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Clinical Impact of Hepatitis B Virus Polymerase Resistance Mutation Testing on the Treatment Decision of Chronic Hepatitis B Patients Who were Refractory to Oral Nucleos(t)ide Analogues

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ABSTRACT

Background: Treatment with oral nucleos(t)ide analogues (NA) are associated with improvement of liver histology and accepted as current standard treatment of patients with chronic hepatitis B (CHB). The emergence of hepatitis B viral (HBV) resistance to oral NA is common and lead to the worsening of liver disease. Detection of HBV mutation may be helpful in clinical management. We aimed to evaluate mutation patterns of HBV polymerase in patients with CHB treating with NA.

Patients and Methods: Patients with CHB receiving one or more of oral NA were enrolled from the Gastroenterology and Hepatitis Clinic, Siriraj Hospital, Thailand during September 2007 to January 2009. Virologic breakthrough is defined by an increase of HBV DNA at least 1 log10 copies/mL (or > 1 log10 IU/mL) from nadir level during treatment. Blood sample was stored at -80˚C until tested. HBV polymerase resistance profile was preformed by line probe assay (INNO-LiPA HBV DR v2).

Results: Twenty-seven patients were enrolled with 21 men and 6 women. Their mean age was 48 ± 11.6 years. Mean initial HBV viral load before treatment was 1×10^7 IU/ml with mean initial AST and ALT of 125 and 165 IU/mL, respectively. Twenty-four patients were initially treated with lamivudine (LMV) and three patients were treated with adefovir dipivoxil (ADV). The mutation patterns of HBV polymerase were refractory to LMV alone (n = 15) and refractory to LMV and other NA (n = 12). Mean HBV viral load at the time of virologic breakthrough was 2.8×10^7 IU/mL and biochemical breakthrough was found in 8 patients (29.6%). The most frequent resistant HBV mutation were M204V/I (88.9%), M180/A181 (77.7%), and L80I 9 (33.3%).

Conclusion: In CHB patients with LMV resistance alone, HBV polymerase mutations were predictable detected in 88% and at least 12% of them had primary ADV resistant mutation. Thus, routine adding of ADV in these patients may result in treatment failure. The usefulness of line probe assay to identify mutation in the patients who have failed to multiple NA is helpful but its sensitivity to detect some mutations is still limited.

Key words : HBV polymerase resistance mutation, chronic hepatitis B, oral nucleos(t)ide analogues

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INTRODUCTION

Chronic hepatitis B (CHB) is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma, especially in Asian population.[1] Treatment of this condition will improve liver morbidity and mortality in these patients.[2] From the current guideline , standard treatment that approved for treating these patients are immunomodulator such as pegylated interferon or oral nucleos(t)ide analogues (NA).[3] Oral NA treatment are associated with improvement of liver histology by decrease HBV DNA level. The problem of prolong use of oral NA is emerging of various drug resistant strains that can be caused flaring of hepatitis and treatment failure in the future. When drug resistance was developed adding on non cross resistance drug is recommended. Despite following this recommendation, the previous study showed that when lamivudine (LMV) resistance was developed, it can increase risk of ADV resistance from 0% at 1 year after treatment in naïve patients to 6.8%.[4,5]

The resistance testing was developed to detected mutation point that located in HBV polymerase gene. Line probe assay is simple method that can be detected known mutation of resistance strain. INNO LiPA Dr version 2 are commercial kit of line probe assays which detected mutation point at 80, 173, 180, 181, 204 and 236 which are LMV and ADV resistance mutation. In former literature it was used for detecting the specific mutation point in the clinical study.[6-8] No study of mutation pattern was reported to be helpful for planning treatment in drug resistance CHB patients. We aimed to determine the pattern of HBV polymerase mutation in CHB patients treated with NA and evaluate the outcome of HBV treatment after developing drug resistance.

PATIENTS AND METHODS

Patients

Twenty-seven adult patients with CHB who were followed by Gastroenterology and Hepatitis Clinic Siriraj Hospital, Bangkok, Thailand during September 2007 to January 2009 were identified. All patient received treatment with oral NA for more than 6 months before enrollment. Patients with presence of more than 1 $\log_{10}$ (IU/mL) of viral breakthrough and/or ALT elevation of more than 2 times of baseline and had HBV DNA more than 10^5 IU/mL were included. We excluded patient who received combination of antiviral therapy and immunomodulator and patients who have co-infection with chronic hepatitis C or HIV. Blood sample was taken and stored at -80˚C for HBV drug resistance line probe assay and HBV DNA PCR.

The patients received rescue treatment regimen depending on physician decision. After rescue regimen was started, HBV DNA will be followed every 6 months. The study was approved by the Research Ethics Board of Faculty of Medicine, Siriraj Hospital, Mahidol University.

HBV drug resistance line probe assay (INNO-LiPA HBV DR V2)

HBV DNA was isolated from 200 μL of serum using High Pure Viral Nucleic Acid Kit (Roche). The INNO-LiPA HBV DR v2 assay (Innogenetics) was performed according to the manufacturer's instructions using Hot Start Taq DNA polymerase (Fermentans, Lithuania). The assay is based on amplification of a part of the viral polymerase gene by the provided primers and reverse hybridization by the probes coated on a strip. It detects HBV polymerase wild-type and known drug-induced mutations associated with LMV and ADV resistance (codons 80, 173, 180, 181, 204 and 236).

Measurement of HBV DNA in serum by real-time PCR

The serum viral load of HBV DNA was determined by COBAS® Taq Man HBV test kit (Roche Diagnostics, USA). The assay covers a linear range from 6 IU/mL to at least 1.1×10^8 IU/mL. Results were presented as IU/mL. Viral loads below 6 IU/mL are reported as less than 6 IU/mL. Biochemical breakthrough is defined by the presence of elevation of ALT for more than 2 times of baseline ALT. Virologic breakthrough is defined by the increase of at least 1 $\log_{10}$ (IU/mL) of viral breakthrough during treatment.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Demographic data were analyzed using descriptive statistical tests. Mann-Whitney *U*-test, Chi-squared test and Fisher's exact test were used for comparison of groups. $P < 0.05$ was accepted as statistically significant. SPSS 10.0 for Windows was used for statistical analysis.

RESULTS

Twenty-seven patients were enrolled with 21 men and 6 women. Their mean age was 48 ± 11.6 years. Mean initial HBV viral load before treatment was 1×10^7 IU/ml with mean initial AST and ALT of 125 and 165 IU/mL, respectively. Twenty-four patients were initially treated with LMV and 3 patients were treated with ADV. The other demographic data and clinical features are summarized in Table 1. Fifteen of 27 patients had history of resistance to treatment with LMV alone and 12 patients resisted to LMV and other NA. Most patients in LMV and other NA resistant group (10 in 12) had history of ADV resistance. No significant difference of demographic data and baseline HBV DNA between LMV resistance alone group and LMV as well as other NA resistant group was found. HBV drug resistance was detected in 19 patients (70.4%) by virologic breakthrough and 8 patients by biochemical breakthrough. One patient with HBV drug resistance had both elevation of ALT and severe clinical flare of hepatitis. Patients in LMV and other NA resistant group had significantly longer duration of treatment before resistance occurred than those in LMV resistance alone (54 ± 26 vs 23 ± 11 months, $p = 0.002$). INNO-LiPA DR V2 assay was successful in all patients. Of 27, three patients (11.1%) had only wild type strain while 24 showed mutation type strain. No significant difference in mutation detection rate between LMV resistance alone group and LMV and other NA resistant group ($p = 0.41$). The patients without resistance mutation had lower number of HBV DNA than those with resistance mutation ($3.0 \pm 3.6 \times 10^7$ IU/mL vs $3.4 \pm 2.9 \times 10^5$, $p < 0.001$). The pattern of resistance mutation was divided into subgroup according to previous drug resistance reported as shown in Table 2. The most common point mutations detected were M204V/I (88%) and M180/A181 (77%). We did not demonstrate the mutation at M180/T181 and M204S. No significant difference in detection of point mutation at 80, 173, 204 and 236 between 2 groups but the

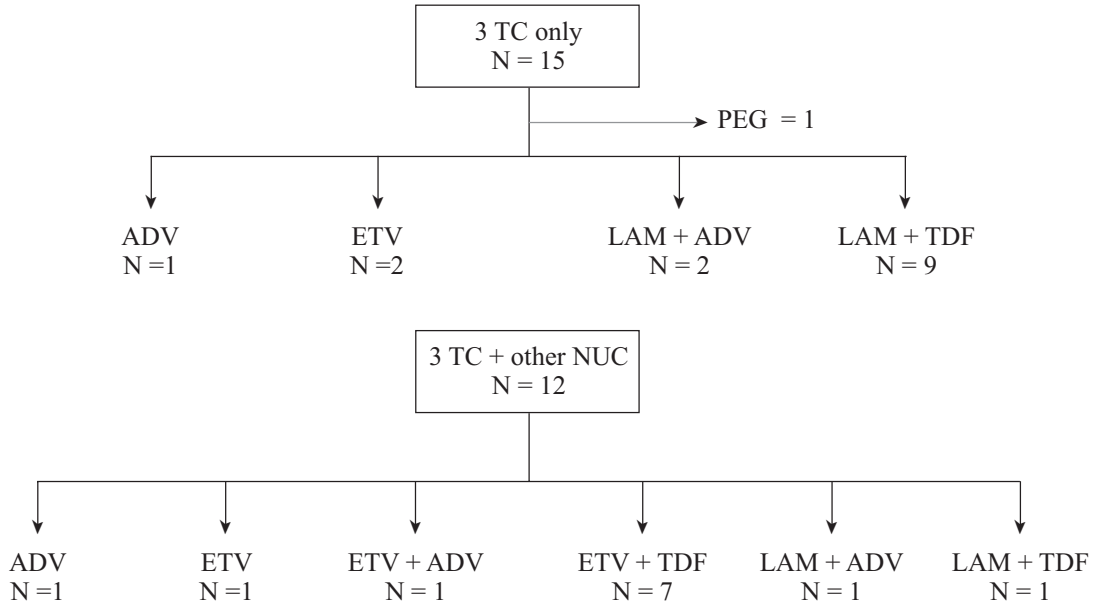
Table 1. Demographic data and clinical features of the study group.

Characteristics	All (N = 27)	LMV resistance alone (N = 15)	LMV + other NA (N = 12)	*p*-value
Age [year: mean (range)]	48 (29-69)	52 (29-63)	44 (30-69)	0.093
Sex (M/F)	21/6	10/5	11/4	0.66
Cirrhosis (%)	13 (48%)	6 (40%)	7 (58%)	1.0
HBeAg (positive/negative)	18/9	11/4	7/5	0.44
Detection of treatment failure*	19/8	12/3	7/5	0.39
Duration before resistance test (months)	37 ± 24	23 ± 11	54 ± 26	0.002
Viral load (IU/mL)	5.1×10^7	4.8×10^7	5.4×10^7	0.87

*treatment failure means virologic breakthrough or biochemical breakthrough.

Table 2. Mutation pattern of 27 CHB patients.

Mutation point	All (N = 27)	LMV resistance alone (N = 15)	LMV + other NA (N = 12)	*p*-value
L80V/I	15 (48%)	7 (46%)	6 (50%)	0.86
L173	3 (11%)	2 (13%)	1 (8%)	0.68
L180/T181	3 (11%)	0	3 (25%)	0.04
L180/V181	1 (3%)	0	1 (8%)	0.25
M180/V181	2 (7%)	1 (6%)	1 (8%)	0.87
M180/A181	21 (77%)	13 (86%)	8 (66%)	0.21
M204V/I	25 (88%)	14 (93%)	9 (75%)	0.18
T236	3 (11%)	1 (6%)	2 (16%)	0.41



3TC, LMV = Lamivudine, ADV = Adefovir dipivoxil, ETV = Entecavir, TDF = Tenofovir, PEG = pegalated interferon

Figure 1. Diagram of rescue treatment regimen.

mutation point at L180/T181 which is LMV and ADV cross resistance mutation was significantly lower in LMV resistance alone group than those in LMV and other NA failure group (0% vs 25%, $p = 0.04$).

Two patients with LMV resistance alone (12% of 15 patients) had primary ADV resistance without history of ADV exposure. In the group of LMV and other NA failure, ADV resistance was detected in 5 of 10 patients.

In patients with LMV resistance alone, the rescue regimen was prescribed as showed in the diagram 1 including ADV monotherapy (1 patient), entecavir (ETV) monotherapy (2 patients), combination of LMV and ADV (2 patients) and combination of LMV and tenofovir (TDF) (9 patients). In LMV and other NA resistance group, the rescue regimen were ADV monotherapy (1 patient), ETV monotherapy (1 patient), combination of LMV and TDF (1 patient), combination of ETV and TDF (7 patients), combination of ETV and ADV (1 patient), and combination of telbivudine and TDF (1 patient).

Of 27 patients, five received ADV or ETV monotherapy and 22 patients received combination regimen. No significant difference in demographic data, clinical features, and initial viral load before receiving rescue regimen were found. Twenty-four patients were followed up for longer than 6 months and their HBV DNA after 6 month of treatment were divided into 2 groups; 19 patients with treatment of combination drugs and 5 patients with monotherapy. The successful viral suppression of less than 2,000 IU/mL was higher in combination drugs (73%) compared to those of 40% in patients with monotherapy without statistical significance ($p = 0.8$)

Fifteen patients were followed up for longer than 1 year and found that the patients who received combination drugs had significantly higher number of undetectable rate of HBV DNA at 1 years (84.6%) than those received monotherapy group (0%) ($p = 0.001$).

After treatment, 9 from thirteen patients (69.2%) in LMV resistance alone group had HBV DNA of less than 2,000 IU/ml at 6 month compared to 8 from 11 patients (72.7%) in LMV and other NA failure group (p = NS).

No difference was found in demographic data, duration of treatment before rescue treatment, viral load at baseline and success rate of viral suppression at 6 months and 1 year between patients with virologic breakthrough and those with biochemical breakthrough.

DISCUSSION

Drug resistance strains mutations are an important factor for the failure of CHB treatment. Emergence of drug-resistant strains is closely related to the

duration of the treatment. LMV resistance occurs at a cumulative rate of 14-20% per year in the previous studies.[9-11] Our data showed that the mean (SD) duration of LMV resistance was 23 (11) months. We also found that the virologic breakthrough was the most common findings for detecting CHB drug resistance (70%) followed by biochemical breakthrough of 30%. However, this information may not be used generally because the measurement of HBV DNA is still limited in many countries.

Genotypic resistance is method which detected the point of mutation in HBV DNA polymerase relies on either DNA sequencing or hybridization. Direct DNA sequencing is typically not able to detect emerging drug resistance because it cannot detect minority populations of mutants. Hybridization-based genotyping methods can detected viral mutants that constitute as little as 5% of the total population.[12] The commercially available INNO-LiPA line probe assay, which relies on differential hybridization of targets to a series of short membrane-bound oligonucleotide probes to discriminate between wild-type sequences and those of known drug-resistant mutants. INNO-LiPA assays can detect developing viral resistance when the mutants responsible constitute only a minor fraction of the total viral population. In previous study, using of INNO-LiPA was well established to early detect resistance mutation in patients during treatment with NA and help for planning the regimen of treatment.[13] In patient who clinical resistance already developed, the data of using of INNO-LiPA method to plan for regimen treatment has not been well studied. Our study showed that INNO-LiPA was successfully performed in 100% of detection of resistance strain of HBV ($>10^5$ IU/mL).

From prior study, mutations that result in replacement of methionine in the tyrosine-methionine-aspartate (YMDD) catalytic site motif by valine, leucine or (rarely) serine at the location of 204th amino acid of polymerase gene are necessary and sufficient to confer resistance to LMV. In vitro studies showed that these mutations decrease sensitivity to LMV by > 100-fold.[14,15] Our study confirmed that fact by detecting YMMD mutation in 89% of patients with history of LMV resistance. The rtL180M was the main compensatory change in our study and showed high coincidence of YMDD and rtL180M in 84% of YMDD mutation. The rtA181T change has been reported to occur in the absence of rtM204I/V and is considered a primary resistance mutation.[16,17] The rtA181T change was also selected during ADV treatment.[18] In present study, we did not find the isolated rt A181T mutation in patient who refractory to LMV alone but we found significantly higher prevalence of this point mutation in patient who refractory to multiple agents and most of them had history of ADV exposure.

The primary ADV-resistance mutations are rtN236T and /or rtA181T/V.[19] These ADV-associated mutations result in only a modest (2-9 fold) increasing in EC_{50} but viral rebound, hepatitis flares and hepatic decompensation had been observed in patients. ADV-associated mutations are partially cross-resistant with TDF but it can be overcome by using high dose of TDF (300 mg). In our study, we found 12% of patients developed of primary ADV-resistance mutations in patients with history of LMV treatment alone. Theses patients may develop treatment failure when receiving the combination regimen of ADV and other NA as rescue regimen. Adding on TDF for these patients should be the best choice. In multiple drugs exposure, we can detect only 50% of primary ADV-resistance mutations in patients that had history of ADV exposure. This finding may be explained by the presence of unknown primary ADV resistance which is not included in the line probe assay or the ADV resistance viral population is too small to detect. According to this finding, identifying of ADV resistance mutation by using this version of INNO-LiPA is insensitive and should not be use for monitoring during ADV treatment to early detect resistance mutation. This version of INNO-LiPA is not included for the primary resistance of ETV and TDF, thus we can not use this mutation pattern to plan for treatment in patients with multiple drugs exposure. Further study is required with using new version of hybridization-based genotyping methods.

Our study has some limitations. First, the variation of patient characteristics; however, we analyzed to compare all characteristics of patients. Second, we selected patients with viral load of more than 10^5 IU/ml for INNO-LiPA test, therefore the success rate of amplification and identifying of mutation my be less than our results when use in patients with the smaller amount of DNA. Last, the number of patients is small thus further study with larger population as well as longer duration of follow up will be need.

In summary, CHB patients with LMV resistance alone, HBV polymerase mutations were predictable

detected in 88% and at least 12% of them had primary ADV resistant mutation. Thus, routine adding of ADV in these patients may result in treatment failure. The usefulness of line probe assay to identify mutation in the patients who have failed to multiple NA is helpful but its sensitivity to detect some mutations is still limited.

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