RESEARCH ARTICLE

Use of ALLGIO Probe Assays for Detection of HBV Resistance to Adefovir in Patients with Chronic Hepatitis B, Kerman, Iran

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Abstract

Hepatitis B virus (HBV) infection is contagious with transmissiobn vertically or horizontally by blood products and body secretions. Over 50% of Iranian carriers contracted the infection prenatally, making this the most likely route of transmission of HBV in Iran. To evaluate the resistance to adefovir (ADV) therapy in patients with chronic hepatitis B infection, a study was conducted on 70 patients (63 males and 7 females), who had received in first line lamivudine and second line adefovir. All were tested for the presence of hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), serum alanine amino transferase (ALT) level and HBV DNA load before and after treatment with ADV. In all samples, resistance to lamivudine and ADV was tested with real time PCR. Among seventy patients with chronic hepatitis B infection, 18 (25.7%) were resistant to LAM and 8 (11.4%) were resistant to ADV. Only one patient was negative for the presence of HBS-Ag (5.6%) and two were negative for HBe-Ag (11.1%). In this study we used a new method (ALLGIO probe assay) that has high sensitivity in detection of adefovir resistance mutants, which we recommend to other researchers. Mutant strains of the YMDD motif of HBV polymerase can be found in some patients under treatment with lamivudine and ADV. ADV has been demonstrated to be efficient in patients with lamivudine resistant HBV.

Keywords: Chronic hepatitis B -adefovir - drug resistance - real time PCR - Kerman - Iran

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Introduction

Hepatitis B virus (HBV) infections are a major cause of acute and chronic hepatitis, and of its long-term complications for example decompensate cirrhosis and hepatocellular carcinoma (HCC) (Hosaka et al., 2010). About 5% of the global population. almost, 350 million persons, are currently infected with HBV. Chronic hepatitis B virus infection is still an important cause of morbidity and mortality, as well as a source of potential new infection (Jeon et al., 2012). Chronic HBV carriers may develop chronic hepatitis, cirrhosis, and HCC with HBV as the most frequent cause (Jayakumar et al., 2012). The goal of therapy of chronic hepatitis B is to achieve a sustained suppression of HBV replication, to obtain remission of the underlying liver disease and thus prevent its progression towards cirrhosis and HCC (Lim et al., 2011). Continuous viral suppression is equally essential in order to avoid the risk of the emergence of antiviral resistance. Chronic hepatitis B (CHB) patients require a prolonged but not a lifelong therapy, therefore data about its long term safety and effect are indispensable for the assessment of its risk and benefit treatment of CHB (Minde et al., 2012). Several nucleoside/tide analogues are available, such as lamivudine (LAM), adefovirdipivoxil (ADV), entecavir (ETV), and telbivudine (LdT), which act as inhibitors of HBV reverse transcriptase and decrease viral load in most cases (Patterson et al., 2011). ADV has become the treatment option for HBV infection due to its effect on lamivudine-resistant mutations occurring upon prolonged treatment. However, viral resistance to ADV develops and increases over time (Segovia et al., 2012). ADV-resistance is mainly associated with rtN236Tand rtA181V/T mutations within the D and B functional domains of HBV polymerase been developed to detect ADV-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 fight-mass spectrometry (MALDI TOF MS) with their respective advantages and disadvantages (Zou et al., 2009). Sequencing remains the best approach to the identification of new mutations. However, it cannot detect rtN236T and rtA181V/T mutations in less than 25% of a 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 manipulations (Liu et al., 2010). INNO-LiPA and MALDI TOF MS are capable of detecting 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 (Osiowy et al.,

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2006; Wang et al., 2009). 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 (Chang et al., 2012). Resistance to lamivudine is well documented and is often associated with mutations in the YMDD of the HBV polymerase B and C domains. Adefovir is effective in suppressing lamivudine-resistant HBV and, therefore, is most commonly used in patients with lamivudine-resistant HBV infection (Chen et al., 2010). While acquired resistance to Adefovir is important, primary resistance to this nucleotide analogue was reported recently (Inoue et al., 2011). Adefovir resistance is associated with increased HBV DNA concentrations, HBeAg positivity and increased alanine transaminase (ALT) concentrations with exacerbation of liver disease during treatment (Tseng et al., 2009). Therefore, detection of mutant resistant to treatment is necessary to prevent disease progression and malignancy and recommended for rapid identification and sensitive treatment monitoring (Kim et al., 2010). In this study described a sensitive method for the detection of ADV-resistant HBV quasi species by ALLGIO real-time fluorescent PCR and evaluation development of primary adefovir resistance in chronic HBV patients that had been long-term lamivudine monotherapy in Kerman, southeast of Iran. So far no study has been done in this area.

AllGlo technology employs two identical reporter dyes that are attached to the ends of an oligonucleotide. Two dyes quench each other when the labeled oligo is in its free form (Liu et al., 2011). Upon hybridization with the target sequence, the labeled oligo becomes stretched and cleaved. This leads to the separation of the two reporter dyes and de-quench occurs. As this process releases two fluorescing molecules instead of one, the signal change is much greater, making AllGlo-based quantitative PCR detections highly sensitive and robust (Miszczycha et al., 2012). Since AllGlo requires only single dye component, it greatly simplifies the manufacturing process. Simple, efficient chemistry also yields high quality and uniform products with much reduced cost. This makes AllGlo probes highly competitive on the market (Yoshida et al., 2008). Because AllGlo does not require a quencher dye, a condition that often limits the choice of reporter dye in traditional fluorogenic probes, AllGlo probes and primers can choose a wide range of absorption and emission wavelengths, virtually cover the entire visible spectrum. With these flexibilities researchers are able to design tailor-made multiplex assays according to their specific needs (Jamnikar, 2012). AllGlo technology is the ultimate choice for fluorogenic probe-based nucleic acid detection. It out performs TaqMan and Molecular Beacon in signal strength, multiplexing capacity, and affordability. It also can be used as fluorogenic primers that compare favourably to current existing primer technologies such as Ampliphore, LUX primer. AllGlo probes is available in six colors, MAR, JUP, SAT, URA, NEP and PLU, which match with the absorption and emission wavelength of FAM, HEX, TAMRA, ROX, Cy5 and Quasar 670, respectively. These six AllGlo dye labels are compatible with most real time PCR instruments and generally require no special calibration (Wu et al., 2010).

Materials and Methods

Patient selection

In retrospective study, seventy patients (63 males and 7 females, mean age 44 years, range 41-66 years) who were diagnosed as Chronic Hepatitis B infection 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. ADV was administered with oral dipivoxil (10 mg) for 53 weeks (range: 38-68 weeks), during which viral breakthrough, viral rebound, partial or inadequate virus response occurred. 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 (Saah et al., 2003) or hepatitis C or D virus.

Molecular test

About 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). Extracted DNA pellets were resuspended in 100µL of prewarmed Elution buffer and stored at -20°C until use. For Quantification of HBV DNA we use Real Time HBV kit (artus HBV kit, Qiagen, Germany). Quantitative determination of the amplified products was done with the Rotor Gene 6,000 (Corbett Research, Australia). Determination of LAM and ADV resistance was done with specific primers and probes from the HBV polymerase region in YMDD motif were design by Dr Mollaie Using Beacon designer software (Version 8, Primer Biosoft, USA). Primers and probes for ALLGIO assay were synthesized by AlleLogic Biosciences (AlleLogic Biosciences Corporation, USA). Primer and probes sequence are shown in Table 1. All of samples were tested with ZNA probe assay (Malekpour and Mollaie, 2012), then all of samples were tested with ALLGIO probe assay, which has been developed for detection of adefovir resistant mutants in our laboratory. To verify the results, determination of ADV and LAM resistance was compared with HBV adefovir and lamividine resistant mutants Real Time PCR Kit (Bioproducts, Mag. Th. Langmann GmbH). Quantitative determination of the amplified products was done with the Rotor Gene 6,000 (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) that were treated with lamivudine as the first drug for 30-86 weeks (Mean= 64 weeks) and adefovir for 12-44 weeks (Mean=26 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 (17.1%) of them showed grade 3-4 fibrosis stage (Table 2). 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 (Figure 2). ALT level and HBV DNA Load were measured in two stages, before and after treatment with Adefovir (Figure 3). Mean of ALT level was 310±12 IU/L and mean of HBV-DNA level was 8.5±1.7 log10 IU/mL. The average load of HBV DNA dropped to 5.6±3.2 log10 IU/mL and mean of ALT level dropped to 260±56 IU/L after treatment with ADV for 86 weeks. Significant differences in HBV-DNA levels were observed between the patients with or without YMDD mutations prior to lamivudine treatment (6.71±0.98 versus 8.5±1.7 log10 IU/mL, respectively, P<0.05)(Figure 4). Of the seventy samples, Eighteen samples had resistance to LAM (25.7%) and eight samples (11.4%) had resistance to ADV therapy. Three types of lamivudine resistance in these samples was determined, Nine patients had a YIDD mutation (50%), Two patients had a YSDD mutation (11.1%) and seven patients had a

Table 1. Primer and Probes Sequences for Detection Adefovir Resistance

Name	N236T	Tm	Position
Forward primer	TTTACCGCTGTTACCAATTTTC	54	151
Reverse primer	CAATGACCCATAACATCCAATG	54	270
Wilde type probe	TATACATTTAAACCCTAACAAAACAAA	54	186
Mutant probe	TATACATTTAACCCCTAACAAAACAAA	55	186
	A181V/T		
Forward primer	GGCTTTCGGAAAATTCCTATG	53	202
Reverse primer	CCAATACCACATCATCCATATAAC	54	337
Wilde type probe	CCGTTTCTCCTGGCTCAGTTTACT	59	238
Mutant probe	CCGTTTCTCCTGGTTCAGTTTACT	61	238
Wilde type probe	CCGTTTCTCCTGGCTCAGTTTACT	59	238
Mutant probe	CCGTTTCTCCTGACTCAGTTTACT	61	238

Table 2. Baseline Characteristics of Patients

Characteristics	Male	Female	Total
Gender (%)	63 (90%)	7 (10%)	70 (100%)
Mean age (years)	43±8	41±9	42±8.5
HBeAg-Positive (%)	30 (47.6%)	1 (14.2%)	31 (44.2%)
F3–F4 (%)*	12 (19.0%)	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%)
Prior Adefovir therapy (%)	54 (85.7%)	4 (57.1%)	58 (82.8%)
Adefovir resistance (%)	8 (12.7%)	0 (0%)	8 (11.4%)
Mean ALT (IU/L)	320±15	300±10	310±12
Mean HBV-DNA (log10 IU/mL)	10±2.1	7±1.3	8.5±1.7

*Classified by liver biopsy examination or clinical radiological findings

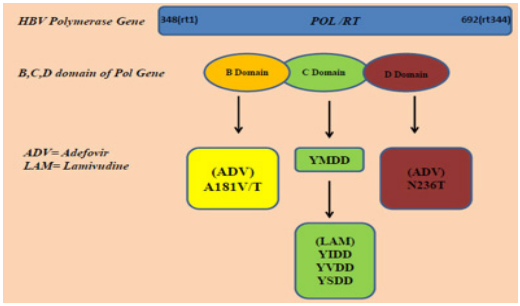

Figure 1. Polymerase Gene Mutations Conferring Resistance to Nucleoside Analogs. Resistance to lamivudine (LAM) is conferred by mutations in the YMDD motif within the C domain of the polymerase. Resistance to Adefovir (ADV) is conferred by an A181V or T mutation, or an N236T mutation

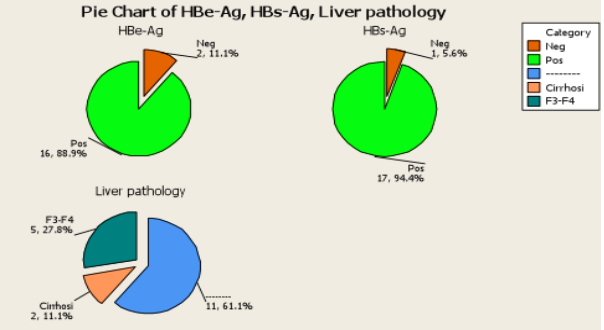

Figure 2. Result and Frequency of HBs-Ag, HBe-Ag and Liver Pathology Findings in CHB Patients in this Study

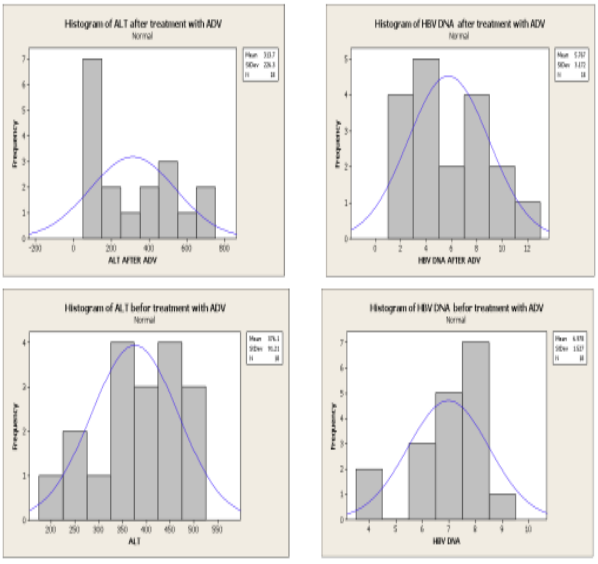

Figure 3. Histogram Results of ALT and DNA HBV Load, Before and After Treatment with Adefovir

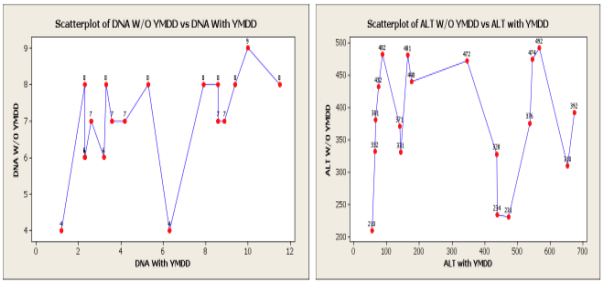

Figure 4. Scatter Plot of ALT Level and DNA Load in Patients With and Without YMDD Motif

YIDD/YVDD mix mutation (38.9%).Also Three types of Adefovir resistance in these samples was determined, , four patients had a rtA181T mutation(22.2%),three patients had a rtN236 mutation(16.7%) , one patients had a rtA181V mutation (5.6%), and Ten samples had no mutations (55.6%). Statistical calculations on the seventy samples showed that there is a significant relationship between gender and drug resistance to lamivudine or Adefovir. (P Value=0.01). No difference was observed between the results of this method and a commercial kit. the positive results was lower ct than commercial kit.

Discussion

Chronic infection with hepatitis B virus represents a global health problem, with continuing new infections worldwide, being an important cause of liver disease, morbidity, and mortality (Husic-Selimovic et al., 2012). The goals of therapy in HBV infected patients are to limit or reverse progression of the disease through sustained suppression of HBV replication. In chronic Hepatitis B infection, patients not able to clear the virus as judged by a positive HBsAg for six months after the acute episode and are known as chronic cases. In these patients, spontaneous clearance of HBs-Ag occurs at an annual rate of only 0.5-2%. Spontaneous loss of HBeAg and serum HBV DNA occurs in 10-15% of cases and Antiviral therapy of CHB patients remains a major clinical challenge (Jayakumar et al., 2012). Lamivudine resistance is associated with mutations in the highly conserved YMDD motif of reverse transcriptase, specifically YMDD mutations including YIDD and YVDD mutations. YMDD mutations reportedly increase from 14% in 1 year to 38%, 49% after 2 and 3 years of treatment, respectively (Jiang et al., 2012). Long-term antiviral treatment is needed in most individuals and therefore, raises the risk of selecting drug-resistant mutants. Drug resistance is associated with Polymerase gene mutations, might be followed by a rise in HBV viral load and ALT levels with exacerbation of liver disease (Veldhuijzen et al., 2012). Adefovir is effective in suppressing lamivudine-resistant HBV and, therefore, is most commonly used in patients with lamivudine-resistant HBV infection (Zhao et al., 2010). The raising potent anti-HBV agents will be important to better combat antiviral drug resistance and improve the current treatment strategies to delay or prevent drug resistance in the long term (Foroutan et al., 2011). Same this study has not been done in Iran, and the information is not available about HBV resistance to ADV. Several studies carried out only on resistance to lamivudine using other methods (PCR-RFLP). In the current study, the rate of YMDD mutations was 25.7% after one year of lamivudine treatment, and 11.4% for ADV, 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 (Wang et al., 2009). 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. HBeAg status at baseline is recognized as an independent factor for YMDD mutations. Thus HBeAg status at baseline is one of the predictors. 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 (Osiowy et al., 2006; Wang et al., 2009; Liu et al., 2010; Zhao et al., 2010; Hu et al., 2012).

The results of this study show that HBV DNA levels during treatment as an indicator of future ADV resistance. ADV-resistant mutation was closely associated with HBV DNA levels during therapy. The risk of developing ADV-resistant mutations in patients with long-term lamivudine monotherapy was high. These findings suggest that combination therapy of LAM and ADV could be used as an option for patients with LAM-resistant HBV infection. In this study we have developed a new method for detection of adefovir resistance mutant in chronic hepatitis B patients. this method was compared with commercial kit and the results show that use of ALLGIO probe assay can be a good alternative to conventional methods and compared to other methods such as TaqMan probe assay has a higher sensitivity. Each AllGlo probe contains two identical fluorogenic reporter dye, therefore it can generate twice as much signal as all other probe systems. Unlike MGB probes, do not inhibit PCR reaction even at very high probe concentrations. We recommend that researchers conduct more studies about this method for detection of single nucleotide mutations on drug resistance.

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