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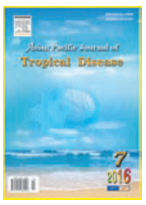

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Evaluation of HBV resistance to tenofovir in patients with chronic hepatitis B using ZNA probe assay in Kerman, southeast of Iran

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ABSTRACT

Objective: To evaluate the mutation rate of polymerase gene and it's correlation with tenofovir resistance in patients with chronic hepatitis B.

Methods: A total of 64 serum samples (36 men and 28 women) were collected from patients with chronic hepatitis B. All of these samples were tested for hepatitis B virus (HBV) DNA level, alanin amino transferase/aspartic amino transferase enzymes and serological markers such as hepatitis B surface antigen (HBsAg)/hepatitis Be antigen (HBeAg) (Electro-chemi-luminescence).

Results: In this study, out of those 64 patients, 13 cases had mutations in the polymerase region (A194T). All mutant cases were HBsAg positive and 5 (38.5%) of them were males and 8 (61.5%) were females, while 6 (46.2%) of the mutants were HBeAg positive and 7 (53.8%) were HBeAg negative and most of the mutants have more than 109 HBV DNA level. Statistical analyses on the 64 samples showed that there was no significant relation between age and HBsAg level but there was a significant relationship between HBV DNA load (P = 0.001) and A194T mutation.

Conclusions: HBeAg had a correlation with A194T mutation (P = 0.02) and tenofovir resistance was seen in 13 patients. Real-time PCR with zip nucleic acid probes is a rapid method to detect mutations in the polymerase region of HBV with high sensitivity and specifity. This method could be used for mutation detection in nt-194 position of polymerase gene for tenofovir resistance and other mutations in drug resistance researches.

1. Introduction

Hepatitis B virus (HBV) is one of the most important viruses, which infects hepatocytes in livers and leads to hepatocellular carcinoma. HBV is spherical virus with 22 nm diameter and has a partially double-stranded and relaxed circular DNA genome[1]. Four open reading frames encode structural proteins of the virion, the central body of the virus, a small transcription activator (X) and a large polymerase (P), which is responsible for the reverse transcriptase and Ribo nuclease H[2]. The C gene contains two start codons that encode hepatitis B core antigen and HBe protein, which will be converting to solution hepatitis Be antigen (HBeAg)[3]. HBV is transmitted through blood transfusion, sexual contact and also can cause acute or chronic diseases, especially in infants, and 5%–10% of people suffering from chronic hepatitis B (CHB) after the primary disease[4,5]. Almost one-third of people with chronic active hepatitis show liver failure and cirrhosis. The other two thirds of people having temporary chronic hepatitis and primary hepatocellular carcinoma can be attributed to chronic HBV infection[6]. The World Health Organization estimates that there are about 350 million carriers of chronic HBV in the world that 15%–40% of their lives are in danger due to the possibility of the disease progression to cirrhosis and liver cancer. In Iran, about 1.2%–9.7% of HBV infection has been reported in different areas[7], and 1.5–2.5 million people in Iran suffer from HBV infection[8]. It should be mentioned that the incidence rate of mutations in HBV infection is ten times more than other DNA viruses. According to different studies, anti-retroviral therapy is the most important factor in the creation of mutations in the virus reverse transcription/polymerase gene (the

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most important target for antiviral drugs[9]. A common treatment for patients with CHB is the combination of interferon alpha and the nucleoside/nucleotide analogous such as lamivudine, entecavir, telbivudine, adefovir and tenofovir. The application of these drugs is a big step in the treatment of CHB patients[10]. However, one problem associated with these drugs is the emergence of drug resistance in mutant strains[11]. Different types of mutations in the polymerase region (POL) have been described, such as mutations in tyrosine-methionine-aspartate-aspartate and leucine–leucine–alanine–glutamine regions, rtN236T, rtM204I, rtV84M and rtM194T. The commonest drug resistance is tyrosine-methionine-aspartate-aspartate motif mutation which possesses lamivudine resistance[12]. Mutations in rtA194T can cause resistance to tenofovir disoproxil fumarate (TDF) which is an acyclic nucleotide analogue relating to adefovir dipivoxil[13]. In 2008, tenofovir got the approve for the treatment of CHB, and it is available for the treatment of HIV infection. Tenofovir is superior to adefovir for the HBV DNA suppression, HBeAg seroconversion and the normalization of alanin amino transferase (ALT)[14]. PCR sequencing, restriction fragment length polymorphisms, INNO-LiPA and matrix-assisted laser desorption/ionization time of fight-mass spectrometry are developed to detect resistant mutations. The zip nucleic acid (ZAN) probe assay was used for detecting rtA194T mutation in POL region of HBV that published before[15]. HBV with rtA194T mutation reduces the susceptibility to tenofovir combining with lamivudine resistance rtM204V and rtL180M mutations *in vitro*[16,17]. Also, rtA181T and rtN236T mutations associated with adefovir resistance in *in-vitro* studies have showed the reduction in the susceptibility to tenofovir[18]. In this study, a sensitive real-time fluorescent quantitative PCR using ZNA probes was used to detect tenofovir-resistant mutants in chronic HBV patients with the history of long-term antiviral therapy in Kerman, southeast of Iran.

2. Materials and methods

2.1. Patients

In retrospective study in our laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, Iran), 64 patients with CHB including 36 men and 28 women with the mean age of 42 years ranging from 21–63 years were enrolled from October 2013 to March 2014. The treatment was based on the combination of the serum HBV DNA level, serum ALT level and the histological grade and stage of the underlying liver disease. Exclusion criteria included having severe illness, organ transplantation, treatment with corticosteroids, any liver disease not due to hepatitis B, and seropositivity for HIV or hepatitis C or hepatitis D virus.

2.2. Serological analysis

ALT and aspartic amino transferase (AST) were tested in blood samples from the patients with CHB. hepatitis B surface antigen (HBsAg) and HBeAg were tested with immunoassay method and ELISA and then both of them were evaluated with electro-chemi-luminescence method (ECL). Our serological markers were analyzed by HBsAgII HBeAg Elecsys and Cobas analyzers (Roche, Germany). ELISA method was carried out with an automatic plate washer (Bioscience, Tian Jin, China) and PETECK96-II Detection System (Bioscience) was used to read relative light units at 450 nm (Figure 1).

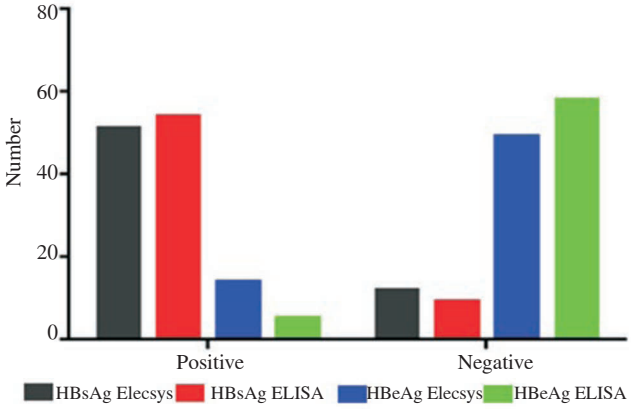

Figure 1. Comparison of serological tests in two different methods (Elecsys and ELISA) [$P < 0.05$, 95% confidence interval (*CI*): 25–45].

2.3. HBV DNA level

By RIBO-Prep kit (ILS, Russia), HBV DNA was extracted from plasma and quantified via real-time PCR by the commercial kit (ILS, Russia). In our method, the real-time PCR had a lower limit of 30 copies/mL.

2.4. Detection of rtA194T mutations

Specific primers and probes designed for HBV polymerase region to determine rtA194T mutation in nt-194 of the POL gene were detected by using Beacon designer software (version 8 primer, Biosoft, USA) (Table 1). Metabion Company (Metabion International AG, Germany) synthesized primers and probes and real time ZNA probe method was developed for tenofovir-resistant mutants. The quantitative real time PCR was done with the Rotor-Gene 6000 (Corbett Research, Australia) and the condition starts with 15 min with hot start Taq DNA polymerase at 95 ˚C followed by 45 cycles at 95 ˚C for 10 s and 60 ˚C for 30 s.

Table 1
Sequence primers and probes for the detection of tenofovir resistance.

Name	Sequence
Forward primer	CCGCTGTTACCAATTTTC
Reverse primer	GCCCATGAAATGTAAAGA
Wild type probe (A)	FAM-TTGGGTATACATTTAACCCCTACCAAA-BHQ1
Mutant probe (T)	JOE-TTGGGTATACATTAAACCCCTACCAAA-BHQ1

2.5. Statistical analyses

To analyze the data, *Chi*-square and Fisher's exact tests were used by SPSS 15.0 software (SPSS Inc, Chicago; USA) and $P < 0.05$ were considered significant.

3. Results

A total of 64 patients with CHB were selected from October 2013 to March 2014. Among them, 36 (56.2%) were men and 28 (43.8%) were women. The age of patients was (42.72 ± 10.90) years. The levels of ALT and AST were (123.30 ± 13.80) and (125.62 ± 15.06) IU/mL, respectively. We categorized HBV DNA load from 30 to more than 109 copies/mL. Twenty-six (40.6%) patients had less than 30 copies/mL DNA load. HBsAg was tested by ELISA method, and