Introduction

Hepatitis B virus (HBV) infection is a serious world health problem. Approximately, 400 million people are chronically infected with the virus worldwide. Liver cirrhosis and hepatocellular carcinoma have been considered as lethal complications of chronic hepatitis B infection. HBV is a member of the family Hepadnaviridae and has partially double-stranded DNA with length of nearly 3.2 kilo base pairs.[1] To date, interferon and 5 nucleos(t)ide analogues (NAs) including: Lamivudine (LAM), telbivudine, entecavir, adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate have been approved for the treatment of chronic hepatitis B (CHB) infection.[2] These antiviral drugs have favorable effects and relatively few side effects, although in some cases, they cannot completely clear the HBV DNA and lead to long-term therapy. Emergence of drug-resistant HBV mutants is an adverse consequence of long-term therapy.[3] High mutational rate of HBV reverse transcriptase (RT) enzyme, in order to its lack of proof-reading activity, is the main cause of HBV mutation occurrence and drug resistance.[4] HBV mutations developing NAs resistance could be divided into primary and secondary (or compensatory). Primary resistance mutations contain an amino acid substitution that significantly decrease susceptibility to an antiviral NA, while secondary resistance mutations have no direct role and restore functional defects of HBV mutant containing primary mutations and enhance resistance to antiviral agents.[5] Most common primary and secondary resistance mutations have been occurred within domain A, B, C and D of HBV RT region.[4] On the basis of HBV RT region overlapping with hepatitis B surface antigen (HBsAg) open reading frame, occurrence of each mutation within RT region can reflect a nucleotide mutation in the HBsAg. These mutations may cause immune escape and accordingly HBV reactivation even in anti-HBs positive patients as well as failure of HBsAg diagnostic assays.[6]

Of note, many clinical studies have reported resistance mutations can naturally occur within HBV RT region in some antiviral-untreated patients. In these cases, mutations are present several months earlier than the phenotypic resistance. These findings suggest that HBV resistant
mutants pre-exist as minor viral population and under conditions of continuous NAs-therapy and selective pressure can gradually emerge as major viral population leading to treatment failure.[7]

To our knowledge, there is no data about mutations pattern of HBV RT region and its variability among chronically infected naive patients in our region. Consequently, the aim of present study was evaluation of mutations occurring spontaneously within HBV RT region among untreated chronic HBV-infected patients in Ahvaz city located in south-west of Iran. It may be helpful for management of chronic HBV infection and prognosis of viral breakthrough.[4,8]

Materials and Methods

Patients and samples

This cross-sectional study (January 2011 till February 2012) was included forty-five chronic HBV-infected patients. Forty patients had HBsAg for more than 6 months and high level of liver enzymes (more than 1.5 times the normal limit) and 5 patients had HBsAg for more than 6 months and pathological signs of chronic hepatitis in their liver biopsies. All patients had not received any antiviral drugs in recent 1 year. Serum samples were immediately separated from patients’ blood and stored at −70ºC until DNA extraction step. HBV DNA and allanine-aminotransferase (ALT) enzyme level were measured for all patients by HBV Quanti cation Advanced kit (PrimerDesign, Southampton, UK) and ALT kit (Pars Azmun, Tehran, Iran), respectively. All samples were tested for hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe) by ELISA. Furthermore, hepatitis C virus antibody (anti-HCV), hepatitis D virus antibody (anti-HDV), human immunodeficiency virus antibody (anti-HIV) were assessed for all samples using ELISA. DiaPro kits (Diagnostic Bioprobes, Milan, Italy) were utilized for ELISA. All protocols of assays, cut-offs and result interpretations were performed according to manufacturers’ instructions.

Primer design and PCR amplification

Three hundred Sequences of HBV polymerase gene were downloaded from the GenBank sequence database (NCBI, U.S. National Library of Medicine, Bethesda, USA, http://www.ncbi.nlm.nih.gov). Then, sequences were aligned by using Mega 5 program.[9] After alignment, on the basis of conserve regions of HBV RT, three primers for semi-nested PCR were designed [Table 1] which encompassed catalytic sites of HBV RT region [Figure 1]. Specificity and characteristics of the primers were evaluated using BLAST (http://www.ncbi.nlm.nih.gov), in silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr) and Oligo Analyzer (http://eu.idtdna.com/ analyzer/Applications- OligoAnalyzer) softwares. Designed primers were synthesized by Bioneer Corporation (Bioneer, Daejeon, South Korea). HBV DNA was extracted from sample sera by using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instruction, and eluted products were stored at −70ºC until PCR performance. All PCR components were purchased from Roche Diagnostics GmbH, Mannheim, Germany. First round of semi-nested PCR assay was performed in 25 µl volume, containing: 5 µl of HBV DNA as template, 0.5 µl of dNTPs 10 mM, 3 µl of MgCl₂ solution 25mM, 0.25 µl Expand High FidelityPLUS enzyme 5 U/µl, 5 µl of 5X Expand High FidelityPLUS reaction buffer, 2 µl of each primers RTMo-F and RTMo-R2 10 pM and 7.25 µl of double-distilled water. Thermal cycler (TC-512, Techne, Staffordshire, UK) was programmed for the first round of semi-nested PCR as follows: Initial denaturation at 94ºC for 5 min, 30 cycles consisting of 94ºC for 30 sec, 67ºC for 40 sec and 72ºC for 70 sec, finally 72ºC for 5 min. Second round of semi-nested PCR was carried out in 50 µl volume, including: 5 µl of first round product, 1 µl of dNTPs 10 mM, 6 µl of MgCl₂ solution 25 mM, 0.5 µl Expand High FidelityPLUS enzyme.

Table 1: Sequences of designed primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTMo-F (sense)</td>
<td>247-271</td>
<td>TCTAGACTCGTGGTGACTTCTCT</td>
</tr>
<tr>
<td>RTMo-R2 (antisense)</td>
<td>1603-1582</td>
<td>CGTGCAGAGGTTGAACGAAGTG</td>
</tr>
<tr>
<td>RTMo-R1 (antisense)</td>
<td>1261-1280</td>
<td>AGTTCCGCAGTTGGATCGG</td>
</tr>
</tbody>
</table>

Figure 1: HBV RT region and primers position. HBV polymerase gene has four regions including: Terminal protein, spacer, reverse transcriptase (RT) and RNaseH. Designed primers surround catalytic sites of RT region.
5 U/µl, 10 µl of 5X Expand High FidelityPLUS reaction buffer, 3 µl of each primers RTMo-F and RTMo-R1 10 pM and 21.5 µl of double-distilled water. PCR conditions for second round of semi-nested PCR were as follows: Initial denaturation at 94°C for 5 min, 25 cycles consisting of 94°C for 30 sec, 68°C for 40 sec and 72°C for 60 sec, finally 72°C for 5 min. Five µl of PCR products of second round stage were subjected to 1% agarose gel electrophoresis. Amplicon length was 1034 base pairs (bp), stained using DNA Safe Stain solution (CinnaGen, Tehran, Iran) and visualized by UV-transilluminator (Vilber Lourmat, Paris, France). HBsAg-positive serum and double-distilled water were used as positive and negative control (no template), respectively.

**Mutation analysis of HBV RT region**

After PCR amplification of HBV RT, PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Purified products were sequenced by Bioneer Corporation (Bioneer, Daejeon, South Korea). Mutation analysis of the sequenced samples were carried out manually and using web-based tools. For manual analysis, the acquired nucleotide sequences were translated and compared with a set of reference sequences for HBV wild-type available at http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi and http://hivdb.stanford.edu/HBV/HBVseq/development/input/isc/RTgenotypes_consensusAA.fasta.txt.[10] On the basis of differences between each sequence and consensus reference sequences, the mutations were determined. Mutation analysis using web-based tools was performed by geno2pheno and HBVseq programs from http://www.geno2pheno.org/ and http://hivdb.stanford.edu/HBV/release Notes/, respectively.[10,11] The submitted sequence was evaluated by these programs and its mutations were detected.

In this study, 14 well-characterized mutations associated with NAs-resistance (rtT169N, rtL180M, rtA181T/V/S, rtT184A/I/S, rtM204V/I/S, rtN236T and rtM250V) and 16 secondary or compensatory resistance mutations associated with reduction of susceptibility to NAs (rtL80I/V, rtN94R, rtV173L, rtS202I, rtV207I/L, rtV214A/E/L, rtQ215S/H/P, rtS219A, rtF221Y, rtI233V and rtN238H) were analyzed.[7,10,11] The sequences of our study have been summarized in Table 2. A 1034 bp fragment was seen for all samples after semi-nested PCR performance.

**Confirmation of novel and multiple amino acid substitutions**

First, the PCR products of the samples of 68-R1, 6-R1 and 16-R1 containing novel amino acid changes (rtV214T, rtQ215M and rtF221V, respectively) and the samples of 89-R1, 62-R1, 6-R1, 22-R1 and 94-R1 containing multiple resistance mutations (rtL80I + rtM204I, rtV207I + rtV214I, rtV214E + rtQ215M, rtQ215P + rtN238H and rtQ215P + rtN238H, respectively) were purified by High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Then, each purified PCR product was cloned into the pPrime Cloning vector using Perfect PCR Cloning kit (5 PRIME GmbH, Hamburg, Germany). The recombinant vectors were transformed into the Escherichia coli (E. coli) DH5α strain (CinnaGen, Tehran, Iran). After blue-white screening, three white colonies of each sample were selected and picked out. These colonies were evaluated by colony PCR and sequencing technique. Colony PCR was performed using standard sequencing primers (T7 promoter primer as forward and SP6 promoter primer as reverse). The recombinant vector was isolated using High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) followed by nucleotide sequencing (Bioneer, Daejeon, South Korea). Nucleotide sequencing was performed by the same standard primers. PCR product purification, cloning and plasmid isolation steps were carried out according to the manufacturers’ instructions.

**HBV genotyping**

HBV genotyping was carried out using NCBI genotyping tool (U.S. National Library of Medicine, Bethesda, USA, http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). NCBI genotyping tool utilizes BLAST to compare each sequence with reference sequences for HBV genotypes and then determines the genotype of submitted sequence.[9]

**Results**

**Patients**

The mean age of patients was 41 years (range of 18-70 years). Out of 45 patients, 32 (71%) cases were male and 13 (29%) cases were female. Patient’s sera were negative for anti-HCV, HDV and HIV. Serological, biochemical and viral parameters tested in our study, have been summarized in Table 2. A 1034 bp fragment was seen for all samples after semi-nested PCR performance.

**Mutation analysis of HBV RT region**

Primary resistance mutations of HBV RT region were found in 3 (6.6%) cases. Of these 3 patients, 2 had rtA181T and rtA181S substitutions and one patient had rtM204I substitution [Figure 2]. While secondary/compensatory resistance mutations were detected in 20 (44.4%) cases. The most frequent secondary resistance mutation was found at position rt215 (10 patients, 50%) which included rtQ215S mutation in 4 patients, rtQ215P mutation in 3 patients, rtQ215H mutation in 2 patients and rtQ215M mutation in 1 patient. The rtN238H mutation was detected in 2 (10%) patients. Finally, each mutation of rtL80I, rtV207I, rtV214I, rtV214E, rtV214T, rtS219A, rtF221V and rtF221Y were found in 1 (5%) patient [Table 3]. In this study, we identified three novel amino acid substitutions rtV214T, rtQ215M and
Following mutations were not detected in patients: rtI169T, rtV173L, rtL180M, rtT184A/I/S, rtM204V/S, rtN236T, rtM250V, rtL80V, rtN94R, rtS202I, rtV207L, rtV214A and rtI233V.

Confirmation of novel and multiple amino acid substitutions

After colony PCR, a band with 1213 bp length was seen for the selected colonies. The results of nucleotide sequencing showed that the selected colonies of the samples of 68-R1, 6-R1 and 16-R1 had rtV214T, rtQ215M and rtF221V amino acid substitution, respectively. Also the sequencing results for the samples containing multiple resistance mutations showed that the samples of 89-R1, 62-R1, 6-R1, 22-R1 and 94-R1 had rtL80I + rtM204I, rtV207I + rtV214I, rtV214E + rtQ215M, rtQ215P + rtN238H and rtQ215P + rtN238H amino acid substitutions, respectively. Accordingly, three novel amino acid changes (rtV214T, rtQ215M and rtF221V) and five multiple resistance mutations were confirmed.

**HBV genotyping**

The results obtained from NCBI genotyping tool revealed that all patients had HBV genotype D.

**Discussion**

In the present study, pretreatment mutations have been analyzed in HBV chronically infected naïve patients. Many studies have shown that mutations occur spontaneously in YMDD motif of HBV RT region among untreated patients, although it has...
been remained controversial. As, AB-Olyae et al.,[12,13] reported no YMDD mutant (0%) among lamivudine-untreated patients, while Wang et al.,[14] determined 31.7% for incidence of YMDD motif mutation. Also, Tan et al., reported that overall incidence of spontaneously occurring YMDD mutation in lamivudine-untreated patients was 12.21%. They suggested that the incidence of YMDD mutant is not associated with gender, age, HBsAg and ALT level.[8]

Most papers have focused on evaluation of mutations in YMDD motif (domain C) for its important role in lamivudine resistance, while occurrence of mutation within other domain of HBV RT region is important and causes drug resistance. In the recent years, different NAs such as adefovir and entecavir other than lamivudine have utilized for treatment of CHB patients. This has led to an increase in drug-resistance patients with broad spectrum of mutation pattern. Accordingly, mutation analysis of more HBV RT domain could help management and treatment of CHB patients.

In this study, the YMDD mutant was detected in one (2.2%) untreated patient in which amino acid methionine has been replaced by isoleucine (YIDD). Other studies have showed the prevalence of 0%-5.3% for YMDD mutant among Iranian lamivudine-untreated patients.[12,15] Also, two mutations (4.4%) at codon rt181 were found, including rtA181T and rtA181S. It has been investigated that amino acid changes at codon rt181 (rtA181T/V/S) confer resistance to adefovir and treatment failure.[16,17] There is no report for detection of rt181 changes in Iranian naïve patients.

We indentified mutation at codon rt215 as the most prevalent mutation (50%) in HBV RT region of untreated patients. The amino acid changes for this codon were as rtQ215S/P/H/M. In addition, mutations at codon rt214, rt221 and rt238 have been found in later frequency among untreated patients. Several studies have described that amino acid changes at codons: rt214, rt215, rt221 and rt238 induce effectiveness reduction to adefovir i.e. these mutation are considered as adefovir secondary resistance mutations.[14,17] HBV genotypes of our patients were also determined. All of patients had HBV genotype D. This result was in quite concordance with other study results of Iran.[15,19] Moreover, some of amino acid changes including rtI169T, rtT184G, rtS202I and rtM250V have not been detected in our study. Interestingly, these changes have direct or indirect role in occurrence of entecavir resistance.[20] Finally, we found 3 novel amino acid changes including: rtV214T, rtQ215M and rtF221V. These changes have not been reported prior to the present study.[15,18,21]

Conclusion

Results of this study indicated that mutation may be pre-existent in HBV RT region among untreated chronically HBV-infected patients. Primary and secondary resistance mutations have been detected in considerable prevalence (51.1%) of our patients. These amino acid changes lead to lamivudine and particularly adefovir resistance. Our findings suggest that untreated patients may be faced with resistance to adefovir and treatment failure; by contrast, entecavir administration could be considered better response to antiviral therapy. Finally, mutation analysis of HBV RT region could be a helpful tool for drug selection and prognosis of treatment of CHB.

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References


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