test to compare the HBV DNA level between baseline and months 3, 6, 9 and 12. Univariate and multivariate logistic regression analysis was performed for the factors associated with complete virologic response. Serum HBV DNA levels were compared after converting to a logarithmic scale. A *p* value <0.05 was considered significant.

Results

Baseline Characteristics

Twenty patients were enrolled. All patients had a history of LMV treatment failure before the switch to ETV 1 mg and subsequent ETV resistance during the rescue therapy. Changes in HBV DNA during the ETV therapy after LMV resistance are demonstrated with available data (fig. 2a). One patient achieved complete virologic response at month 21, but virologic breakthrough occurred. All the remaining patients were partial virologic responders to ETV. They developed ETV resistance before enrollment in the present study.

Seventeen patients were HBeAg positive (85%). Seven patients had cirrhosis (35%). The baseline mean serum HBV DNA level was 5.59 ± 1.28 log₁₀ IU/ml (median 5.66, range 3.36–7.45). Seven patients (35%) had HBV DNA less than 5 log₁₀ IU/ml; in 5 patients (25%) it was between

5 and 6 log₁₀ IU/ml, and in 8 patients (40%) it was over 6 log₁₀ IU/ml at baseline (fig. 2b). Resistance-associated mutations were evaluated. All the ETV resistance mutations had background LMV resistance mutations, of which the rtM204V + rtL180M mutation was the most common (85%). The rtT184L/I/A/F mutation was the most common ETV resistance mutation (50%), followed by the rtS202G mutation (25%), rtT184I + S202G mutation (15%) and rtI169T + rtT184L ± rtM250V mutation (10%; table 1). No ADV-resistant mutations (rtA181V/T or rtN236T) were detected at baseline. Forty percent of patients had elevated ALT (>45 IU/l). ALT was higher in patients with a higher HBV DNA level, and there was a significant correlation between the baseline HBV DNA level and the ALT level ($\rho = 0.519$, $p = 0.019$). Seventeen patients completed the 12-month follow-up.

Responses to Therapy

Virologic Responses

Mean HBV DNA levels at baseline and 3, 6, 9 and 12 months were 5.59 ± 1.28 , 3.49 ± 1.51 , 3.44 ± 1.57 , 3.33 ± 1.57 and 3.19 ± 1.40 log₁₀ IU/ml, respectively (fig. 2b). The mean reductions in serum HBV DNA levels from baseline to 3, 6, 9 and 12 months were -2.10 ± 1.28 , -2.14 ± 1.43 , -2.15 ± 1.32 and -2.37 ± 1.06 log₁₀ IU/ml, respectively. Repeated-measurement ANOVA showed a significant reduction in HBV DNA level from baseline throughout 12 months of ADV plus LMV combination therapy ($p < 0.001$). Post hoc analysis showed a significant difference in HBV DNA level between baseline and each follow-up visit (all $p < 0.001$). At month 12, complete virologic response was observed in 4 patients (23.5% by per-protocol analysis, 20% by intention-to-treat analysis).

Biochemical and Serologic Responses

After initiation of ADV and LMV combination therapy, serum ALT levels were gradually stabilized up to 94.4% by 9 months. Thereafter, the rate decreased to 82.4%. Three patients lost HBeAg (21.4%), but no patient achieved HBeAg to anti-HBe seroconversion (table 2).

Antiviral Resistance

Primary Nonresponse

Primary nonresponse, which was determined at 3 months of rescue therapy, was observed in 2 patients (10.5%). One patient was lost to follow-up at month 12, and the other patient did not achieve complete virologic response. The former had the rtL180M + rtM204V + rtT184T/I + rtS202G mutation and the latter patient had

Fig. 2. Serum HBV DNA levels during the two treatment periods. **a** Changes in HBV DNA levels before enrollment in the present study. Patients were receiving ETV 1.0 mg due to LMV resistance. Available data from 16 patients are shown. Virologic breakthroughs were observed at 12 months of ETV therapy or later. Patients were enrolled into the present study after genotypic resistance was confirmed. **b** Changes in HBV DNA after enrollment in the study. Patients received a combination of ADV 10 mg and LMV 100 mg due to ETV resistance. Mean serum HBV DNA levels at baseline and 3, 6, 9 and 12 months were 5.59, 3.49, 3.44, 3.33 and 3.19 log₁₀ IU/ml, respectively. There was a significant reduction in HBV DNA from baseline to 12 months ($p < 0.001$, repeated-measurement ANOVA). Subject identification numbers are shown on the right of each graph.

the rtL180M + rtM204V + rtS202G mutation at baseline. The mutational patterns did not change during ADV and LMV combination therapy. Also, there was no new development of any mutation associated with ADV resistance until the end of follow-up.

Antiviral Resistance during Treatment

A search for resistance-associated mutations to LMV, ADV and ETV was performed every 3 months to detect the emergence of ADV resistance and to monitor changes in the preexisting mutational pattern. RFMP as-

say revealed genotypic resistance to ADV (rtA181T) in 2 out of 17 patients (11.7%) by month 12. One patient developed rtA181T at month 3 (rt181A:rt181T = 1:1), and the other patient developed the same mutation at month 9 (rt181A:rt181T = 9:1). The proportion of mutants was not high. Virologic breakthrough did not develop in either patient, and HBV DNA continued to decrease.

LMV resistance mutations continued to be detected in all patients. The mutational pattern changed during treatment in 4 patients, from rt180M + rt204V to rt180M/L +

Table 1. Baseline characteristics of the patients

Parameter	
Age, years	47 (29–74)
Male, n	15 (75)
HBeAg-positive, n	17 (85)
AST, IU/l	31 (17–72)
ALT, IU/l	39 (11–185)
HBV DNA, log ₁₀ IU/ml	5.66 (3.36–7.45)
Duration of ETV treatment, months	31 (12–39)
Cirrhosis, n	7 (35)
LMV-resistant mutation, n	
M204V + L180M	17 (85)
M204V/I + L180M	1 (5)
M204I	2 (10)
ETV-resistant mutation, n	
T184L/I/A/F	11 (50)
S202G	5 (25)
T184I + S202G	3 (15)
T184L + I169T	1 (5)
T184L + I169T + M250V	1 (5)

Data are given as the median (range) or number (percentage) of patients. AST = Aspartate aminotransferase.

rt204V/I in 2, from rt180M + rt204V to rt180M/L + rt204M/V in 1, and from rt204I to rt204I/V in 1. Two patients developed the rtA181T mutation.

Among the 17 patients who completed follow-up, 13 patients had detectable HBV DNA at month 12. The ETV-resistant mutation (rtT184F) disappeared in 1 patient, and the proportion of ETV-resistant mutations decreased in 6 patients. The remaining 6 patients did not show any changes in the mutational pattern or proportion of ETV-resistant mutations (table 3; fig. 3).

Virologic Breakthrough

No patient showed an increase in HBV DNA of more than 1 log₁₀ IU/ml, although there was fluctuation of the HBV DNA level past month 6 in several patients.

Factors Associated with Complete Virologic Response

Univariate analysis showed the EVR (undetectable HBV DNA at month 3) as a single significant factor for complete virologic response at month 12 (OR 36.0, 95% CI 1.7–757.8; $p = 0.021$). Baseline HBV DNA level, HBeAg, ALT, baseline ETV-resistant mutation types and presence of liver cirrhosis were not significant factors. EVR still remained as a significant factor for virologic response after adjusting HBeAg and baseline ALT (OR 43.5, 95% CI 1.5–100.0; $p = 0.028$) by multivariate analysis.

Adverse Events

An increase in serum creatinine above the normal range (>1.5 mg/dl) or to 1.5 times the baseline level was not noted. No significant hypophosphatemia was noted. No hepatocellular carcinoma was newly detected at the end of the study.

Discussion

ETV is a deoxyguanosine analogue with potent antiviral activity against HBV. Previous phase 3 clinical trials have shown ETV to be efficacious for the reduction of HBV DNA in the treatment of CHB patients [3, 4]. The resistance rate was only 1.2% in treatment-naïve patients up to 5 years [5]. However, susceptibility to ETV decreases by 3.1- to 20-fold when rtL180M and rtM204V are present [20]. Further reduction is noted if there are additional changes at positions rt180M, rtS202 or rtM250 [20]. Therefore, the presence of LMV resistance mutations should be a high risk factor for subsequent ETV resistance, although multiple site mutations are required for the development of ETV resistance. In this context, the rate of ETV resistance is increased during long-term ETV treatment for LMV-refractory patients [5, 21].

ETV-resistant mutants are susceptible to ADV. Previous phenotypic analyses from isolates of ETV-treated patients with virologic rebound revealed the unchanged susceptibility to ADV compared with baseline [13, 22]. However, clinical experience of ADV for the treatment of ETV-resistant CHB is limited. A case report suggested that switching to ADV monotherapy was effective [23]. However, ADV monotherapy may put a patient at high risk of ADV resistance, so combination therapy is certainly needed. We chose LMV as the combination drug with ADV because of the low risk of adverse events, low cost and good accessibility. In addition, another case report [13] prompted us to further evaluate the ADV plus LMV combination.

In our study, ADV plus LMV significantly reduced the HBV DNA level through 12 months compared with baseline ($p < 0.001$). HBV that retained ETV-resistant mutations became suppressed, so their proportion in sera decreased after treatment. However, the complete virologic response rate was lower than we had expected (20–23.5% at month 12). In addition, the mutation resistant to both ADV and LMV (rtA181T) was newly detected in 2 patients. Although virologic breakthrough did not develop in these patients, they would still be at

Fig. 3. Changes in the antiviral resistance mutational pattern during treatment. The evolution of ETV-resistant (ETV-R) mutants during treatment among the study population is demonstrated. At 12 months, 4 patients had undetectable HBV DNA. Among 13 patients who had detectable HBV DNA, 1 patient showed disappearance of ETV-resistant mutants, 6 patients showed reductions in the proportion of ETV-resistant mutants among viral quasi-species and the remaining 6 patients did not show any changes in the mutational pattern or proportion of ETV-resistant mutations.

Table 2. Virologic, biochemical and serologic response to ADV and LMV combination therapy

	3 months	6 months	9 months	12 months
HBV DNA undetectable by PCR (<20 IU/ml), n	4/19 (21.1)	5/19 (26.3)	4/18 (22.2)	4/17 (23.5)
Normal ALT, n	14/19 (73.7)	16/19 (84.2)	17/18 (94.4)	14/17 (82.4)
HBeAg loss, n	4/12 (33.3)	5/16 (31.6)	5/14 (35.7)	3/14 (21.4)
HBeAg seroconversion, n	1/12 (8.3)	0/16 (0)	0/14 (0)	0/14 (0)

Data are presented as the number of cases out of the number of patients being followed up (percentage in parentheses).

Table 3. Comparison of the ETV resistance mutational pattern between baseline and month 12

Subject No.	Amino acid		Ratio		Proportion of ETV-resistant mutants
	position	wild type:mutant	baseline	month 12	
A02	rt184	Thr:Leu	0:1 (100)	1:2 (67)	decreased
A03	rt169	Ile:Thr	0:1 (100)	2:1 (33)	decreased
	rt184	Thr:Leu	0:1 (100)	1:1 (50)	decreased
C02	rt184	Thr:Leu	0:1 (100)	0:1 (100)	NC
C03	rt184	Thr:Phe	1:4 (80)	1:0 (0)	disappeared
C04	rt184	Thr:Ile:Ala	2:1:8 (82)	1:0:1 (50)	decreased
C05	rt184	Thr:Ile	5:1 (17)	1:0 (0)	decreased
	rt202	Ser:Gly	0:1 (100)	1:5 (83)	decreased
C06	rt202	Ser:Gly	0:1 (100)	0:1 (100)	NC
D01	rt184	Thr:Ile	0:1 (100)	0:1 (100)	NC
E01	rt184	Thr:Leu	0:1 (100)	0:1 (100)	NC
F01	rt202	Ser:Gly	1:3 (75)	1:2 (67)	decreased
F02	rt202	Ser:Gly	0:1 (100)	0:1 (100)	NC
H01	rt184	Thr:Leu:Ala	0:1:0 (100)	3:7:1 (72)	decreased
J02	rt202	Ser:Gly	0:1 (100)	0:1 (100)	NC

Subjects with undetectable HBV DNA at month 12 and cases lost to follow-up were not included in this comparison. Figures in parentheses represent the percentage of mutants. rt = Reverse transcriptase; NC = no change.

high risk of a poor response to the ADV plus LMV combination. Interestingly, the LMV-resistant mutation did not disappear in all patients, suggesting that selection pressure for the LMV-resistant mutant was being exerted. In this study, the high rate of partial virologic response to the ADV and LMV combination could be attributable to HBeAg positivity, HBV genotype C, which is prevalent in Korea, and relatively high levels of baseline HBV DNA. The only factor associated with complete virologic response at month 12 was EVR, so an early response to this therapy is mandatory for successful treatment. Moreover, modification of the treatment strategy may be required in the absence of EVR. Another ADV combination, such as ADV plus ETV, could be an alternative therapy [24–26], but the cost will be increased. In addition, the efficacy of ADV plus ETV needs to be demonstrated compared with that of the ADV plus LMV combination because a previous retrospective study failed to show its benefit [27].

Current guidelines recommend a switch to or addition of TDF [7, 8]. Indeed, TDF-based rescue therapy seems very effective for treating ETV-resistant or multi-drug-resistant CHB [28, 29]. However, TDF is not widely available in many Asian countries, and we were not able to prescribe TDF in Korea during the period of the present study. In the absence of TDF, ADV-based therapy could be an option for the management of ETV resistance. However, as the efficacy of ADV is limited, it would not be an optimal choice. Once TDF is available, TDF should be considered the first-line therapy for ETV resistance based on the current guidelines [7, 8].

In this study, we examined the changes in mutation profiles, including the proportion of each resistant mutant by RFMP. Previous RFMP results were well correlated with clonal analysis [17]. So, it would be relevant to evaluate the evolutionary change in resistant mutants with this assay. In more than half of the patients who had detectable HBV DNA at month 12, ETV-resistant mutants decreased in the proportion of viral quasispecies or disappeared. These patients tended to show a more marked decrease in the HBV DNA level than patients with no changes in the proportion of viral quasispecies (data not shown), suggesting an association of viral evolutionary changes and the replication fitness under the therapy [14].

This study has several limitations. It was a single-arm study and so could not assess superiority or inferiority compared to other treatment regimens. However, considering that a significant reduction in HBV DNA level for ETV-resistant CHB was evident, future comparative

studies could be designed using our treatment regimen as a reference arm. Another limitation is the small number of study subjects. As ETV resistance is not very common in clinical practice, we were not able to enroll a large number of subjects even though we conducted a multicenter study. Future larger studies comparing ADV plus LMV with other treatment options for the treatment of ETV resistance are warranted. Lastly, the method for detection of antiviral resistance mutations could be an issue. RFMP assay cannot detect new molecular changes which may confer resistance to the given drug, although very sensitive and quantitative detection of mixed populations is possible.

However, our study has several strong points. This is the first prospective evaluation of the efficacy of ADV plus LMV for ETV-resistant CHB. There have been only case reports or small retrospective chart reviews for this combination previously; these were unable to evaluate the exact complete virologic response rate of ADV plus LMV therapy for ETV resistance. Secondly, this study was performed in a well-defined population. Past medical treatment history and characteristics were very homogeneous. All the patients received LMV first and subsequently ETV due to LMV resistance. All the patients had confirmed ETV resistance by RFMP assay at baseline. Lastly, we analyzed resistance-associated mutations regularly at 3-month intervals and observed the evolutionary changes in ETV-resistance mutants. This revealed the decreasing proportion of ETV-resistant mutants among viral quasispecies and also their disappearance in sera.

In conclusion, ADV plus LMV combination therapy significantly reduced serum HBV DNA levels in CHB patients with resistance to ETV although the degree of reduction was modest. ETV-resistant mutants were suppressed with the combination therapy, and the proportion of ETV-resistant mutants was decreased. However, the incidence of a resistant mutation to both ADV and LMV was not negligible.

Acknowledgements

The authors thank Dr. Eileen L. Yoon, Dr. Jeong Han Kim, Prof. Soon-Sun Kwon, Ms. Young Eun Park, Ms. Su Hyun Kim (Korea University Ansan Hospital) and clinical research coordinators at all study sites for their dedicated assistance in collecting and analyzing the data.

This study was partially supported by a grant of the Korean Healthcare Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A 102065 to Y.H.J., S.Y.S. and U.S.H.) and partly by a research fund from Glaxo-Smith-Kline Korea. Lamivudine was kindly supplied by Glaxo-Smith-Kline Korea.

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