Hepatitis B virus (HBV) genotype and YMDD motif mutation profile among patients infected with HBV and untreated with lamivudine

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Received 31 October 2006; received in revised form 9 May 2007; accepted 16 May 2007

Corresponding Editor: Jane Zuckerman, London, UK

Introduction

Hepatitis B infection has long been a serious public health problem in the world, and is one of the main causes of acute and...
chronic hepatitis infections in man. Nearly 400 million persons are infected chronically with the hepatitis B virus (HBV) worldwide, and it has been demonstrated that chronic infection with this virus is linked to the development of hepatocellular carcinoma (HCC).

Genotypically, HBV has been grouped into eight genotypes, from A to H, which show distinctive geographical distribution across the entire world. It has been shown that HBV genotype is associated with certain prognoses, the clinical picture, and especially the outcome of response to treatment.

Lamivudine, a nucleoside analogue, has been shown to be very effective for the treatment of hepatitis B; however, viral mutants resistant to this medicine have emerged. Lamivudine-resistant HBV has been reported frequently and is associated with mutations of the YMDD motif in the HBV polymerase gene. It has also been demonstrated that YIDD and YVDD are the two main types of mutations among HBV-infected lamivudine-resistant patients. However, interestingly, some reports have indicated that YIDD and YVDD HBV-drug resistant mutants can also be detected in infected individuals not treated with lamivudine.

Our previous surveys have shown genotype D of HBV and the wild type YMDD pattern among Iranian populations infected with the virus, although the number of patients enrolled in these studies was small (ca. thirty subjects). In this study, the study population was increased to 147 HBV-infected patients to investigate HBV genotype distribution. Also, the YMDD drug-resistant mutation profile among infected patients not treated with lamivudine was explored in order to better understand the variability of the YMDD motif before antiviral therapy.

Materials and methods

Study population and clinical samples

The study population consisted of individuals referred to different hospitals in Tehran for various types of operations. In Iran, according to public health guidelines, those individuals who undergo any surgical procedures have to be examined for blood-borne viruses such as HBV, hepatitis C virus (HCV), and human immunodeficiency virus (HIV). In order to categorically obtain HBV-infected subjects not treated with lamivudine, only those operation candidates who were accidentally positive for HBV markers and negative for HCV and HIV markers were enrolled in this survey. The serological tests were carried out using available ELISA commercial assays (Abbott Laboratories, North Chicago, IL, USA). The majority of these individuals were not aware of their infection, thus they had not been treated. One hundred forty-seven HBV-infected blood samples not treated with lamivudine were collected from several hospitals in Tehran from 2002 to 2006. Blood samples were centrifuged immediately, and the sera separated, aliquoted, and then kept frozen at −20 °C until testing.

Gap-PCR and artificially created restriction site-PCR (ACRS-PCR)

Sera were thawed and centrifuged at 12,000 rpm for 10 minutes; HBV DNA was extracted by the DNG™-plus DNA extraction kit (CinnaGen, Tehran, Iran). Genotype D of HBV among Iranian patients was determined according to Eroglu et al. (the gap-PCR method with minor modification in primers and thermal-cycling program). The primers in this method specifically distinguish genotype D of HBV from non-D, based on the specific deletion in the pre-S1 region that occurs naturally in genotype D of HBV. This can be a simple and cost-effective method for assessing the molecular epidemiology of HBV, especially in those areas of the world where the genotype D of HBV has high prevalence, such as the Middle East and the Mediterranean countries. Briefly, a pair of primers was designed (for-D: 5’-TGG GAA CAA GAG CAT CAG CAT GG-3’ and rev-D: 5’-CAA CTG GTG GTG GGG AAA GAA TC-3’) in the two sides of deletion in the genotype D of HBV pre-S1 region to discriminate genotype D from non-D (Figure 1). After PCR optimization, the PCR reaction consisted of the following: 1 × PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs (Fermentas, Vilnius, Lithuania), 0.5 μM primer for-D and primer rev-D, 1 U Taq DNA polymerase (CinnaGen), and 5 μl HBV DNA. The following PCR thermal-cycling program was carried out: 95 °C for 3 min, 45 cycles consisting of 94 °C for 25 s, 65 °C for 25 s, and 72 °C for 25 s, followed by 72 °C for 3 min. The plasmids containing the full genome of HBV genotype D and A were utilized as controls for the gap-PCR method. Control plasmids for genotype D were achieved from five complete genome sequences of Iranian isolates, and full-length genome control plasmids for genotype A of HBV, kindly provided by Dr Frank Tacke, RWTH-University Hospital Aachen, Germany, were used as a reference control for non-D (A, B, C, E, F, G, and H) genotypes. In each run, positive and negative controls were included, and the validity of the results was assessed by comparing with the values of controls.

ACRS-PCR was carried out by an in-house developed method with an internal-control digestion that distinguishes mutations in the YMDD motif as previously described by our group. In brief, three conserved and diagnostic primers were utilized as controls for the gap-PCR method. Control plasmids for genotype D were achieved from five complete genome sequences of Iranian isolates, and full-length genome control plasmids for genotype A of HBV, kindly provided by Dr Frank Tacke, RWTH-University Hospital Aachen, Germany, were used as a reference control for non-D (A, B, C, E, F, G, and H) genotypes. In each run, positive and negative controls were included, and the validity of the results was assessed by comparing with the values of controls.

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Figure 1 A schematic diagram of the gap-PCR method. The position of the primers at the HBV pre-S1 region is depicted. Lines show deletions; dots point to similar nucleotides. Each sequence is presented as the consensus of each genotype. Genotype A of HBV is shown in detail. The size of amplicon length in genotype D of HBV is around 80-bp and for non-D (A, B, C, E, F, G, and H) is around 110-bp.
were generated to identify YMDD, YVDD, and YIDD strains with certain restriction endonuclease enzymes, and their reverse primers were also modified in order to monitor enzyme correctness and to avoid a false outcome. In this survey, plasmids containing YMDD, YIDD, YVDD, and seven additional known positive samples with certain YMDD mutations confirmed by INNO-LiPA assay were also applied, as reported in our previous study.\textsuperscript{15}

**Sequencing and phylogenetic analysis**

Eleven samples were randomly selected for sequencing and phylogenetic analysis. Seven samples were the positive controls for YMDD mutants and four samples were selected from untreated patients. The HBV polymerase gene was intentionally chosen for sequencing, not only to verify the YMDD motif mutation and then ACRS-PCR accuracy, but also to check the HBV genotype and the validity of the gap-PCR method. After the amplification of polymerase gene, the amplicons were purified by Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and bi-directionally sequenced using the BigDye\textsuperscript{10} Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) at the Sequence Laboratories Göttingen GmbH (SEQLAB) in Germany. To perform the phylogenetic analysis, Iranian HBV genome sequences were compared with those of different defined HBV strains retrieved from GenBank as reference genes, as reported previously by Rozanov et al.\textsuperscript{16} Alignment was performed by CLUSTAL X program; genetic distance was calculated using the Kimura two-parameter algorithm; the phylogenetic tree was constructed by the neighbor-joining method and bootstrap re-sampling, and reconstruction was carried out 1000 times to confirm the reliability of the phylogenetic tree. In this study, MEGA2 software was utilized for phylogenetic and evolutionary analysis. The HBV gene sequences used in this study were deposited in GenBank under accession numbers DQ839509—DQ839519.

**Results**

The study population consisted of 107 males and 40 females with a mean age of about 41 years (age range 19—75 years). Of 147 samples, 125 were successfully amplified for both genotyping (gap-PCR) and YMDD mutant detection (ACRS-PCR). Liver function tests were normal as AST and ALT values were less than 45 U/l; other liver parameters such as bilirubin and total protein also had normal values. Laboratory results revealed that all of the 147 untreated patients could be categorized as asymptomatic HBV carriers. Serological data showed that all patients had positive HBsAg status and had negative serological markers for HCV and HIV.

To determine the genotype, the simple gap-PCR method was applied, which could readily distinguish the genotype D of HBV from non-D. The gap-PCR method showed that all HBV strains isolated from Iranian patients had an 80-bp amplicon, the specific length for genotype D of HBV, and not the 110-bp amplicon length of non-D genotype. To confirm the gap-PCR results, some samples were randomly sequenced and deposited in GenBank under accession numbers DQ839509—DQ839519.

**Figure 2** The neighbor-joining phylogenetic analysis of 11 Iranian HBV isolates reveals that Iranian isolates are grouped with the other genotype D reference genes branch with 99% bootstrap value (1000\times replicates). The bootstrap value below 70% is not shown. Iranian isolates have the IR-TMU prefix. The scale bar indicates 2% nucleotide divergence. Other HBV genotypes are shown in capital letters.
analyzed. The phylogenetic analysis results confirmed that Iranian isolates were clustered in the genotype D branch with other genotype D HBV reference genes (Figure 2). These isolates branched with genotype D reference isolates with a 99% bootstrap value (1000× replicates). The phylogenetic analysis data were completely in agreement with the gap-PCR results.

In this study, no evidence of mutation in the YMDD motif among 147 Iranian HBV-infected patients not treated with lamivudine was determined by the ACRS-PCR assay. The amino acid comparison results deduced from the polymerase gene sequences were also in agreement with those of this method for all 11 patients (seven patients with mutation (controls) and four untreated patients with wild-type pattern in the YMDD motif).

Discussion

HBV possesses distinct geographical distribution and clinical outcomes worldwide. Genotype A is pandemic, but most prevalent in northwest Europe, North America, and Central Africa; genotypes B and C are found in East Asia, Korea, China, and Japan; genotype D is also more or less pandemic, but is predominant in the Mediterranean area and the Middle East expanding into India; genotype E is typical of Africa; genotype G has been found in the USA and France; genotype H has been reported from Nicaragua, Mexico, and California; and the origin of genotype F is proposed to be in the New World because genotype F of HBV is predominant in Central and South America.17 In the Mediterranean region,18 the Middle East,19 and in particular, in Iran,12,13 genotype D of HBV is highly predominant. In this study, a simple and cost-effective gap-PCR method was developed and utilized to determine HBV genotype D from non-D. The gap-PCR results on 147 patients revealed that all Iranian HBV-infected subjects were unsurprisingly infected with genotype D of HBV, similar to the results of our previous studies with smaller population sizes.

The advantage of the gap-PCR method is that it is a very simple and cost-effective method in comparison with the expensive sequencing method, and can be done in all laboratories performing routine testing. Of course, it has some limitations as well. This method is useful only for areas where genotype D of HBV is predominant, because it distinguishes HBV genotype D from non-D. In the case of genotype non-D detection of HBV by this method, the sample requires further sequencing in order to determine the HBV genotype. However, in the endemic genotype D regions, the rate of non-D is normally very low; hence, only a few samples would need to be sequenced, leading to a considerable reduction in costs.

It has been demonstrated that HBV genotype can affect the clinical outcome of chronic HBV infection.1 For instance, genotype D of HBV is associated with more severe liver disease than genotype A and may lead to HCC in young patients.6,20 It has also been reported that genotype D of HBV has a lower response rate to lamivudine in comparison with genotype A of HBV.21 Therefore, understanding of HBV genotype distribution seems to be highly important for the better management of HBV infection by medical personnel and for the improvement of public health.

Lamivudine inhibits viral DNA polymerase in HBV and can be effective for rapidly reducing the serum HBV DNA titer.7 However, lamivudine resistance among HBV-infected patients during therapy has been reported (as much as 10–45% during the first year of treatment).22 It has been demonstrated that the emergence of lamivudine-resistant HBV is associated with mutations of the YMDD motif within the conserved sub-domain C in the polymerase gene.9 Interestingly, lamivudine-resistant mutants have been reported among patients not treated with lamivudine with a range of 1–27%.10,11,23–26 It has been shown that there exists a relationship between HBV genotypes and YMDD mutant profile in untreated patients. Horgan and coworkers revealed that among untreated patients, genotype D of HBV had no mutation in the YMDD motif, whereas untreated patients with genotype C of HBV had mutations in the YMDD motif.26

Antiviral drug resistance is currently a major problem in the management of patients with chronic HBV infection. It has been demonstrated that the mutation in the YMDD motif can directly affect HBV replication and may affect the outcome of the disease. For example, it has been verified that the precore (A1896A) and, in particular, basic core promoter mutations (A1762T/G1764A) result in increased replication capacities together with lamivudine-resistant mutations compared with those of wild-type virus. Likewise, it has been revealed that the combination of lamivudine-resistant mutations with the ‘a’ determinent mutation has enhanced the replication of the virus as well.27 Moreover, because of the proximity of the pol-gene and the S-gene in HBV genome; mutations in the pol-gene can also alter the envelope gene sequence and consequently may result in vaccine escape or diminished HBsAg secretion.28 Therefore, because of the importance of drug-resistant strains in the treatment strategy, we explored the rate of YMDD mutants among untreated patients in this study; the result of the ACRS-PCR method revealed no mutation among Iranian subjects not treated with lamivudine. Hence, further investigations need to be carried out to understand whether lamivudine-resistant HBV can naturally exist in other populations of HBV untreated patients.

This survey revealed that HBV genotype D is dominant in Iran, and no YMDD mutation was found among HBV-infected patients not treated with lamivudine in Iran, using simple and cost-effective methods. These methods can easily be employed in general clinical laboratories for the screening of large numbers of individuals who are living in HBV genotype D prevalent areas of the world.

Acknowledgments

The author would like to express their gratitude to Dr Behzad Poupak, Dr Masoud Parsania, and Ms Maryam Radpour for their assistance in sample collection.

Conflict of interest: No conflict of interest to declare.

References

Genotype D and YMDD pattern of HBV among untreated patients


