



Drug-Related Mutational Patterns in Hepatitis B Virus (HBV) Reverse Transcriptase Proteins From Iranian Treatment-Naïve Chronic HBV Patients

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A B S T R A C T

Background: Immunomodulators and Nucleotide analogues have been used globally for the dealing of chronic hepatitis B virus (HBV) infection. However, the development of drug resistance is a major limitation to their long-term effectiveness.

Objectives: The aim of this study was to characterize the hepatitis B virus reverse transcriptase (RT) protein variations among Iranian chronic HBV carriers who did not receive any antiviral treatments.

Materials and Methods: Hepatitis B virus partial RT genes from 325 chronic in active carrier patients were amplified and directly sequenced. Nucleotide/amino acid substitutions were identified compared to the sequences obtained from the database.

Results: All strains belonging to genotype D.365 amino-acid substitutions were found. Mutations related to lamivudine, adefovir, telbivudine, and entecavir occurred in (YMDD) 4% (n = 13), (SVQ) 17.23% (n = 56), (M204I/V + L180M) 2.45% (n = 8) and (M204I) 2.76% (n = 9) of patients, respectively.

Conclusions: RT mutants do occur naturally and could be found in HBV carriers who have never received antiviral therapy. However, mutations related to drug resistance in Iranian treatment-naïve chronic HBV patients were found to be higher than other studies published formerly. Chronic HBV patients should be monitored closely prior the commencement of therapy to achieve the best regimen option.

Keywords: Therapy; Drug-Resistance; Hepatitis B Virus; Iran

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▶Implication for health policy/practice/research/medical education:

Hepatitis B virus DNA polymerase primary resistance mutations in treatment-naïve patients have been reported in variable frequencies and with unclear clinical significance in different studies. Understanding Drug-Related Mutation among Treatment-Naïve Chronic HBV Patients can help to prevent viral breakthrough after treatment. Chronic HBV patients should be monitored closely prior the commencement of therapy to achieve the best regimen option.

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1. Background

Nearly 400 million individuals worldwide have been infected with chronic hepatitis B virus (HBV) (1), and of these, 75% are of Asian origin. These patients are at risk of developing progressive liver diseases including fibrosis, cirrhosis, or hepatocellular carcinoma and may require liver transplantation (2). Approved HBV therapies include immune modulators interferon alpha (IFN- α), peginterferon alpha (PegIFN- α 2a), and nucleotide analogues (NAs) such as lamivudine, adefovir, entecavir, telbivudine, tenofovir, etc. Therapy reduces HBV DNA levels as much as possible, ideally less than the lower limit of detection of molecular assays, followed by biochemical remission, histological improvement, and prevention of complications. Although the initial effect of NA in suppressing HBV replication and reducing alanine aminotransferase activity is promising, the emergence of drug-resistant variants considerably reduces the benefit of therapy (3, 4). Drug resistance has been associated with the emergence of polymerase gene mutations that are localized within the RT region that consists of 6 functional domains (G, F, A, B, C, D and E) and 5 interdomains (F-A, A-B, B-C, C-D and D-E) (Figure 1, Tables 1 and 2). Among NAs, lamivudine and adefovir are the most commonly studied in the literature. Viral resistance emerges with both drugs, with frequen-

cies as high as 30 and 80% after 4 years, respectively (5). Evidence now increasingly indicates that drug-related mutation does occur naturally and can be found in HBV carriers who have never received both therapies (6-8). The reported incidence of prevalence of YMDD mutations in treatment-naïve patients varies, ranging from 1 to 18% (9-12) in patients with chronic hepatitis B. The prevalence of HBV in Iran ranges from 1.7 to 2.5% in the general population (2, 13) and both lamivudine and adefovir have widely been used in Iran.

2. Objectives

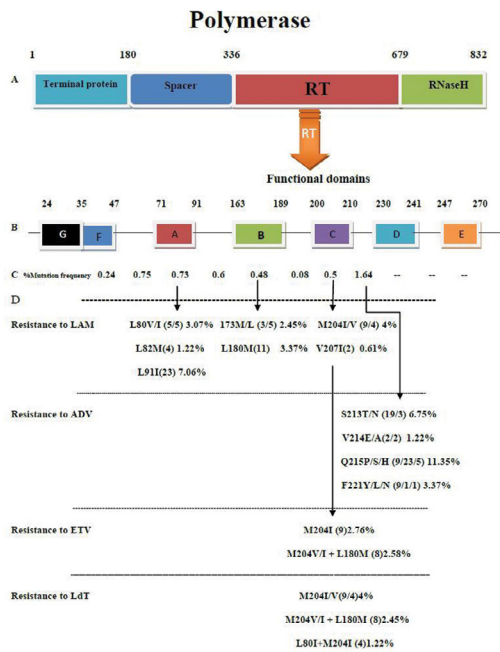
The aim of the present study was to explore the RT protein variations between Iranian HBV chronic carriers who had not received any type of HBV therapy.

3. Materials and Methods

3.1 Patients and Samples

Three hundred and twenty-five HBS Ag-positive patients who were referred to the Iranian Hepatitis Center (Tehran IR Iran) between April 2004 and April 2010, were enrolled in a cross-sectional study. All patients were given their informed consent and the study protocol was approved by the Iranian Hepatitis Network ethics committee. To cover

Figure 1. Schematic Figure Showing Polymerase Protein



A) Four main domains include the RT region, B) RT region including domains and inter domains, C) Mutation frequency in the F domain to the C-D inter-domain, D) Individual drug-resistance mutation to LAM (Lamivudine), ADV (Adefovir), ETV (Entecavir), and LdT (Telbivudine).

all ethnic groups in the country, we studied 10 regions based on population and geographical zones. Exclusion criteria included hepatitis C virus, hepatitis D virus, and human immunodeficiency virus infection. All patients were interviewed and examined by gastroenterologists to evaluate the clinical findings and the results of the investigative workup (liver histology, ultrasonography, and laboratory tests such as serologic, biochemical, and virological tests) to determine the clinical status of the patient. All of the patients were chronic carriers of HBV. Next, 5 ml aliquots of whole blood samples were drawn from each participant. Serum was aseptically separated in the field by centrifugation at 4,000/rpm for 5 minutes and was stored at -20°C until tested. HBV serological markers including HBS Ag and anti-HBS were examined by ELISA kits manufactured by Organon Technika, Holland.

3.2. DNA Extraction and Polymerase Chain Reaction and DNA Sequencing

HBV DNA was extracted from 200 µl of sera using a Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was stored at -20°C. First-round amplification was conducted by RTF CCT GCT GGT GGC TCC AGT TC as the sense primer and RTR CCA CAA TTC (K) TT GAC ATA CTT TCC A as the antisense primer.

Table 1. Mutations at AA Positions of HBV Reverse Transcriptase for Drug Resistance Reported With Known Phenotypic Data ^a

Type	Drugs	References
L80V/I	LAM	(7,14)
V84M	ADV	(32)
S85A	ADV	(32)
I169T	ETV	(50)
V173L	LAM	(7,14)
L180M	LAM, ETV, LDT	(7,14)
A181T/V	LAM, ETV, LDT	(14,25)
T184G	ETV	(7,33)
A194T	TNF	(32,34,50)
S202I/G	ETV	(32,50)
M204I/V	LAM,ETV, LDT	(34,50)
V214A	ADV	(32,51)
Q215S	ADV	(32,51)
I233V	ADV	(32)
N236T	ADV	(32,34,51)
P237H	ADV	(7,32)
M250V	ETV	(32,51)

^a These types of AA changes have been reported to be selected during NA therapy and associated with replication of hepatitis b virus in vitro

Table 2. Mutations at AA Positions of HBV Reverse Transcriptase Reported Genotypic Resistance for Drug Resistance

Type	Drugs	References
S53N	LAM	(7)
T54N	ADV	(7)
L82M	LAM	(31)
I91L	LAM	(7)
Y126C	ADV	(7)
T128I/N	LAM	(31)
N139D/K/H	LAM	(7)
W153Q	LAM	(7)
F166L	LAM	(7)
V191I	LAM,ADV	(31)
A200V/P	LAM	(7,52)
L217R	ADV	(7,52)
V207I	LAM	(31)
S213T	ADV	(7)
S/C256G	LAM,ETV	(7)
L229G/V/W	LAM	(25)
I233V	ADV	(7)
N238K/D/S/T	ADV	(7)
Y245H	ADV	(7)

Second-round PCR was performed by RTNF GCA CAC GGA ATT CCG AGG ACT GGG GAC CCT G as the sense primer and RTNR GAC ACC AAG CTT GGT TAG GGT TTA AAT GTA TAC C as the antisense primer. The first-round PCR was run at 94°C for 4 minutes, followed by 35 cycles at 94°C for 30 seconds, 62°C for 35 seconds, and 72°C for 30 seconds, followed by 72°C for 10 minutes. A similar program was applied for the second-round PCR. Direct sequencing of PCR products was carried out (Perkin Elmer ABI-3130XL DNA Sequencer, Foster city, CA, USA) using 0.5 µl of appropriate primers RTF and RTR for the first cycle, an RTNF and RTNR for the second-round PCR products.

3.3. Mutational Analysis

Amino acid variations within two hundred and thirty amino acid RT regions were compared with reference sequences obtained from different HBV genotype and sequences from Iranian isolates obtained from Gen Bank and NCBI. Comparing to the former, any amino acid changes defined as “variant”. With regards to the latter (Iranian database sequences), amino acid differences defined as “mutation”. The sequences were analyzed using Bio Edit Software version 7.0.5.3. Drug-related mutations in hepatitis B virus reverse transcriptase sequences within RT referred and are listed in *Tables 1* and *2*. The amino acid mutation frequencies were obtained and by drug-resistance mutation found in individual RT domains (A, B, C and F) divided by the number of amino acid residues in

that particular domain. The sequences sent to Gen bank under accession numbers from JX565097 to JX565421.

3.4. HBV Genotyping and Phylogenetic Analysis

HBV genotyping was determined using NCBI Viral Genotyping Tool (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>). Phylogenetic analysis was conducted MEGA version 4. Briefly, sequences of HBV partial RT gene sequences (approximately 700-bp, 325 isolates in this study) were block aligned by the CLUSRAL X program and corrected visually, the Kimuratwo-parameter algorithm was used for genetic distance calculation. A phylogenetic tree was generated along with a different HBV genotype (A to H) sequences retrieved from the Gen Bank as reference genes by the neighbor-joining method, and bootstrapped-sampling and reconstruction was carried out 1000 × to confirm the reliability of the phylogenetic tree.

4. Results

4.1. Patient Characteristics

Three hundred and twenty five HBS Ag-positive chronic patients who were all were chronic carriers enrolled in this study, which were native residents of diverse regions of Iran, HBV DNA positive and treatment-naïve. Of total, 46.47% (151/325) were female and 53.53% (174/325) were male with a mean age of 36 years (SD ± 20). The mean ALT

levels were 29 IU/L (SD \pm 5) and 38% (123/325) were HBeAg positive.

4.2. Genotypic and Phylogenetic Analysis

The phylogenetic tree was constructed using the alignment of HBVRT region of patients (325 isolates from this study) along with different HBV genotype (A to H) sequences retrieved from the Gen Bank as reference genes. The phylogenetic tree revealed that all Iranian isolates were branched with other genotype D of HBV reference isolates with a high boot strap value, 99%, 1000 \times replicates (Figure 2).

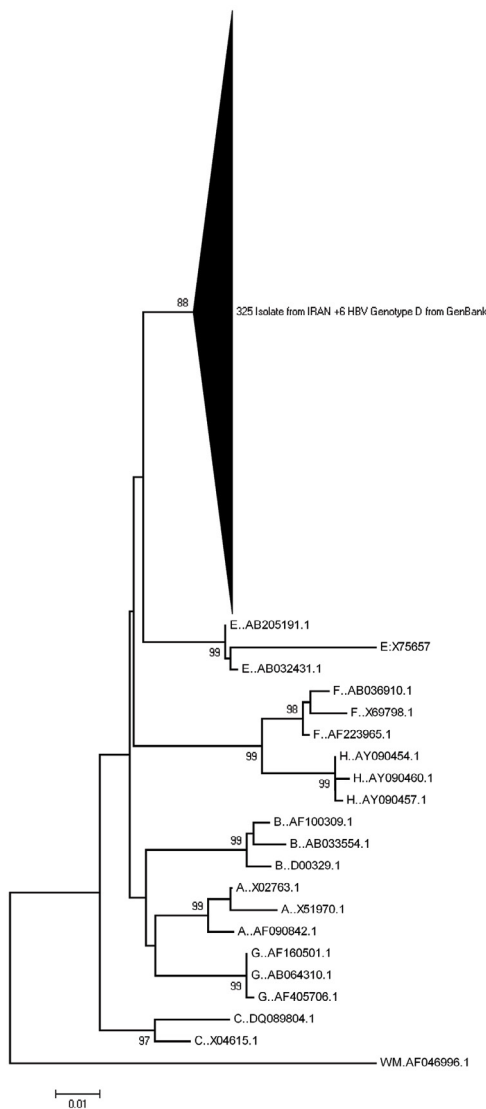


Figure 2. Neighbor joining phylogenetic analysis based on the alignment of the RT gene region (approximately 750-bp) of 325 HBV isolates from Iran as well as other HBV genotypes from Gen Bank as reference. Boot-strap values indicate 1000-fold replicates. Due to clarity, the 325 isolates from Iran and 6 reference genes of HBV genotype D was collapsed. Woolly monkey HBV was used as the out-group sequence.

4.3. Analysis of Amino Acid Substitutions

Compared to the reference genotypes D and according to the above-mentioned criteria for differentiation between variants and mutants, all patients contained at least one amino-acid mutation in the RT region. The total number of amino-acid mutations was 365, of which 14.5% (n = 53), 11.2% (n = 41), and 4.9% (n = 18) were found within the A, B and C domains, respectively. The mutation frequencies in the functional domains of the partial RTs were 0.73% (53/6825) in Domain A, 0.48% (41/8450) in Domain B, and 0.50% (18/3575) in Domain C. The average amino-acid substitution was 0.44%, indicating the probability of changes per position. In the mutational analysis of the RT inter domains, the substitutions and mutation frequencies were found in A-B, 0.60% (141/23400), B-C, 0.08% (3/3575), and C-D, 1.64% (107/6500) inter domains (Figure 1).

4.4. Lamivudine-Related Resistance Mutations

Common and frequent lamivudine-related amino acid substitutions were found in this study including N53K 3.68% (12/325), L80V/I 3.07% (each 5/325), L82M 1.22% (4/325), L91I 7.06% (23/325), T128I 1.53% (5/325), L180M 3.37% (11/325), and M204I 2.76% (9/325), M204V 1.23% (4/325) see Figure 1.

4.5. Adefovir-Related Resistance Mutation

RT sequence changes implicated in adefovir-resistance were detected, including S213T 5.83% (19/325), S213N 0.9% (3/325), V214E 0.61% (2/325), V214A 0.61% (2/325), Q215P 2.75% (9/325), Q215S 7.06% (23/325), Q215H 1.53% (5/325), and F221Y 2.76% (9/325) of isolations that previously reported adefovir-resistance mutation (Figure 1).

4.6. Other Drug-Related Resistance Mutation and Anti-Viral Cross-Resistance Mutations

Cross-resistance refers to the situation in which a decreased susceptibility to more than one antiviral drug is conferred by the same amino acid substitution or combination of amino acid substitutions. Cross-resistance mutations to lamivudine and telbivudine were: M204I 2.76% (9/325), M204V 1.23% (4/325), M204V/I + L180M 2.45% (8/325), and L80I + M204I 1.23% (4/325). Cross-resistance mutations to lamivudine and entecavir were: M204I 2.76% (9/325) and M204V/I + L180M 2.45% (8/325) (Figure 1).

5. Discussion

Hepatitis B virus DNA polymerase primary resistance mutations in treatment-naïve patients have been reported in variable frequencies and with unclear clinical significance in different studies (9-12). The aim of this study was to characterize the mutational patterns of RT protein in chronic HBV carriers. All 325 patients included in this study were treatment naïve. All had been infected with genotype D. The results showed that viral mutations related to lamivudine, adefovir, telbivudine, and

entecavir resistance were discovered in patients who had never been treated with these drugs, with various mutation frequencies ranging between 1.23% (for M204V) and 7.06% (for L91I). However in the present study, the molecular cloning was not performed, and the results of HBV DNA polymerase mutations were obtained from direct sequencing techniques; therefore, the actual number of mutations might be underestimated (14). Lamivudine was the first effective oral HBV-replication suppressive agent to enter widespread clinical use; however, its use had been challenged with the emergence of resistant mutations, which increased from 23% to 80% following the first and fourth years, respectively (15, 16). The most commonly described mutations are the substitution of valine or isoleucine for methionine at residue 204 (rtM204I/V). These YMDD motif mutations are necessary and sufficient to confer a high level of lamivudine resistance. For this specific mutation, the rtL180M is the main compensatory change. Other compensatory mutations include the rtV173L and rtL80I changes (17, 18). The rtA181T change has been reported to occur in the absence of rtM204I/V and is considered a primary resistance mutation (19, 20). In this study we found that L180M 3.37% (11), M204I 2.76% (9), and M204V 1.23% (4) were more common than had been reported previously in Iran (21, 22). Our results fall between the extremes reported by other studies around the world (7-12, 23). A Adefovir dipivoxil has been shown to be effective against both wild-type and lamivudine-resistant HBV both in vitro and in vivo (18-20). Compared to the lamivudine, resistance to ADV usually occurs less frequently and takes longer to emerge. Adefovir-resistant HBV mutations have not been detected in patients up to 48 weeks into therapy and increase to 3%, 11%, 18%, and 29% following 2, 3, 4, and 5 years of therapy, respectively (22, 24). HBV genotype D is associated with an increased risk of adefovir resistance (11, 25). Adefovir resistance has been associated with a primary mutation in the D domain at rtN236T and rtA181T/V in the B domain. In addition, a number of other mutations have been detected in our study and were clustered into three distinct regions of the RT: the D and A domains (rtP237H, rtN238T/D, rtV84M, and rtS85A) and the C-D interdomain (rtS213T/N, rtV214A, and rtQ215S) (26-31). The latter finding had a mutation frequency of 1.64% and was higher than the other domains studied (Figure 1). These mutations may be regarded as secondary resistance mutations, as they have been associated only with very low-level resistance in vitro (22, 26). These secondary mutations have also been detected in the absence of rtN236T (both alone and in combination) in patients who have either not responded to or have had a virological breakthrough during adefovir treatment (32-34). The high number of adefovir-related mutation in this region may be indicative of an increased risk of adefovir resistance in genotype D-infected patients if they receive this drug (11, 25). Preliminary evidence indicates that the primary lamivudine-resistance mutation

rtM204V/I seems to be a prerequisite for the development of entecavir and telbivudine resistance (35). Telbivudine and entecavir-related RT mutations were also found in this study. M204I/V and M204V/I/L180M are related to entecavir resistance (36-39). Recently, the GLOBE-Trial demonstrated that in vitro telbivudine resistance is conferred by either rtM204I or rtM204V+rtL180M and L80I/M204I but not by the rtM204V mutation alone, while in vivo telbivudine resistance is almost exclusively due to the presence of M204I. The reasons for the presence of such mutations in treatment-naïve chronic patients are not clear. Polymerase is an essential structural protein with a high error rate resembling RNA viruses. As a quasi-species in the hepatitis B virus pool, a cocktail of variants could be found in a single patient even in each body compartment with distinguished sequences from the master sequence (wild type). Due to a relatively high mutation rate of HBV (a rate between cellular and RNA virus replication), a tremendous number of mutations are produced and accumulate as spectra within the host. Some of these variants are not necessarily infectious (40). Furthermore, intensive investigation on RNA viruses (with a mutation rate close to HBV) shows that even in the absence of selective pressure, a minority of sequence variants exist as "memory," which is a property of the population as a whole contained within the components of the mutant spectra (41, 42). This memory provides an adaptive advantage to the viral populations (43) and is eliminated when severe bottlenecks occur (44, 45). They gain fitness and can constitute a reservoir of new variants that display increased replication capacity in the environment together with the dominant genome populations (46). Together, these preexisting variants, which have less replicative ability than wild-type variants, can be reorganized in the presence of drugs in vivo and in vitro (like LAM, ADV, etc.) and encompass the wild-type variants, which has led to the drug-resistance phenomenon as it has shown for HCV (47) and HIV (47, 48) viruses. From a clinical perspective, the treatment-naïve patients in our study may have been infected with strains from other patients who had been treated with the corresponding nucleotide analogues. In this regard, differences in the distribution of HLA antigens or other immune genes across diverse geographical areas probably contributed to the selection of amino-acid variations. However, primary data need to be accumulated to prove this hypothesis (49). In conclusion, the clinical impact of pretreatment mutations on the efficacy of antiviral therapy should be better characterized. For the desired benefits in a cost-effective manner in the management of chronic HBV patients, genotypic and phenotypic screening before the decision to pursue antiviral therapy (as a genotypic approach) seems to be advisable.

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Authors' Contribution

All authors have been contributed either in the process of sampling or serological and molecular analysis as well as results interpretation.

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