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Original Article

Identification of Drug Resistant Mutants of HBV (Hepatitis B Virus) by Direct Sequencing in Iranian Patients Treated with Lamivudine

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Abstract

Background and Aims: lamivudine is amongst the antiviral for drug chronic hepatitis B treatment. During therapy with lamivudine, variants may emerge with YMDD mutation in the reverse transcriptase (RT) region of polymerase gene. This mutation might have a role in drug resistant for HBV.

Materials and Methods: HBV DNA extraction from serum sample of 88 patients, were subjected to nested PCR for surface gene that overlaps the RT region of polymerase. Then, direct sequencing was carried out on the PCR products to identify the possible mutation.

Results: 32 samples were positive for PCR. Direct sequencing analysis showed point mutations in YMDD motif in 9 (28%) of the specimens. These mutations were I/V replacing for the wild type (M). Also, there were some compensatory mutations within the overlapping surface gene.

Conclusion: Mutations inside YMDD motifs may influence drug attachment to the virus, hence causing drug-resistance.

Keywords: Lamivudine; HBV (Hepatitis B Virus); Drug Resistant

Introduction

Hepatitis B virus (HBV) infection has long been a serious public health problem around the world. HBV is one of the main causes of acute and chronic hepatitis in humans. Nearly 400 million people are infected chronically with HBV worldwide. It has been demonstrated that chronic infection with this virus is linked to the development of cirrhosis and hepatocellular carcinoma, accounting for 0.5–0.75 million deaths per year.

Antiretroviral treatment is the main clinical treatment of chronic hepatitis B (CHB). Lamivudine, a nucleoside analogue, has been widely used because of its high effectiveness. However, long-term use of lamivudine may lead to the emergence of lamivudine-resistance in some HBV infections. The resistant generation is closely associated with mutations in the highly conserved YMDD motif, which is in the catalytic domain C of viral DNA polymerase. The YMDD motif has an amino acid sequence of tyrosine (Y)-methionine (M)-aspartic acid (D)-aspartic acid (D) and is both the binding and functional site of lamivudine. Recent reports revealed that besides the mutations caused by lamivudine therapy, some YMDD mutations can occur spontaneously in lamivudine-untreated CHB patients. Because HBV reverse transcriptase has no proofreading activity during the replication process, mutations can naturally occur due to random nucleotide misincorporation. The most

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common substitutions are methionine at amino acid position-204 to either isoleucine (rtM204I, YIDD mutant) or valine (rtM204V, YVDD mutant). Many studies have reported the incidence and characteristic of spontaneous YMDD-motif mutation in untreated, lamivudine-naive CHB patients. However, the reported incidences were quite different (0% to 31.58%) or even were contradictory with each other. This paper reviews the available publications, aiming to comprehensively provide not only the incidence but also associated factors of natural YMDD-motif mutation among lamivudine-untreated CHB patients. The results should provide scientific evidences for clinical treatment and future research of YMDD mutation.

Methods

88 hepatitis B surface antigen (HBsAg)-positive serum samples were collected from Iranian patients and were stored at -70°C until DNA extraction. QIAamp MinElute Virus Spin Kit (QIAGEN, Germany), the extraction procedure was performed according to the manufacturer's instructions. 200µl buffer AL was added into the tube including 200µl serum and 25µl QIAGEN protease and incubated at 56°C for 15 min in a heating block.

250µl of absolute ethanol was added and incubated at room temperature for 5 min. 500µl of buffer AW1 was added. After centrifuging at 6000g for 1 min, 500µl buffer AW2 was added and centrifuged at 6000g for 1 min. 500µl of absolute ethanol was then added and centrifuged at 6000g for 1 min. The column was dried at 56°C for 3 min. In later step, 100µl buffer AVE was added and centrifuged at 20000g for 1 min and then stored at -20°C until using.

Nested PCR was done using specific primers for HBV s gene sequences according to previously described method by Zeng *et al.* by some modifications.The sequences of the primers are shown Nested PCR, in first- and second-round PCR, was performed for 3 min at 94°C, following of denaturation at 94°C for 45 sec, annealing for 60 sec at 55°C and extension at 72°C for 90 sec. Final extension was done at 72°C for 6 min. PCR solution contained 2.5µl of extracted DNA, 0.5µl dNTP mix, 2.5µl 10x Taq polymerase buffer, 0.75µl MgCl2, 0.2µl Taq polymerase and 1µl of each primer.

Results

Direct sequencing was carried out on PCR products.In first group 40 sample (45.5%) were positive for PCR and in second group 33 samples (37.5%) were positive. Direct sequencing analaysis showed point mutations in YMDD motif in 12 (30%) of samples for 12 month after treatment and 23 (70%) of samples

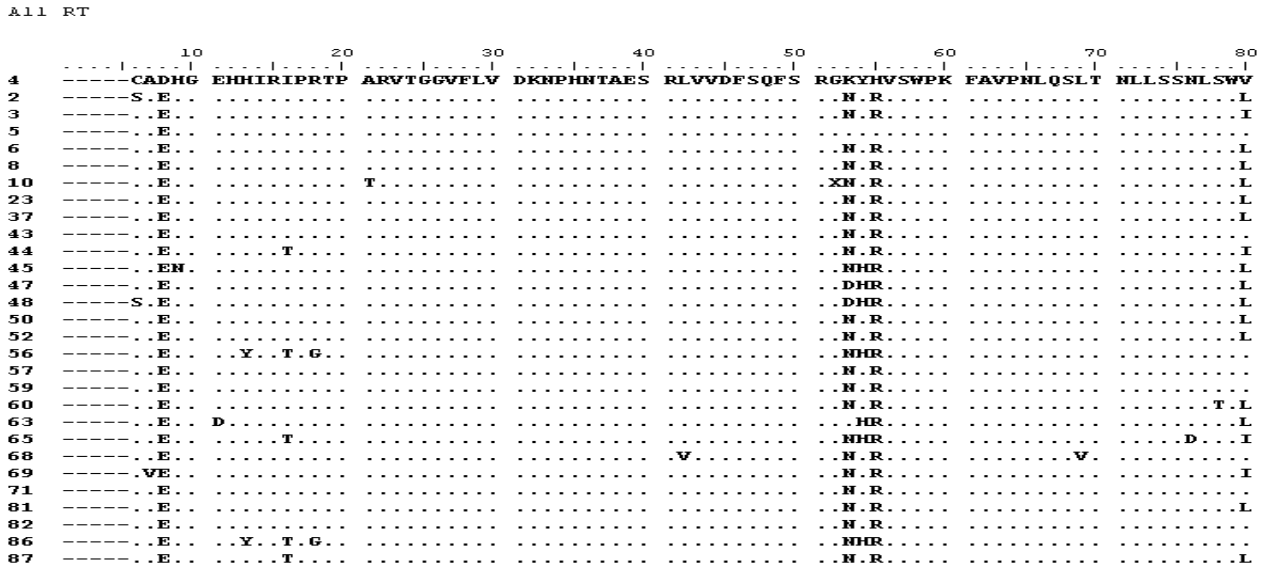


Fig. 1. YIDD, YVDD Mutations in positive samples.

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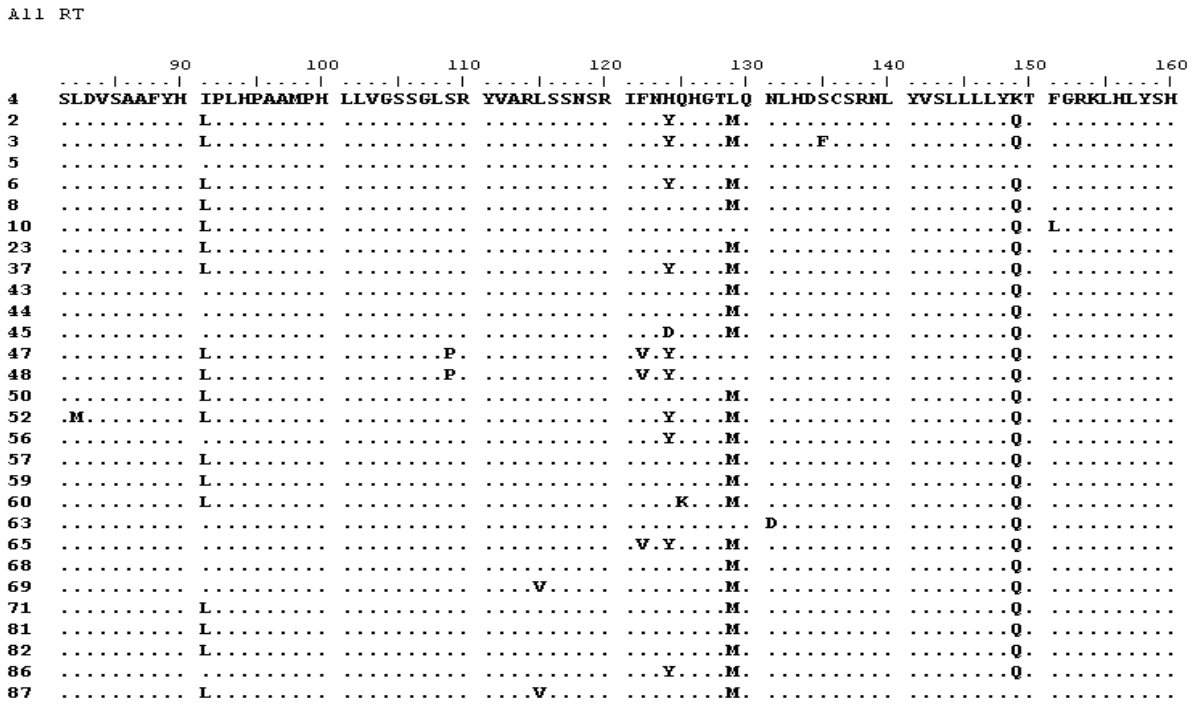


Fig. 2. Mutations and new Amino Acid.

for 24 month after treatment. These mutation were Valin amino acid (10% for 12 month and 36% for 24 month) and Isoleucine amino acid (20% for 12 month and 33% for 24 mnth) replacing for the wild type (Methionine).

Discussion

Lamivudine is an effective drug for hepatitis B patients, but the appearance of resistants after long course of treatment has brought about some problems. It is extremely important to explore the impact of lamivudine on hepatitis B patients, including the seroconversion of HBeAg. It was believed that the disappearance of HBeAg and the lower level of HBV DNA in serum during the period of treatment are the identical markers for the clearance of HBV. Seroconversion of HBeAg is useful index for evaluation of the efficacy of treatment. Researchers have suggested that the patient could cease the course of treatment if HBeAg becomes to negative after one year of treatment. If HBeAg is still positive after 6 months of treatment, acute hepatitis B might be induced if the patient discontinues the course of treatment. There for, detecting the level of HBV DNA and the seroconversion of HBeAg are particularly important in the course of lamivudine treatment.

In ourstudy, the levels of HBV DNA in HBeAg positive and anti-HBe seroconversion patients were lower than 100 pg/ml; no conversion happened in the patients with a HBV DNA level of >100 pg/ml. The results suggest that the lower level of HBV DNA may lead to the seroconversion of HBeAg and anti-HBe, which causes easy cleanness of HBV.

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