Detection of rtN236T mutation associated with adefovir dipivoxil resistance in Hepatitis B infected patients with YMDD mutations in Tehran

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ABSTRACT

Background Objectives: The risk of adefovir dipivoxil resistance emergence has increased in lamivudine-resistant hepatitis B infected patients. The mutations known as causing adefovir resistance, rtN236T and rtA181V/T, are detected within the D and B functional domain of the HBV polymerase, respectively. In this study, we intended to determine the pre-existing adefovir-resistance mutations in patients infected with LAM resistant mutants prior to starting adefovir therapy.

Material and Methods: The study included 30 patients with chronic hepatitis B with lamivudine resistance mutations in the YMDD motif that experienced viral breakthrough.

Results: After alignment of protein coding sequences, the rtN236T mutation was observed in two (6.6 %) patients, while twenty-eight others had neither rtN236T, nor rtA181V/T mutation. All 30 patients were infected with genotype D of hepatitis B virus.

Conclusions: The early detection of LAM-resistance mutations may allow a timely chance of therapy to avoid hepatitis flare-up. This data suggests that monitoring of ADV-resistance mutations in ADV naïve patients can be considered in selecting the appropriate anti-viral regimen.

Keywords: Hepatitis B virus, adefovir dipivoxil, Lamivudine, resistance, mutation

INTRODUCTION

Chronic Hepatitis B (CHB) remains a major health problem world-wide and can lead to hepatocellular carcinoma and cirrhosis (1, 2). Several therapeutic agents including pegylated interferon and nucleotide/nucleoside analogues such as lamivudine, adefovir, telbivudin, entecavir and tenofovir (3, 4) are available.

Lamivudine has been widely used as the first-line therapy in patients with chronic hepatitis B with well-documented efficiency and safety profile (5, 6). Long term usage of lamivudine leads to development of drug resistance which is mainly associated with mutations in the YMDD motifs, substitution of methionine by either valine (rtM204I) or isoleucine (rtM204I) in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase C domain (7-9). The rtL180M mutation may occur concurrently with YMDD mutations and serves as a compensation for better replication fitness (10). The risk of emerging LAM resistance is 14-32% in the first year and rises up to 70% by the fifth year (6).

Adefovir dipivoxil (ADV) is a nucleotide analogue that interferes with the reverse transcriptase activity of hepatitis B virus (HBV) polymerase upon viral replication. ADV has become an alternative treatment for HBV infection regarding the high rate of lamivudine resistance during prolonged viral therapy.
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(11). However, viral resistance to ADV has been shown after either switching to or adding adefovir to previous regimen (ADV add-on therapy) in patients with LAM resistance (12,13). The risk of developing adefovir resistance was 2% in the first year and 25-30% by the second and fourth years of mono-therapy in treatment-naïve cases respectively (12-14). The risk of ADV resistant emergence increased to 20% in LAM-resistant patients with switching to ADV mono-therapy within 1.5 years of ADV treatment (3). The mutations known as causing adefovir resistance, rtN236T and rtA181V/T (15, 16), are detected within the D and B functional domain of the HBV polymerase, respectively (17).

The aim of the present study was to determine the prevalence of the pre-existed ADV-resistance mutations in patients infected with LAM resistant mutants of HBV prior to starting ADV therapy.

MATERIALS AND METHODS

Thirty chronic hepatitis B patients with the documented presence of genotypic resistance mutation to lamivudine were identified between October 2010 to December 2011. After at least 6 months lamivudine administration; the emergence of YMDD mutations was identified in those patients using restriction fragment length polymorphism (RFLP) methods (18).

Biochemical tests and viral markers. Laboratory tests were performed at baseline and on a monthly basis during treatment. Serum ALT levels, HBeAg, anti-Hbe and HBsAg were determined using ELISA kits (Dia.Pro. Diagnostics, Italy) according to manufacturer’s instructions. HBV DNA level in serum was measured using COBAS Amplicor (Roche Diagnostic, USA), this assay has a detection range between $2 \times 10^2-10^9$ copies/ml.

HBV DNA was extracted from 140 μl of serum using the QIAamp DNA mini kit (Qiagen Ltd. Germany) according to the manufacturer’s instructions. Extracted DNA eluted in 100 μl of elution buffer and stored at -20°C until use.

Direct sequencing. To amplify the HBV polymerase gene by the nested PCR, we employed the primers and PCR program of Osiowy et al. (19). Briefly, the primers: spr1F (5’-GTTCTACGACAGTACGCCC-3’) and the reverse primer spr1R (5’-GAAAGGGCTTGAAGTTGGCG-3’) were used in the first round PCR. The inner primers: sense, spr2F (5’-GGTTGACCTCTCTCAATTGTCTAGG-3) and antisense spr2R (5’-ACTTTCAAATCAATAGGCC-3) were used for nested PCR. The following PCR thermal-cycling program was performed: 35 cycles consisting of 94°C for 30s , 56°C for 30s (first round) or 50°C for 30s (second round), and 72°C for 40s. The intended fragment were amplified using 2X PCR master mix solution (i-tag, iNtRON Biotechnology, Inc. Korea) with 5 μl of DNA extract and 2 μl of the first round PCR product. After the amplification of polymerase gene, the amplicons (730 bp) were visible after agarose gel electrophoresis and gel purified using High Pure PCR Product purification kit (Roche Diagnostic Gmbh). The purified PCR products were bi-directionally sequenced commercially (SEQLAB, Germany) using inner primers.

To perform the phylogenetic analysis, obtained sequence data were used to identify the genotype of each sample, using the NCBI genotyping tool (20). HBV polymerase encoded protein sequences were translated from each sample nucleotide sequences using Bioedit software (The BioEdit Sequence Alignment Editor software, Department of the Microbiology, North California State University) and confirmed by visual inspection. Sequences were then aligned with HBV polymerase coding sequences from the Gene Bank data base using CLUSTAL W program (http://www.ebi.ac.uk/Tools/msa/clustalw2).

RESULTS

Baseline characteristics. Baseline characteristics of enrolled patients prior to starting ADV therapy are listed in Table 1. All 30 patients were already infected with genotype D of hepatitis B virus. All of the patients showed viral breakthrough after at least 6 months of LAM therapy, the mean serum HBV-DNA level was $1 \times 10^6.2$ copy/ml. HBeAg was positive in 28 (93%) of cases. The median ALT level was 177 IU/L (Table 1). LAM resistance analysis using RFLP method identified the type of YMDD mutations; 24(80%) patients had the rtM204I mutation, 2 (6.6%) had the rtM204V and 4(13.3 %) had both.

Detection of ADV resistance mutations by direct sequencing. After alignment of protein coding sequences, the rtN236T mutation was observed in
The results of alignment of protein coding sequences indicated the rtN236T mutation was observed in two (6.6%) patients, while 28 others had neither rtN236T, nor rtA181V/T mutation. 

There have been a number of studies to support the correlation between clinical response to antiviral agents and the HBV genotype, and yet there are still controversial reports (21, 22). It appears that genotype A develop LAM resistance more frequently or quite rapidly in comparison with genotype D (24). However, several reports have shown no association between genotype and the emergence of LAM resistance (25, 26).

In 2005, a report indicated an association between existence of HBV genotype D and an increased risk of ADV resistance (27). In present study since all patients were infected with HBV genotype D, the incidence of ADV resistance does not reflect true association between genotype D and the emergence of ADV resistance rate. Thus, a larger study with inclusion of other HBV genotypes is necessary to deeply understand the relationship.

Recent studies suggest new alternative strategies to conquer the rapid and frequent development of drug resistant mutant viruses. Newly approved antiviral agents which are able to suppress viral replication robustly, will probably decrease the likelihood of selecting resistance (12, 13).

Pre-existing ADV-resistance mutations such as rtN236T, prior to starting ADV mono-therapy or ADV add-on treatment may adversely affect its antiviral efficacy. Moreover the early detection of LAM-resistance mutations may allow a timely chance of therapy to avoid hepatitis flares. This study suggests that monitoring of ADV-resistance mutations in ADV naïve patients can be of a great importance.
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REFERENCES

1. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol 2008; 48: 335-352.
2. Ghany MG, Doo E. Assessment and management of chronic hepatitis B. Infect Dis Clin North Am 2006; 20:63-79.
3. Keeffe EB, Marcellin P. New and emerging treatment of chronic hepatitis B. Clin Gastroenterol Hepatol 2007; 5: 285-294.
4. Marcellin P, Chang TT, Lim SG, Sievert W, Tong M, Arterburn S, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. Hepatology 2008; 48: 750-758.
5. Dienstag J, Schiff E, Wright T, Perrillo RP, Hamm HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999; 341: 1256-1263.
6. Lai C, Chien R, Leung N, Chang TT, Guan R, Tai DI, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61-68.
7. Allen M, Deslauriers M, Andrews C, Tipples GA, Walters KA, Tyrrell DL, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Hepatology 1998; 27: 1670-167.
8. Delaney W IV, Yang H, Westland C, Das K, Arnold E, Gibbs CS, et al. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol 2003; 77: 11833-11841.
9. Ogata N, Fuji K, Takigawa S, Nomoto M, Ichida T, Asakura H. Novel patterns of amino acid mutations in the hepatitis B virus polymerase in association with resistance to lamivudine therapy in Japanese patients with chronic hepatitis B. J Med Virol 1999; 59: 270-276.
10. Bartholomeusz A, Locarnini SA. Antiviral drug resistance: clinical consequences and molecular aspects. Semin Liver Dis 2006; 26: 162-170.
11. Locarnini, S. 2005. Molecular virology and the development of resistant mutants: implications for therapy. Semin Liver Dis 25(Suppl. 1): 9-19.
12. Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, Ayres A, Bartholomeusz A, Locarnini S. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003; 125: 292-297.
13. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Marcellin P, Lim SG, Borroto-Esoda K, Arterburn S, Chuck SL. Adefovir Dipivoxil 438 Study Group: Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. Gastroenterology 2006; 131: 1743-51.
14. Delaney WE. Progress in the treatment of chronic hepatitis B: long-term experience with adefovir dipivoxil. J Antimicrob Chemother 2007; 59: 827-832.
15. Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology; 125: 292-297.
16. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic Hepatitis B. N. Gastroenterology 2006; 131(6).
17. Bartholomeusz A, Thai NG, Chalmers DK. Comparisons of the HBV and HIV polymerase, and antiviral resistance mutations. Antivir Ther 2004; 9: 149-160.
18. Yang DH, Liang WF, Xie YJ, Zhao NF, Fan J. PCR restriction fragment length polymorphism in detection of YMDD variants of viral polymerase in hepatitis B virus patients treated with lamivudine. Hepatobiliary Pancreat Dis Int 2002; 1: 232-237.
19. Osiowy C, Villeneuve JP, Heathcote EJ, Giles E, Borlang J. Detection of rtN236T and rtA181V/T mutations associated with resistance to adefovir dipivoxil in samples from patients with chronic hepatitis B virus infection by the INNO-LiPA HBV DR line probe assay. J Clin Microbiol 2006; 44: 1994-1997.
20. Rozanov M, Plikat U, Chappey C, Kochergin A, Tatusova T. A web-based genotyping resource for viral sequences. Nucleic Acids Res 2004; 1: W654-W659.
21. Guettouche T, Hnatyszyn HJ. Chronic hepatitis B and viral genotype: the clinical significance of determining HBV genotypes. Antivir Ther 2005; 10: 593-604.
22. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepatitis 2005; 12: 456-464.
23. Wai CT, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg (+) chronic hepatitis than genotype C. Hepatology 2002; 36: 1425-1430.
24. Zöllner B, Petersen J, Puchhammer-Stöckl E, Kletzmayr J, Sterneck M, Fischer L, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology 39: 42-50.
25. Sun J, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, et al. Clinical and virological characteristics of lamivudine resistance in chronic hepatitis B patients: a single center experience. J Med Virol 2005; 75: 391-398.
26. Moskovitz DN, Osiowy C, Giles E, Tomlinson G, Heathcote EJ. Response to long-term lamivudine treatment (up to 5 years) in patients with severe chronic hepatitis B, role of genotype and drug resistance. J Viral Hepat 2005; 12: 398-404.
27. Fung S, Chae R, Fontana H, Conjeevaram J, Marrero K, Oberhelman M, et al. HBV genotype D and switch
to adefovir (ADV) monotherapy are associated with increased risk of ADV resistance in chronic hepatitis B (CHB) patients. Hepatology 2005; 42(Suppl. 1): 590A-590A.

28. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16: 111-20.

29. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4: 406-25.