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Changes of DNA Sequence in Polymerase Gene after Adefovir Treatment for Lamivudine Failure in Chronic Hepatitis B

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ABSTRACT

Background: Adefovir dipivoxil (ADV) is a new nucleotide analogue against HBV with low incidence of resistance mutation in naïve chronic hepatitis B (CHB) and has no cross resistance to lamivudine resistance mutation.

Objectives: To characterize genotypic mutation of HBV polymerase gene after 1 year of ADV treatment in lamivudine failure CHB and to correlate polymerase mutation with response to ADV treatment.

Methods: The serum HBV DNA was quantified by a real time PCR at entry of study, 3 month and 12 month after initiation of adefovir dipivoxil. Then DNA sequencing was performed to demonstrate mutation.

Results: The DNA sequencing confirmed YMDD mutation at entry. HBV DNA viral load level was dramatically reduced winhin the first 3 and at 12 month in almost of the patients. There were only 2 of 32 patients who initially decreased of HBV DNA viral load at 3 month but then gradually rising at the end of study.

Conclusion: Adefovir is effective in recurrent chronic hepatitis B patients with YMDD mutation. Most of the patient treated with lamivudine showed viral recurrent. (31 of 32 patients, 96.87%) However, only one patient showed a rebound of HBV DNA after 12 months of treatment by ADV.

Key words : adefovir, lamivudine, chronic hepatitis B, DNA sequence

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BACKGROUND

Infection with hepatitis B virus (HBV) represents a global public health problem, with a major impact in South-East Asia including Thailand. It has been proved to be responsible for chronic liver disease that causing burden to both the patient and public heath system. In Thailand, the Ministry of Public Health (MOPH) controls HBV infection as part of its seventh 5-year health development plan (1992-1996). This results to overall decreasing number of HBsAg carrier rate from 3.4 to 0.7% (among the 1-18 year-old.)[1] But the survey in

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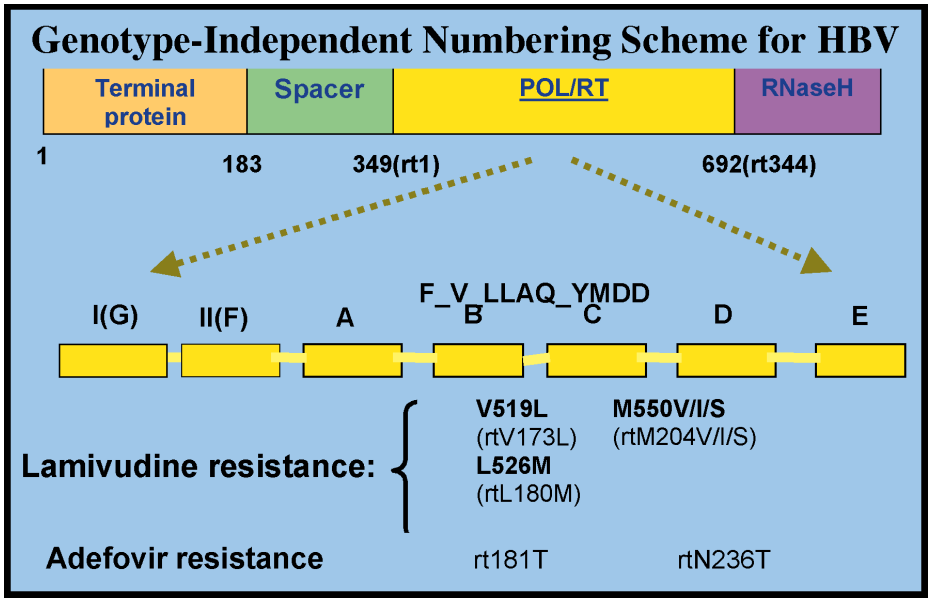
Thai prevalence of chronic HBV infection is still high at 3-6% and the age group with the highest number of carrier is 20-40 years. The estimated Thai HBV carrier in 2002 was 3 million people[2].

Currently, Interferonα is one of the standard treatments for chronic hepatitis B. Loss of HBsAg occurred in 11.4%[3] of treated patients. But its benefit is still limited due to variety of side effects and only selected group of patient will respond. One caveat is that Child's B and C cirrhotic patients who undergo treatment may develop fatal decompensation after hepatic flare. Moreover, in genotype C that more common in Thai 2); seems to be associated with the lower response rate than genotype A and B.[3]

Lamivudine, also known as 3TC, is a nucleoside analog group and the first oral antiviral drug, that used for treatment of chronic hepatitis B even in decompensated liver with little of side effect. Reports of the multicenter Asian study showed that HBeAg seroconversion rates increased with increasing the duration of treatment from 17% at 1 yr to 27%, 33%, 47%, and 50% at 2, 3, 4, and 5 yr respectively. The principal limitation of lamivudine therapy is the development of lamivudine resistance, that occurred in 14%, 38%, 53%, 67% and 69% at year 1, 2, 3, 4 and 5 respectively.[4]

Adefovir dipivoxil, a nucleotide analog; is the latest antiviral for the treatment of chronic hepatitis B. It has been approved for use in Thailand by Thai Food and Drug administration on 30 June 2004. The inhibition of DNA polymerase that cause suppression of viral replication is the main action. It can beused effectively in both positive and negative HBeAg patients including lamivudine resistance (YMDD mutation). At the end of 48 weeks treatment, the result demonstrated significantly reduction in HBV DNA viral load, normalization of serum alanine aminotransferase and improvement in liver histology[5-7]. The 10 mg daily administration (recommended dose) showed no significant changed in serum creatinine or phosphorus levels for 64 weeks when compared to placebo (1% vs <1%)[5,8]. Concerning drug resistance; there was no resistance mutation found at 60 week[9]. At 96 week of treatment in HBeAg negative group, two patients (1.7%) had mutation at the different site to YMDD mutation and associated with ALT flare. Moreover, all of the adefovir-resistant strains remained susceptible to lamivudine[5,6]

At the time of study, there is limited data regarding outcome of adefovir treatment in Thai. Almost all studies involved only naïve CHB patient. The present study was focusing in lamivudine failure CHB patients for many aspects including; the changes of HBV DNA level, viral mutation, response of treatment in many parameters (clinical, biological and virological data). (Figure 1)



Adapted from Stephen Locarnini, M.D., Ph.D. Seminars in Liver Disease. 24 (2004).

Figure 1

PATIENT AND METHOD

This is a prospective descriptive study conducted from October, 2004 until September, 2005. The study population was recruited from hepatitis clinic at Siriraj Hospital and using the following criteria; 1) male or female subjects ≥18 years of age. 2) either HBeAg positive or negative CHB who resist to lamivudine [at least 6 month and must be currently on lamivudine until Day 1 plus: a) 2 episode rising of serum ALT ≥2 × UNL at least 4 week apart. and b) breakthrough viremia ≥1 log10 of at least 4 week previous HBV DNA level]. The exclusion criteria were 1) previously received adefovir for treatment of CHB. 2) co-incidental with hepato and/or nephrotoxic drugs. 3) severe co-morbid disease. 4) pregnant or lactating female. 5) allergy to adefovir dipivoxil. The study protocol was approved by the Ethical committee, Faculty of Medicine, Siriraj Hospital, Mahidol University. Informed consents were obtained from all enrolled patients.

Data Collection

Comprehensive medical history and physical examination were performed at the beginning and throughout the study. Serum samples were collected at the beginning of the study before adefovir was administered. Then Adefovir was started at the dose of 10 mg daily overlaping with a few months of lamivudine. Subsequent serum samples were taken at 3 and 12 months after starting adefovir treatment. These samples were subjected to hematological and biochemical tests for complete blood count, serum ALT, blood urea nitrogen (BUN), serum creatinine and serum phosphorus. Virologic assay for HBV DNA viral load (Roche HBV Amplicor test, Roche Molecular Systems, Inc., Branchburg, NJ, USA) was performed. Nucleotide sequence within polymerase gene of HBV genome was determined by direct sequencing of amplified PCR product. Briefly, part of polymerase gene covering regions previously reported to confer resistances to lamivudine and adefovir was amplified by using sense YMDD3L (5'TTC CTC TTC ATC CTG CTG CT 3') and antisense YMDD6R (5'-AAC T(AGCT)C CAA T(AGCT)A CAT A(AGCT)C CC) primers. Primary PCR products were then subjected to second round of amplification using sense YMDD5L (5'TCG GAC GGA AA(CT) TGC AC(CT) TG) and antisense YMDD6R primers. PCR profile was consisted of 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 40 ˚C for 1 min and extension at 72 ˚C for 1 min, followed by final extension for 10 min. PCR products were detected by agarose gel electrophoresis and samples with positive results were further subjected to PCR product purification using QIAGEN PCR purification (kit cat NO. 28104 QIAGEN USA). Nucleotide sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit P/N 4336917 Applied Biosystems USA according to manufacturer's instruction. Nucleotide and deduced amino acid sequences were analyzed using ClustalX software.

Statistical Analysis

Demographic data (age, sex, duration of lamivudine, HBV DNA level) were reported as mean ± SD, median, range, frequencies. Wilcoxon signed rank test was used to compare HBV DNA viral load between the entry and the end of study serum.

Using SPSS version 10.0. (Chicago, IL,USA).

RESULTS

A total of 32 participants (25 male, 7 female) were enrolled with the mean age of 51 years old (range 25-71). Median duration of treatment before diagnosis of lamivudine failure is about 41 months. The demographic data of the study populations at entry is shown in Table 1.

Overall, serum transaminase (AST and ALT) was gradually decreased during the follow up period. HBV DNA viral load was rapidly decreased from the median of 29,050,000 copies/ml to 71,200 and 3,282 copies/ml at 3 months and 12 months, respectively (as shown in Figure 2). Obviously, HBV DNA viral load

Table 1 Baseline parameter of studied participants

Parameters	Mean (min-max)
Sex M : F	25 : 7
Age (yr)	51 (25-71)
Duration of lamivudine treatment before entry (month)	41.23 (11-71)
Serum AST (U/L)	107.28 (24-774)
Serum ALT (U/L)	127.37 (22-774)
HBeAg+ : anti HBe+	24 : 8
HBV DNA viral load (copies/ml)	81×106 ($4530 - >200 \times 10^6$)

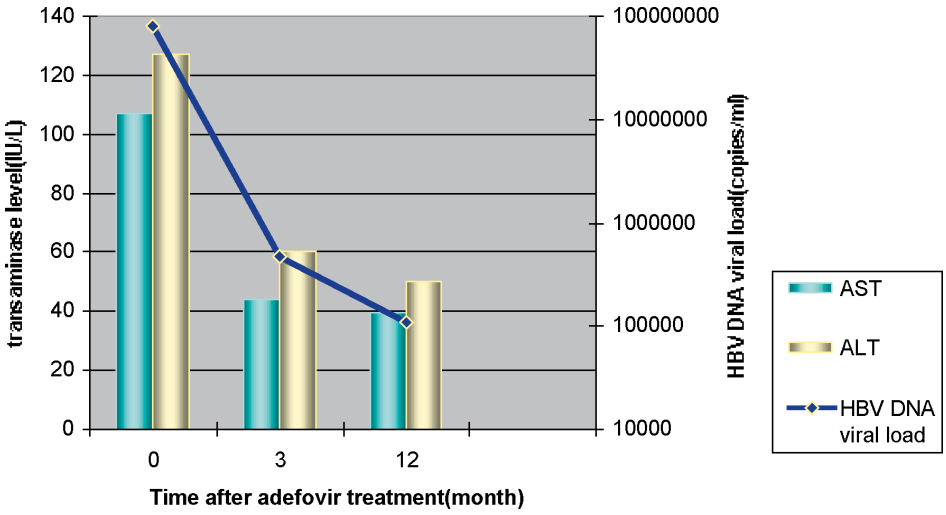


Figure 2 Reduction in serum transminase and HBV DNA level.

decreased when present in log, from entry to the third month and to the end of study (2.41 log and 3.19 log respectively.) These reflect an appropriate response in adefovir rescue therapy for lamivudine resistance chronic hepatitis B patients.

Only one patient; 51 years old man with HBeAg positive initially had a failed response. He was first treated with Interferon α2b 5 mu three times a week for 24 weeks with subsequent HBe seroconversion. However at 6 month post interferon treatment, AST and ALT still elevated with a high HBV DNA viral load. He was then diagnosed as chronic hepatitis B; HBeAg negative and treated with lamivudine continuously for four and a half years before he faced an evidence of flare with elevation of ALT and HBV DNA level. His HBV DNA level before initiation of adefovir was 857,000 copies/ml (5.93 log) at 3 month and increased to 14,044,420 copies/m l(7.157 log) at 12 months. His HBV DNA sequencing is not demonstrated in this study.

The result of DNA sequencing was demonstrated in Figure 2. This information was complete in 10 patients. All are at entry specimens. The mutation was noted in position 526. (patient no. 6, 7, 8, 9 and 10 developed this mutation) and patient no. 6, 7, 8 and 9 had "M" mutation. While patient no. 10 had "Q" mutation. As "M" is known to associate with lamivudine resistance that compatible with L526M. (rtL180M). Another site of mutation was observed in position 550. Patient no. 3, 4, 5, 6, 7, 8, 9 and 10 had mutations at this point. This confers to M550V/I/S (rtM204V/I/S); another known point mutation of lamivudine resistance

Discussion

It has been demonstrated that adefovir has demonstrated a remarkable efficacy in the therapy and prophylaxis of a number of retrovirus infections in animal models and also proven to be more effective than lamivudine in suppressing viremia and intrahepatic viral DNA in ducks (experimental infection by hepatitis B virus). Although adefovir could induced a substantial reduction of viremia, but it was unable to prevent the initial formation of covalently closed circular DNA (cccDNA). (Neither was lamivudine).[10]

Many clinical studies of adefovir in CHB treatment showed growing evidence in the effectiveness of the drug (mainly conducted in naïve CHB)[11,12].

Viral resistance to nucleoside analogs poses an increasing major problem in the management of chronic hepatitis B overtime. Again, even many studies showed lower incidence of adefovir resistance when compared with lamivudine[9,13,14] and the site of mutation did not cross react to lamivudine resistance[15]. In lamivudine failure CHB, there are very scant data of adefovir treatment outcome in this group. Although, theoretically, adefovir is effective for treatment in this group but the incidence of resistance in this population is not well recognized.

To answer the question regarding adefovir treatment in patient who failed lamivudine has any changes in DNA sequencing. Our study was conducted by determination of DNA sequencing that may confer to mutation at the entry of study and serially followed up at 3 and 12 month after initiation of adefovir. Unfortu-

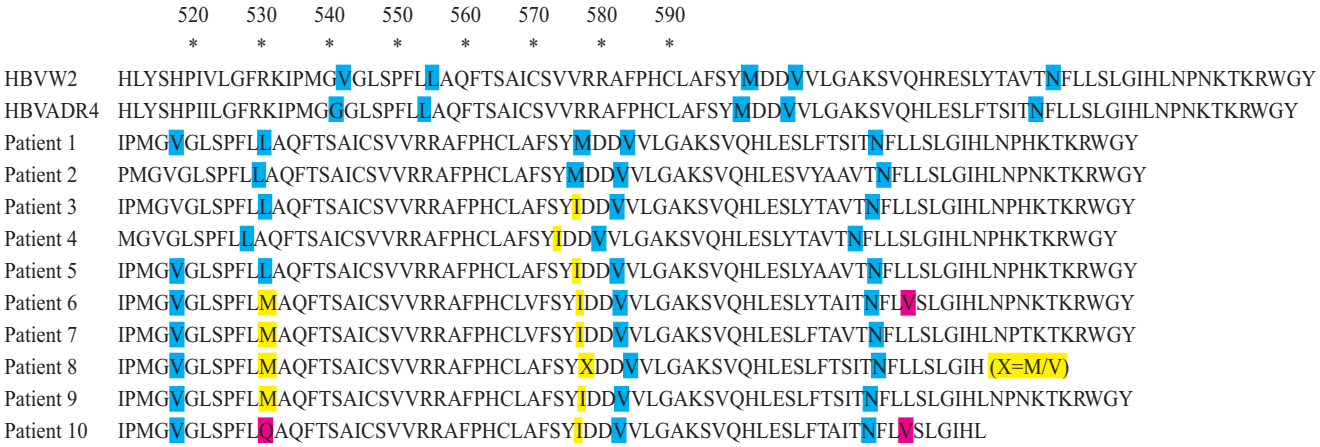


Figure 2 Alignment of amino acid sequence.
Blue highlights = positions # 519, 526, 550, 553, 572
First two lines = HBV from database as reference viruses
Yellow highlights = changes of amino acid at the site of known mutation
Pink highlights = change of amino acid that unknown/unreported mutation

nately, due to technically limitation that may contribute from low sensitivity of the assay, mismatched primers and the error in condition of sample collection and storage. Therefore, we successfully sequenced in only 10 specimens; all were at entry serums. Almost of the sequences were compatible with known lamivudine resistance; L526M in patient no. 6-9 and M550V/I/S in patient no. 3-10. The two remaining patient were no. 1 and 2 that the DNA sequence did not show any lamivudine resistance mutation at entry. Patient No. 1 had clinically and biological response to adefovir. In addition, this patient had clinical and biological response too. In virological response, it seemed to response well in the first 3 month but tended to increase in viremia at the end of the study.

There is only one patient with declining of viremia in the third month of treatment but subsequemtly rebounded at the end of the study. This may confer to new mutations but we could not demonstrate their DNA sequencing.

In vitro study,[15] the combination of rtL180M + M204V and rtN236T mutations impaired HBV replication and confered resistance to both 3TC and ADV. This suggested that the emergence of the triple mutant might be delay and associated with viral resistance in patients treated with 3TC + ADV.

From the recent study,[16] which characterize the genotypic and phenotypic mutation profiles to adefovir in lamivudine resistant CHB patients. It demonstrated 11 amino acid substitutions developed in the reverse transcriptase (rt) domain of the HBV polymerase in 9 out of 67 patients. The rtA181V, rtN236T and rtA181T mutations were detected in 5, 4 and 2 of the patients at treatment months 12~17, 3~19 and 7~20, respectively. This result supported that the emergence of the ADV mutation in LMV resistant patients who are treated with ADV appeared to present earlier and more frequent than previous reports.[17] Scott K. *et al.* reported a similar result. They monitored for virologic response and adefovir resistance and were able to show cumulative probability of adefovir resistance at 2 years to be as high as 22% (79% of studied population took lamivudine prior to enrolment).

CONCLUSION

The emergence of HBV strains which resist to antiviral treatments is still going on and becomes a major clinical concern. This study demonstrates that adefovir is effective for treatment of recurrent chronic hepatitis B patients with YMDD mutation. Only one patient showed rebound of HBV DNA after 12 months of treatment. But the data is limited and unable to demonstrate the precise mutation point. The present study demonstrated an increasing evidence of earlier resistance to adefovir in prior lamivudine treatment patients compared to naïve CHB. This alerts us to consider a combination or sequential therapy that may prevent, reduce or delay emergence of new drugs resistance. More studies are needed to confirm this postulation.

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