

RESEARCH ARTICLE

Detection of HBV Resistance to Lamivudine in Patients with Chronic Hepatitis B Using Zip Nucleic Acid Probes in Kerman, Southeast of Iran

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Abstract

HBV infection is contagious and may be transmitted vertically or horizontally by blood products and body secretions. Over 50% of Iranian carriers have contracted the infection prenatally, making this the most likely route of transmission of HBV in Iran. This study assesses the resistance to Lamivudine in patients with chronic hepatitis B infection using a new ZNA probe Real Time PCR method. To evaluate the effectiveness of Lamivudine therapy for chronic hepatitis B infection, a study was conducted on 70 patients (63 men and 7 women), who had received the drug first line. All patients were tested for the presence of HBsAg and HBeAg, the serum ALT level and the HBV DNA load before and after treatment. In all samples resistance to Lamivudine was tested with the ZNA Probe. Our results showed that ZNA Probe Real Time PCR method could detect wild type, YMDD, and its mutants, tyrosine-isoleucine-aspartate-aspartate and tyrosine-valine-aspartate-Aspartate. Among an estimated seventy patients with chronic hepatitis B infection, 18 (25.7%) were resistant to lamivudine. Only one patient was negative for presence of HBS-Ag (5.6%) and two patients were negative for HBe-Ag (11.1%). Real-time PCR with Zip nucleic acid probes is a sensitive, specific and rapid detection method for mutations in the YMDD motif, which will be essential for monitoring patients undergoing Lamivudine antiviral therapy.

Keywords: Chronic hepatitis B - Lamivudine - zip nucleic acids - real time PCR

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Introduction

Hepatitis B virus (HBV) is one of the major causes of liver disease worldwide, and chronic HBV infection can progress to cirrhosis and hepatocellular carcinoma. About 5% of the global population (350 million persons), are currently infected with HBV (Elefsiniotis et al., 2005). Chronic HBV can be treated with a nucleoside analogue, lamivudine, which in the short term can inhibit HBV replication by blocking the viral polymerase activity. However, during long term treatment, lamivudine resistant strains have been detected. Based on a previous study, this resistance has been associated with variations in the YMDD motif of the HBV polymerase which is the catalytic site (Degertekin et al., 2009) and after some study on this part of gene we designed our primers and probes for amplification. Lamivudine resistant HBV strains with YMDD mutations are an important factor for the failure of chronic hepatitis B treatment. Some patients with mutation even went worse, and eventually died. However, it has been rarely reported whether YMDD mutations have natural existence (Geng et al., 2006).

Treatment with lamivudine leads to the development of lamivudine-resistant virus which is associated with a gradual reversal of the drug's antiviral effects as early

as the first year of therapy. This resistance is shown with increased HBV DNA concentrations and HBeAg-positivity and/or increased alanine transaminase (ALT) concentrations (Zhou et al., 2012) and in the same way in our study the patients had shown these variations. During lamivudine therapy, for those patients with persistently elevated ALT and HBV DNA concentrations it is vital to determine the presence of lamivudine resistant mutations. Detection of drug resistance mutation is recommended for rapid identification and effective treatment monitoring before HBV DNA and ALT concentrations increase (Wu et al., 2012). In Islamic Republic of Iran (I.R. Iran), recent studies reported the range of HBV infection between 1.2 to 9.7 percent in different regions. Generally, it is estimated that about 1.5 to 2.5 million people are suffering from HBV infection in I.R. Iran, and some of them are carriers who may transmit infection to others (Fallahian et al., 2010). HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses. The replication and mutation rate is an important factor in the viral diversity. HBV uses a reverse transcription step in its replication cycle and the rate of evolution in the range of 10^4 - 10^5 nucleotide substitutions per site per year is shown (Kamar et al., 2004). The main aim of therapy of chronic hepatitis B is to achieve a sustained suppression of HBV replication,

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to obtain remission of the underlying liver disease and thus prevent its progression towards cirrhosis and HCC (Brunelle et al., 2005). Continuous viral suppression is equally essential in order to avoid the risk of the emergence of antiviral resistance (Buti et al., 2007). Consequently, patients should be considered for antiviral treatment only when (a) serum HBV DNA is above 2,000 IU/ml (that is, approximately 10,000 copies/ml), and/or (b) serum ALT levels are above the upper limit of normal (ULN), and (c) liver biopsy shows moderate to severe active inflammation and/or fibrosis using a standardized scoring system (e.g. $\geq A2$ and/or $\geq F2$ when using the METAVIR score) (Kamar et al., 2004). Many current therapeutic approaches for treating HBV infection focus on the DNA polymerase as a target to inhibit viral replication, including nucleoside analogs that terminate viral DNA synthesis and decrease viral load in most cases (Rizzetto et al., 2005). Resistance to lamivudine, is invariably associated with mutations in the highly conserved YMDD motif, which is part of the catalytic site of the HBV polymerase (Arslan et al., 2008). Many methods were developed to detect LAM-resistant mutations, such as direct polymerase chain reaction (PCR) sequencing, INNO-LiPA, restriction fragment length polymorphism (PCR-RFLP), matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI TOF MS) with their respective advantages and disadvantages (Moosavy et al., 2011). Sequencing remains the best approach to the identification of new mutations. However, it cannot detect total mutations in less than 25% of total viral population and is not appropriate for large-scale use in large cohort studies or clinical laboratories because of its labor intensive and time consuming (Aghasadeghi et al., 2011). Manipulations INNO-LiPA and MALDI TOF MS are capable to detect variants, but more strict experiment conditions and equipment's are required. PCR-RFLP could only detect mutations in a high proportion and is also labor intensive and time consuming (Aberle et al., 2001). Monitoring of viral load will play an important role in assessing treatment. Prolonged treatment with antiviral agents can lead to the emergence of drug-resistant virus (Chen et al., 2010). Zip nucleic acids (ZNAs) are oligonucleotides conjugated with cationic spermine units which increase affinity for their target by decreasing electrostatic repulsion between negatively charged anionic single strand Nucleic Acids to improve hybridization, thus enhancing and accelerating target recognition (Noir et al., 2008). The possibility of modulating the global charge of the ZNA oligonucleotide-oligocation conjugates by the number of cationic spermine moieties attached to the Nucleic Acid oligomer, is a key to predict melting temperature of ZNA-DNA/ZNA-RNA hybrids easily. T_m increases linearly with the length of the oligocation. ZNAs were shown to enable specific and sensitive reactions when used as primers for PCR and Reverse Transcription. Moreover ZNA probes provide broad flexibility in assay design and represent an effective alternative to Minor Groove Binder (MGB) and Locked Nucleic Acid (LNA) containing oligonucleotides (Voirin et al., 2007). Lamivudine resistance is associated with increased HBV DNA concentrations and HBeAg-

Positivity and increased alanine transaminase (ALT) concentrations with exacerbation of liver disease during treatment (Li et al., 2005). Thus, detection of mutant resistant to treatment is necessary to prevent disease progression and malignancy and recommended for rapid identification and sensitive treatment monitoring (Fasano et al., 2012). In this study described a sensitive method for detection of LAM-resistant mutants by Real-time fluorescent quantitative PCR using ZNA-probes in Chronic HBV patients who had been long-term lamivudine monotherapy in Kerman, southeast of Iran. So far no study has been done in this area with same method. This method can be used to detect YMDD and its mutants, tyrosine-isoleucine-aspartate-aspartate (YIDD) and tyrosine-valine-aspartate-aspartate (YVDD) in a single multiplex reaction simultaneously.

Materials and Methods

Patients

In retrospective study, Seventy patients (63 men and 7 women, mean age=44 years, range 41-66 years) who were diagnosed as Chronic Hepatitis B infection (CHB) from October 2010 to December 2011 in our Laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, IRAN) following the guidelines of prevention and treatment of chronic hepatitis B were enrolled in this study. The decision to treat is primarily based on the combination of three criteria: a) serum HBV DNA levels, b) serum alanine amino transferase (ALT) levels and c) histological grade and stage of the underlying liver disease.

Exclusion criteria included a coexisting severe illness, organ or bone marrow transplantation, recent treatment with systemic corticosteroids, immunosuppressant's or chemotherapeutic agents, liver disease not due to hepatitis B, and seropositivity for human immunodeficiency virus (HIV) or hepatitis C (HCV) or hepatitis D virus.

Serological tests

The blood from the patients with chronic hepatitis B was assayed for alanine transaminase at our clinical laboratory using an automated analyzer. HBsAg and HBeAg as well as Anti-HCV and Anti-HIV were determined using commercial radioimmunoassay kits (Abbott Laboratories, Chicago, IL). Hepatitis D antigen was detected using enzyme immunoassay kits (Abbott Laboratories, Chicago, IL).

HBV DNA level

5 ml of peripheral blood were collected from each patient into EDTA-containing vacutainer tubes. Plasma was separated and stored at -70°C . HBV DNA was extracted from 200 μL of plasma with High Pure Viral Nucleic Acid kit (Roche Diagnostics GmbH, Mannheim, Germany). Serum HBV-DNA levels were quantified via quantitative real-time polymerase chain reaction with a commercial detection kit (artus HBV kit, Qiagen, Germany).

The Real-time PCR had a lower limit of 35 copies/mL.

Detection of YMDD mutations

Determination of LAM resistance was done with specific primers and probes from the HBV polymerase region in YMDD motif that were design by Dr Mollaie Using Beacon designer software (Version 8 Primer, Biosoft, USA).Primers and probes synthesized by Metabion company (Metabion International AG, Germany).All of samples were tested with Real Time ZNA probe method, which has been developed for detection of lamivudine- resistant mutants in our laboratory, and this is a patent test for us. 5 μ l of DNA sample combined with 15 μ l reaction mixture of fermentas TaqMan Master mix (Thermo fisher Scientific, USA) contain primers and probe were subjected to Real-time PCR. The condition for the ZNA mediated probe assay was initially 10 min with hot start Taq DNA polymerase at 95°C followed by 45 cycles at 95°C for 15 sec and 60°C for 40 sec. Of each cycle at the extension step, the fluorescent signals of Probe YMDD, YVDD, YSDD and Probe YIDD were measured at Green, Yellow, Orange and Red channel, respectively. All of samples also were tested via quantitative Real Time PCR with a commercial Lamivudine Resistance HBV detection kit (Bioproducts, Mag. Th. Langmann GmbH). Quantitative determination of the amplified products was done with the Rotor Gene 6000 (Corbett Research, Australia).

Statistical analyses

Chi square and Fisher's exact Tests were used to analyze the data obtained by SPSS 11.5 software (SPSS Inc, Chicago; USA). The differences or association with $p < 0.05$ were considered statistically significant.

Results

Seventy patients with CHB were selected during about one year (2010-2011) who were treated with lamivudine as the first drug for 30-86 weeks (Mean= 64 weeks). Eighteen (25.7%) of whom developed LAM resistance mutations who six experienced virological relapse after discontinuation of LAM treatment. Of the seventy samples Sixty-one percent of patients were HBeAg Positive. Liver biopsy was available in 23 patients and 12 (52%) of them showed grade 3-4 fibrosis stage. Because the study was on patients who their sample had been previously collected, all liver biopsy samples were not available. Additionally, three patients without liver biopsy had been classified as having cirrhosis based on clinical, analytical and ultra sonographic findings. Thus, 15 (21.4%) of 70 patients were classified as having severe fibrosis or cirrhosis. Mean ALT level was 485.4 ± 28.7 IU/L and mean HBV-DNA level was 8.311 ± 0.269 log₁₀ IU/mL (Table 1). Three types of lamivudine resistance in these samples were determined, Nine patients had a YIDD mutation (50%), Two patients had a YSDD mutation (11.1%) and seven patients had a YIDD/YVDD mix mutation (38.9%). Serological and pathological details of the eighteen patients are shown in Figure 2. ALT level and HBV DNA Load were measured in two stages, before and after treatment with Lamivudine. Statistical calculations on the seventy samples showed that there is a significant relationship between gender and

Table 1. Baseline Characteristics of Patients

Characteristics	Male	Female	Total
Gender (%)	63 (90%)	7 (10%)	70 (100%)
Mean age (years)	43 \pm 8	41 \pm 9	42 \pm 8.5
HBeAg-Positive (%)	30 (47.6%)	1 (14.2%)	31 (44.2%)
F3-F4 (%) (Classified by liver biopsy examination or clinical radiological findings)	12 (19%)	0 (%)	12 (17.1%)
Cirrhosis (%)	3 (4.7%)	0 (%)	3 (4.28%)
Prior lamivudine therapy (%)	63 (100%)	7 (100%)	70 (100%)
Lamivudine resistance (%)	17 (26.9%)	1 (14.2%)	18 (25.7%)
Mean ALT (IU/L)	320 \pm 15	300 \pm 10	310 \pm 12
Mean HBV-DNA (log ₁₀ IU/mL)	10 \pm 2.1	7 \pm 1.3	8.5 \pm 1.7

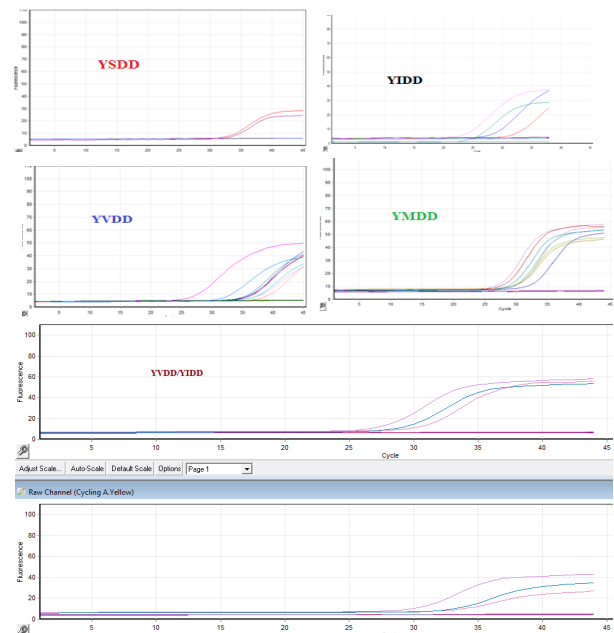


Figure 1. Results of ZNA Probes in Four Channels and YIDD/YVDD Mix Mutations.

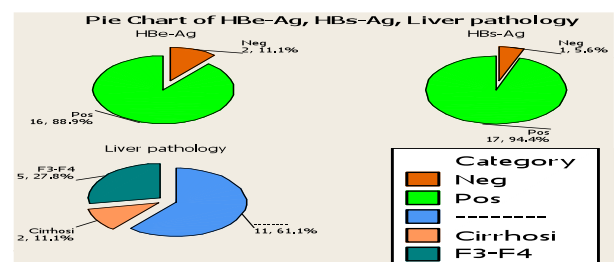


Figure 2. Serological and Pathological Details of the Eighteen Patients.

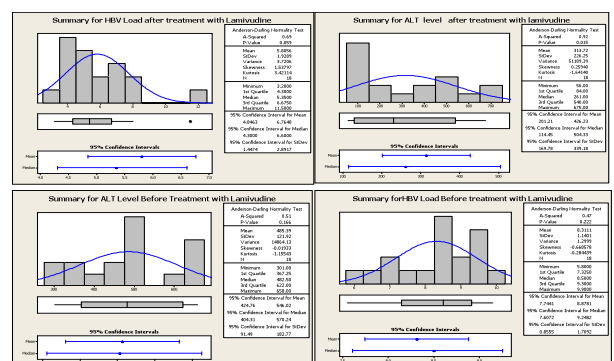


Figure 3. Histogram Results of ALT and HBV DNA Load, Before and After Treatment with Lamivudine.

drug resistance to lamivudine. (P Value=0.01). Histogram results of ALT and DNA HBV load, before and after treatment with Lamivudine is compared and is shown in the Figure 3. To evaluate the real-time PCR using ZNA mediated probe, each DNA template and each probe were paired in separate reactions, for example using YMDD template with Probe and primers for YMDD. Signals were showed but not matched the respective template/probe pair and it represents either non-specific reactions or cross reactions between probes and templates. If the signal only appears when probe and template match, it ensures the probe is specific for its template. All samples were typed blindly to each variant by standard Real Time PCR kit, without any conscious bias. There was no difference between the results of two methods (Figure 4).

Discussion

To date, many assays have been used for detection of lamivudine-resistant mutants in patients with Hepatitis B. Differences in sensitivity, specificity, cost, and time required, exist in these methods (Liu et al., 2011). Real-time PCR is able to quantitatively detect a small portion of resistant mutants in HBV populations and ZNA probe detection method is a newly developed method for detection of low abundant mutants in the background of wild-type HBV (Liu et al., 2010). So we used these methods and we could detect single mutation about YSDD and YIDD. Interestingly we reported YIDD, YSDD and YVDD mutations. Another thing which was mentioned is gender. In our study most of drug resistance cases were in men. Sequence analysis, on the other hand, has already proven to be a useful technique for mutation detection, however, it takes more time and cannot be used everywhere and also cannot detect any mixtures of variant and wild type virus (Aberle et al., 2001) but sequencing results of this study were same to lamivudine detection kit which used in this study and PCR-RFLP (Data not shown). Using ZNA probe method is quick and inexpensive comparing to other probe methods for detection of YMDD mutation; and ZNA probe method is more trustable than other methods. The results obtained with both methods were completely concordant in all serum samples. ZNA was able to detect as low as 35 copies/mL of YMDD mutants, while other method only detected 1000 copies/mL of YMDD Mutants. In addition, the cost of ZNA is slightly lower and is much more rapid so it requires less manual work than PCR-RFLP. The total assay time for ZNA real-time PCR was 1.5 hours, respectively. Another advantage of the real-time PCR method is it is able to calculate the ratio of mutants to total virus in samples. This will be useful in clinical studies on the dynamics of resistant mutants during lamivudine therapy. In the current study, the rate of YMDD mutations was 25.7% after two year of lamivudine treatment, which is higher than in a previous study. However, it is lower than that in another study, which had YMDD mutations in 57.4% of Japanese patients with chronic hepatitis B after one year of lamivudine treatment. The current study also aimed to determine the association of pretreatment HBV-DNA levels and HBeAg status at baseline with the occurrence of YMDD mutations. Logistic regression

analysis demonstrated that high pretreatment HBV-DNA levels are independent factors for the occurrence of YMDD mutations. These findings are in accordance with the results of other studies (Amini-Bavil-Olyae et al., 2008). HBeAg status at baseline is recognized as an independent factor for YMDD mutations. Thus HBeAg status at baseline is one of the predictors (Fasano et al., 2012). But the results are not consistent with those of another study suggesting that the rate of YMDD mutations in HBeAg positive patients was similar to that in HBeAg negative patients. The present study suggests that pretreatment HBV-DNA levels and HBeAg status should be considered for patients with chronic HBV after lamivudine treatment. High ALT levels are thought to be related to a more rapid selection of YMDD mutations. The current study revealed no significant differences in ALT levels in patients with or without YMDD mutations prior to lamivudine treatment. Logistic regression analysis in our study also showed that pretreatment ALT levels are not predictors of YMDD mutations. Studies including the current one showed that high ALT levels are not related to the rapid selection of YMDD mutations. Thus, there are different findings concerning the correlation of ALT levels with YMDD mutations. However, ALT levels are very important parameters for host factors, and they should be monitored carefully during lamivudine treatment. In conclusion, virological and biochemical markers (HBeAg, HBV DNA load, and serum ALT level) are used in the diagnosis and monitoring of HBV disease. A combined end point of biochemical response (ALT normalization) and virologic (serum HBV-DNA suppression) response is used frequently. In this study, of seventy patients, thirty eight (54.2%) was HBeAg negative. We did not study other markers include liver covalently closed circular DNA (cccDNA), HBV genotype, and genotypic resistance markers. Also, lack of define of a target serum HBVDNA level, and different assays for a patient to make the interpretation difficult. We calculated the amount of mutations according to rise of serum HBVDNA titer or ALT level and showed it in some divided times. However, in many clinical trials, resistance is calculated by about the cumulative probability of HBV polymerase mutations. In present study, most chronic hepatitis B patients on lamivudine treatment developed mutations in mean 36 ± 10 months. In CHB patients without YMDD mutation and high HBVDNA titer, analysis for possible new mutants should be performed. Elevated HBVDNA titer might be supposed as a clue to drug resistant, regardless of ALT level or mutation report. The YMDD-motif mutations can occur spontaneously with a fairly high incidence in CHB patients untreated with lamivudine or adefovir. These mutations might be the result of accumulated base mismatch caused by viral polymerase. More basic and clinical studies are needed to clarify the influence of YMDD mutations in hepatitis B progression and antiviral treatment. Lamivudine therapy would increase the risk of mutations 5.23 times higher than the untreated patients. A higher HBV DNA copy number was associated with higher incidence of natural YMDD mutation. No significant difference was found in YMDD mutation incidence between groups of different gender, age, HBeAg

status and ALT level.

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