Low efficacy of entecavir therapy in adefovir-refractory hepatitis B patients with prior lamivudine resistance

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-ABSTRACT-

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We determined the virologic response, incidence of entecavir resistance, and evolution of lamivudine and adefovir-resistant mutants during entecavir (ETV) therapy in adefovir-refractory patients with prior lamivudine resistance. Forty adefovir-refractory chronic hepatitis B patients with prior lamivudine resistance who had received entecavir for ≥6 months were included and monitored for virologic response and entecavir resistance. Ten percent of patients achieved HBV DNA < 50 copies/mL by PCR after 24 weeks of ETV therapy, and an initial virologic response (IVR) was observed in 12 out of 40 patients (30%). Higher pretreatment ALT (p=0.039) and the presence of the rtL180M mutation (p=0.038) were associated with IVR. During a mean follow-up of 11.4 months, four patients (10%) experienced virologic breakthrough, while ETV-resistant mutants were detected in six patients (15%). YMDD and adefovir-resistant mutants were detected in 57% and 35% of patients at baseline, respectively. At 48 weeks of therapy, 96% and 4 % of patients had YMDD and adefovir-resistant mutants, respectively. These data suggest an early development of ETV resistance and low antiviral response during ETV therapy in adefovir-refractory patients with prior lamivudine resistance.

Key words: Hepatitis B, Entecavir, Lamivudine, Adefovir, Drug resistance

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I. INTRODUCTION

Chronic hepatitis B virus (HBV) infection is an important health problem throughout the world, and leading frequently to cirrhosis, liver failure, and hepatocellular carcinoma (Ganem and Prince, 2004; Wright and Lau, 1993). Nucleos(t)ide analogues have been found to suppress HBV replication and to improve biochemical and histological status of hepatitis B patients (Lai et al, 1998; Dienstag et al, 2003; Leung et al, 2001). Prolonged antiviral therapy in patients with chronic HBV infection can prevent progression to cirrhosis and hepatocellular carcinoma (Liaw et al, 2004); however, it also often results in the emergence of drug-resistant mutants and an ensuing treatment failure (Lai et al, 2003).

Prolonged lamivudine (LAM) therapy is associated with a high rate of selection for LAM-resistant HBV, at approximately 24% and 70% after 1 and 4 years of therapy, respectively (Lai et al, 2003). Mutations in the YMDD catalytic motif in the C domain of HBV polymerase (rtM204V/I) are responsible for LAM resistance (Melegari et al, 1998). Although adefovir (ADV) has shown to be effective against both wild-type and LAM-resistant HBV (Marcellin et al, 2003; Schiff et al, 2003), suboptimal viral response has been frequently observed in LAM-resistant patients (Fung et al, 2006) and ADV-resistant mutants were found to appear more frequently in LAM-resistant patients than in treatment-naive patients (Lee et al, 2006). Combination therapy with ADV and LAM is considerable by international guidelines as the standard of care options for LAM-resistant patients. The selection of the rtN236T or rtA181V/T mutants was associated with ADV resistance (Lee et al, 2006; Hadziyannis et al, 2006). Entecavir (ETV) is

another drug that displays potent antiviral activity against wild-type HBV (Innaimo et al, 1997; Zoulim, 2006). LAM-resistant mutants exhibit an intermediate susceptibility to ETV as administration of a high dose of ETV is required to suppress these mutants (Levine et al, 2002; Tenney et al, 2007). Althought ETV resistance seems to be rare in treatment-naive patients (Colonno et al, 2006; Gish et al, 2007), it does emerge with a rate of 6%, 15% and 51% after 1, 2 and 5 years therapy, respectively, in LAM-resistant patients (Tenney et al, 2007; Tenney et al, 2006). The emergence of rtT184, rtS202, and rtM250 mutations is associated with viral rebound in LAM-resistant patients (Tenney et al, 2004).

Sequential nucleos(t)ide analogue monotherapies increase the risk of selection of multi-drug resistant strains (Fung et al, 2006) and the development of multi-drug resistance to LAM and ADV is becoming a common problem. Combination therapy with ETV and tenofovir has been recommended for the treatment of patients with resistance to LAM and ADV (Lok et al, 2007); however, tenofovir has not yet been available for the treatment of chronic HBV infection in many countries, and the ministry of Health, Welfare, and Family Affairs does not reimburse for combination therapy in Korea.

In vitro studies have shown that ETV is effective in suppressing ADV-resistant mutants [23]. Although ETV has been reported to be effective in suppressing HBV DNA levels in two ADV-resistant patients with prior LAM resistance (Fung et al, 2006), the antiviral effect of ETV in this setting has not been fully investigated. Furthermore, studies on the emergence of ETV resistance in ADV-refractory patients with prior LAM resistance are limited. In the present study, we determined the virologic response and

emergence of ETV-resistant mutants in ADV-refractory patients with prior LAM resistance during ETV therapy, and the evolution of LAM and ADV-resistant mutants was observed.

II. PATIENTS AND METHODS

The study subjects included consecutive 40 ADV-refractory chronic hepatitis B patients with prior LAM resistance. All patients had LMV resistance documented by virologic breakthrough defined as an increase in the level of HBV DNA of at least 1 $\log_{10}$ copies/mL from the lowest point during therapy ,genotypic analysis of rtM204 sequences, and LAM was switched to ADV monotherapy. Patients were considered to be ADV refractory if they had alimited virological response with or without documented ADV mutations while on ADV. Fourteen patients developed ADV resistance and another 26 patients experienced suboptimal virological response to ADV monotherapy. These were switched to ETV monotherapy from ADV. Patients were positive for HBsAg at least 1 year before LAM therapy. None of the patient had co-infections(HCV,HIV) or other concomitant liver disease such as alcoholic liver disease or autoimmune liver disease. All patients had HBV DNA level > 5$\log_{10}$ copies/mL before ETV administration and received 1.0 mg ETV once daily. Biochemistry and HBV DNA levels were tested before and every 3 months during ETV therapy. Serial blood samples were taken before and every 3 months during therapy and stored at -70 ℃ until used for HBV molecular analyses. This study was approved by the Institutional Review Board of our institution, and all the patients gave their informed consent.

A. Analysis of virological markers

Routine biochemical tests were performed using standard procedures

during therapy. HBsAg, HBeAg, and anti-HBe were tested with a commercial radioimmunoassay kit (Abbott Laboratories, Chicago, IL, USA). HBV DNA was determined quantitatively by branched DNA (bDNA) assay (versant^{TM}3.0, Bayer Healthcare LLC Diagnostic Division, New York, USA), which has a detection limit of 2000 copies/mL. In samples showing undetectable HBV DNA by bDNA assay, detection of HBV DNA was done by the COBAS TaqMan^{TM} HBVtest (TaqMan test; Roche Diagnostics, Branchburg, NJ), which has a detection limit of 50 copies/mL (or 12 IU/mL).

B. Genotypic analysis

We performed restriction fragment mass polymorphism (RFMP) to detect LAM-resistant mutations (rt180, rt204), Adefovir-resistant mutations (rt181, rt236), and ETV-resistant mutations (rt169, rt184, rt202, rt250 plus rt204) at baseline and every 3 months in all patients during ETV administration, as previously described [24,12]. The genotypic analysis by RFMP was confirmed in some patients by sequencing analysis. This analysis was performed using primers with the sequences 5′-TCC TAC GAC CCC TGC TCG TGT TAC-3′ (nucleotide 177-200) and 5′-CTG TAA ATA GAC CTA TTG ATT GGA-3′ (nucleotide 959-982).

C. Statistical analysis

Statistical testing was performed using SPSS version 13 (SPSS Inc., Chicago, IL). Results are reported as mean ±SD or median (range). HBV DNA levels were logarithmically transformed for analysis. Continuous variables were compared using the independent sample's *t*-test. Categorical data were compared using the Pearson χ^2 test or Fisher's exact test. Factors

associated with a initial virologic response were analyzed by univariate analysis. A *P*-value of less than .05 was considered statistically significant.

III. RESULTS

A. Baseline characteristics

The study population comprised 40 adefovir-refractory patients who had previously shown LAM resistance. The baseline characteristics of the patients studied are shown in Table 1. Thirty-five patients were men and the mean age was 45±10.48 years. Ten patients (25%) had cirrhosis and 36 patients (90%) were positive for HBeAg. Fourteen patients (35%) were treated with ETV at the time of virologic breakthrough due to ADV resistance, and the remaining 26 patients were treated due to suboptimal response to ADV. The mean duration of ETV therapy was 11.4±3.2 months. At the commencement of ETV therapy, 23 patients (57.5%) had YMDD mutants; seven with rtM204V, 13 with rtM204I, and three with rtM204V/I. Nineteen patients (47.5%) had rtL180M. ADV-resistant mutants were found in 14 patients.

Table 1. Baseline characteristics of patients (n=40)

	Patients	
Mean age, years (SD)	45.1	(10.48)
Male (%)	35	(87.5)
HBeAg-positive (%)	36	(90)
Mean ALT, IU/L (SD)	83.75	(155.72)
Mean AST, IU/L (SD)	84.28	(252.83)
HBV DNA, log_{10}copies/mL(SD)	6.68	(0.93)
Mean duration of ADV prior to ENT, mos (SD)	17.13	(7.72)
Mean duration of ENT, mos (SD)	11.40	(3.21)
Cirrhosis (%)	10	(25)
LAM-resistant mutation		
rtM204I (%)	13	(32.5)
rtM204V (%)	7	(17.5)
rtM204I+rtM204V (%)	3	(7.5)
rt204M (wild) (%)	17	(42.5)
rtL180M (%)	19	(47.5)
rt180L (wild) (%)	21	(52.5)
ADV-resistant mutation		
rtA181T (%)	6	(15)
rtA181V (%)	4	(10)
rtA181T + rtA236T (%)	1	(2.5)
rtA181V + rtA236T (%)	3	(7.5)
rt181A + rt236A (wild) (%)	26	(65)

ADV, adefovir; ETV, entecavir; LAM, lamivudine.

B. Virologic and biochemical response to entecavir

At the start of ETV therapy, all patients had HBV DNA> 5 $\log_{10}$ copies/mL and 23 patients had elevated ALT levels. ETV redeuced HBV DNA levels to undetectable by PCR (<50copies/mL) in 10 % and 12 % of patients by week 24 and week 48, respectively, and initial virologic response (IVR) defined as HBV DNA < 4 $\log_{10}$ copies/mL after 6 months of therapy was observed in 12 of 40 patients (30%)(Table2). Patients who achieved IVR had higher baseline ALT and AST levels(80 vs 44 IU/L, p=0.039; 51 vs 31 IU/L, p=0.036, respectively) compared to those who did not achieve IVR. The rtL180M mutations were significantly more detected at baseline among patients with IVR (75 vs. 35%, p=0.038). However, there was no difference in baseline HBV DNA levels, HBeAg positivity, presence of YMDD mutation or ADV-resistant mutation between patients with and without IVR (Table3). Serum ALT levels were normalized in 13 of 23 patients (56%) with high baseline ALT level at 6 months of therapy. Among 36 HBeAg-positive patients ,four (11.1%) achieved HBeAg loss (n=2) or HBeAg seroconversion (n=2) during ETV therapy (mean 11.4 months) (Table2).

Tabel 2 Virologic, serologic and biochemical response to entecavir

	Week 12 (n=40)	Week 24 (n=40)	Week 36 (n=34)	Week 48 (n=33)	Total
HBV DNA, log_{10} copies/mL,n(%)					
Undetectable by PCR*		4 (10)		4 (12.1)	
< 3.3	1 (2.5)	8 (20)	7 (20.6)	5 (15.2)	
3.3-3.9	5 (12.5)	4 (10)	2 (5.9)	4 (12.1)	
4.0-4.9	8 (20)	7 (17.5)	7 (20.6)	6 (18.2)	
≥ 5.0	26 (65)	21 (52.5)	18 (52.9)	18 (54.5)	
HBeAg seroconversion /loss (n=36) (%)	0/1	2/1			2 (5.5) /2 (5.5)
Virologic breakthrough (n=40) (%)			1	3	4 (10)
Emergence of ETV resistance (n=40) (%)		1	1	4	6 (15)

ETV, entecavir

* Detection limit of COBAS TaqMan™assay is < 50 copies/mL (or 12 IU/mL).

Table 3. Baseline factors associated with an initial virologic response

	Patients with IVR (n=12)	Patients without IVR (n=28)	p-value
Mean age,years (SD)	49 (11) 02	43 (9)	0.102
Male (n=35)	10	25	0.627
HBV DNA, log_{10}copies/mL			
<7 (n=24)	9	15	0.297
≥7 (n=16)	3	13	
Median ALT,IU/L	80 (12-1007)	44 (19-136)	0.039
Median AST,IU/L	51 (18-1594)	31 (19-61)	0.036
HBeAg-positive (n=36) (%)	11 (30.5)	25 (69.4)	1.000
Cirrhosis (n=10) (%)	5 (41.7)	5 (17.9)	0.133
rtM204V/I (n=23) (%)	7 (58.3)	16 (57.1)	0.738
rtL180M (n=19) (%)	9 (75)	10 (35.7)	0.038
rtA181T/V, rtA236T (n=14) (%)	4 (33.3)	10 (35.7)	1.000

IVR, initial virologic response Initial virological response defined as HBV DNA < 4 log10 copies/mL after 6 months of entecavir therapy.

C. Emergence of ETV-resistant mutants

During a mean follow-up of 11.4±3.2 months, four patients (10%) experienced virologic breakthrough. ETV-resistant mutants emerged in six out of 40 patients (15%). Among the six patients with ETV-resistant mutants, four had virologic breakthrough and ETV-resistant mutants transiently appeared in two patients. ETV-resistant mutants emerged in one, one and four patients at 6, 9, and 12 months of therapy, respectively. Among the six patients with ETV-resistant mutants, four patients had the rtS202G and two had the rtT184L mutants (Table2,4).

D. Evolution of LAM and ADV-resistant mutants in patients who developed ETV-resistant mutations

Among the six patients with ETV-resistant mutants, four had wild-type YMDD before ETV administration. YMDD mutations were found to emerge at 12 weeks of ETV therapy in all four patients. The rtM204V mutation was detected in three patients, and the rtM204I mutation in one at the time of emergence of ETV-resistant mutants. ADV-resistant mutants (rtA181V/T, rtA236T) were detected in three patients before ETV therapy. ADV-resistant mutants were replaced with wild-type HBV within 24 weeks of therapy in all three patients and were not detected at the time of emergence of ETV-resistant mutants (Table 4).

Table 4. Evolution of ETV, LAM, and ADV-resistant HBV during ETV therapy in 6 patients who developed ETV-resistant mutant

Patients	Time to resistance (weeks)	HBV DNA level (log_{10}copies/mL)andgenotypicresistance				
		Baseline	week 12	week 24	week 36	week 48
1	24	5.7 rt202S rtM204V rt181A,rt236A	5.0 rt202S rtM204V rt181A,rt236A	ND† **rtS202G**>rt202s rtM204V rt181A,rt236A	ND rt202S rtM204V rt181A,rt236A	ND not detected not detected not detected
2	36	> 8.0 rt202S rt204M rt181A,rt236A	5.2 rt202S rtM204V rt181A,rt236A	ND rt202S rtM204V rt181A,rt236A	5.4 rt202S>**rtS202G* rtM204V rt181A,rt236A	7.9 rt202S<**rtS202G* rtM204V rt181A>rtA181T, rt236A
3	48	6.1 rt202S rt204M rtA181T,rt236A	5.0 rt202S rt204M>rtM204I rt181A,rt236A	ND rt202S rt204M>rtM204I rt181A,rt236A	ND rt202S rtM204I rt181A,rt236A	ND **rS202G** rtM204I rt181A,rt236A
4	48	> 8.0 rt184T rt204M rtA181V,rtA236T	6.4 rt184T rtM204V>rt204M rt181A>rtA181V, rt236A	6.9 rt184T rtM204V rt181A,rt236A	6.8 rt184T rtM204V rt181A,rt236A	7.4 rt184T>**rtT184L* rtM204V rt181A,rt236A
5	48	6.3 rt184T rtM204V rt181A,rt236A	6.2 rt184T rtM204V rt181A,rt236A	6.2 rt184T rtM204V rt181A,rt236A	6.0 rt184T rtM204V rt181A,rt236A	7.2 **rtT184L* rtM204V rt181A,rt236A
6	48	6.0 rt184T,rt202S rt204M rt181A>rtA181V, rt236A>rtA236T	4.1 rt202S rtM204I>rt204M rt181A,rt236A	3.6 rt202S rtM204I>rt204M rt181A,rt236A	3.3 rt202S rtM204V rt181A,rt236A	7.1 **rtS202G***>rt202S rtM204V rt181A,rt236A

* Combined with virologic breakthrough

† not detectable, <3.3 log_{10}copies/mL

ETV, entecavir; LAM, lamivudine; ADV, adefovir.

Entecavir-resistant mutations are rtS202G, rtT184L, lamivudine-resistant mutations are rtM204V/I, and adefovir-resistant mutations are rtA181T/V, rtA236T.

E. Evolution of LAM and ADV-resistant mutants during ETV therapy

Among the 40 patients studied, 23 (57.5%) had rtM204V/I and 19 (47.5%) had rtL180M mutants before ETV administration. RFMP analysis of the position rtM204 in patients receiving ETV therapy showed that rtM204V/I mutants were detected in 87.5% (35/40) and 96% (25/26) of patients at 24 and 48 weeks of therapy, respectively. In addition, rtL180M mutants emerged in 62.5% (25/40) and 73.0% (19/26) of patients at 24 and 48 weeks of ETV therapy. These results suggest that ETV therapy selects for LAM-resistant mutants. ADV-resistant mutants (rtA181V/T, rtA236T) were detected in 14 of 40 patients (35%) before ETV administration. ADV-resistant mutants remained positive in five (12.5%) patients at 24 weeks and one patient (3.8%) at 48 weeks of ETV therapy (Table 5).

Table 5. Evolution of lamivudine and adefovir-resistant mutants during entecavir therapy

Genotype	Baseline (n=40) (%)	week 12 (n=40) (%)	week 24 (n=40) (%)	week 36 (n=35) (%)	week 48 (n=26)(%)
YMDD, wild-type	17 (42.5)	5 (12.5)	5 (12.5)	3 (7.5)	1 (2.5)
YIDD, YVDD	23 (57)	35 (87)	35 (87)	32 (91)	25 (96)
rt181A, rt236A	26 (65)	30 (75)	35 (87.5)	33 (94)	25 (96)
rtA181T/V, rtA236T	14 (35)	10 (25)	5 (12)	2 (6)	1 (4)
rt180L	21 (52.5)	12 (30)	15 (37.5)	11 (31)	7 (26.9)
rtL180M	19 (47)	28 (70)	25 (62)	24 (68)	19 (73)

III. DISCUSSION

Multi-drug resistance to LAM and ADV is becoming prevalent due to sequential treatment of LAM followed by ADV. ETV displays antiviral activity against both LAM-resistant and ADV-resistant HBV (Innaimo et al, 1997; Zoulim, 2006; Vileneuve et al, 2003). A previous study showed that 79% of LAM-resistant patients had undetectable HBV DNA levels by bDNA assay at 24 weeks of ETV therapy and that HBV DNA was undetectable by PCR assay in 26% of patients at 48 weeks (Chang et al, 2005). A preliminary study of 12 patients showed ETV administration reduced HBV DNA levels in patients with a limited virological response to adefovir but only 33% of patients achieved HBV DNA levels of less than 3 $\log_{10}$ copies/mL at 24 weeks (Reijnders et al, 2007). These results suggest a low response to ETV in patients with LAM resistance and in those with a limited response to ADV. In our study investigating the efficacy of ETV in ADV-refractory patients with prior LAM resistance, IVR was observed in 30 % of patients and HBV DNA levels were undetectable by PCR assay in 10 % of patients after 6 months of ETV therapy. These findings demonstrated that the antiviral activity of ETV is low in ADV-refractory patients with LAM resistance.

In this study, high baseline ALT/AST levels were found to be associated with IVR on ETV in ADV-refractory patients with LAM resistance. Previous studies with LAM therapy have shown that baseline ALT is the most important predictor of HBeAg seroconversion (Chien et al, 1999). Thus, the results of this study confirmed that nucleos(t)ide analogues

are more effective in patients who have high pretreatment ALT levels. Among 40 study subjects who had had YMDD mutations previously, YMDD mutations were detected in 23 subjects (57.5%) before ETV therapy. The presence of YMDD mutations was not associated with IVR on ETV. However, the presence of the rtL180M mutation appeared to be associated with IVR. The role of rtL180M as a predictor of IVR needs to be validated in further studies with larger numbers of patients.

It had been reported that viral rebounds due to ETV resistance were detected in 10% of LAM-resistant patients after 48 weeks and an additional 9% after 96 weeks (Tenney et al, 2007). In the present study, virologic rebounds were observed in four (10%) out of 40 ADV refractory patients with prior LAM resistance during a mean follow-up of 11.4 months, suggesting that ETV-resistant mutations develop early during therapy in these patients. We observed emergence of ETV-resistant mutants in six out of 40 patients. Among those six patients, 4 had a rtS202G mutant and 2 had a T184L mutant.

Virologic rebound occurs in nucleoside-naive patients receiving ETV treatment due to selection of a LAM-associated mutation (Colonno et al, 2006). We investigated the emergence of LAM-associated mutations during ETV treatment in ADV-refractory patients who had had previous LAM resistance. Fifty-seven percent of patients had YMDD mutants before ETV treatment, and ETV treatment increased the rate of emergence of YMDD mutants to 96% of patients at 48 weeks. This suggests that YMDD mutants had reappeared in almost all patients within one year of ETV therapy. ETV has been shown to be less effective against LAM-resistant mutants than wild-type HBV (Chang et al, 2005). Thus, ongoing ETV treatment may

confer a selective advantage to YMDD mutants over wild-type HBV in patients infected by a mixture of wild-type and LAM-resistant mutant HBV. Early emergence of YMDD mutants, coupled with suboptimal response to ETV, can increase the risk of selection for ETV-associated mutations in ADV refractory patients with LAM resistance.

On the other hand, reversion from ADV-resistant mutants to wild-type HBV occurred in nine (64.2%) out of 14 patients at 24 weeks of ETV therapy and most patients had reverted wild-type HBV by 48 weeks of therapy. These findings suggest that ETV may suppress ADV resistant mutants more effectively than wild-type HBV and that ETV could be effective for the treatment of those ADV-refractory patients with no prior exposure to LAM.

In conclusion, only 30% of ADV-refractory patients with prior LAM resistance achieved IVR on ETV and high pretreatment ALT level and the presence of rtL180M mutation were associated with IVR. We also found that YMDD mutants reappeared in the majority of patients within one year of therapy, even in the absence of YMDD mutants before therapy. However, ETV was efficacious in suppressing the replication of ADV-resistant mutants. A suboptimal response to ETV, coupled with early emergence of YMDD mutants, led to early and frequent development of ETV resistance in these patients.

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- 국문요약 -

라미부딘 및 아데포비어 내성인 만성 B형 간염 환자에서의 엔테카비어 치료의 효용성

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본 연구에서는 이전에 라미부딘 및 아데포비어 내성을 보였던 환자를 엔테카비어로 전환하여 치료하여 이후 발생된 바이러스 반응, 엔테카비어 내성 및 라미부딘과 아데포비어 내성변종의 발현을 관찰하였다. 대상 환자는 예전에 라미부딘 내성을 보였던 아데포비어 내성 환자 중에서 6개월 이상 엔테카비어를 복용한 40명의 환자를 대상으로 하였으며 이들 환자에서의 바이러스 반응 및 엔테카비어 내성을 모니터링하였다. 전체 10% 환자에서 24주 동안의 엔테카비어 치료로 HBV DNA가 50 (copies/mL) 이하가 되었으며 40명의 환자 중 12명(30%)이 초기 바이러스 반응(initial virologic response, IVR)을 보였다. 치료전 높은 ALT수치(p=0.039) 및 rtL180M 변이(p=0.038)가 초기 바이러스 반응과 연관이 있었고 평균 11.4개월 추적 기간 동안 4명(10%)의 환자에서 바이러스 돌파 현상을 보였으며 엔테카비어 내성은 6명(15%)의 환자에서 관찰되었다. YMDD 및 아데포비어 내성 변이는 치료전 각각 57%, 35%를 보였고 48주 동안의 치료 후 전체 환자의 96% 와 4%에서 YMDD 및 아데포비어 내성변이를 보였다. 본 연구 결과 이전에 라미부딘 내성을 보였던 아데포비어 내성 환자에서의 엔테카비어 치료는 낮은 항바이러스 반응 및 엔테카비어 조기 내성을 유발하는 것으로 사료된다.

핵심어 : B형 간염, 엔테카비어, 라미부딘, 아데포비어, 약제 내성