Rate of YMDD motif mutants in lamivudine-untreated Iranian patients with chronic hepatitis B virus infection

Amitis Ramezani a,b, Ali Akbar Velayati c, Mohammad Reza Hasanjani Roshan d, Latif Gachkar b, Mohammad Banifazl e, Hossein Keyvani f, Arezoo Aghakhani a,*

a Clinical Research Department, Pasteur Institute of Iran, No. 69, Pasteur Ave., Tehran, Iran
b Infectious Diseases Research Center, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
c Masih Daneshvari Hospital, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
d Babol University of Medical Sciences, Babol, Iran
e Iranian Society for the Support of Patients with Infectious Diseases, Tehran, Iran
f Keyvan Virology Laboratory, Tehran, Iran

Received 22 April 2007; received in revised form 31 July 2007; accepted 22 August 2007
Corresponding Editor: Jane Zuckerman, London, UK

Summary

Background: Lamivudine is used for the treatment of chronic hepatitis B patients. Recent studies show that the YMDD motif mutants (resistant hepatitis B virus) occur as natural genome variability in lamivudine-untreated chronic hepatitis B patients. In this study we aimed to determine the rate of YMDD motif mutants in lamivudine-untreated chronic hepatitis B patients in Iran.

Patients and methods: A total of 77 chronic hepatitis B patients who had not been treated with lamivudine were included in the study. Serum samples from patients were tested by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for detection of YMDD motif mutants. All patients were also tested for liver enzymes, anti-HCV, HBeAg, and anti-HBe.

Results: Of the 77 patients enrolled in the study, 73% were male and 27% were female. Mean ALT and AST levels were 124.4 ± 73.4 and 103.1 ± 81 IU/l, respectively. HBeAg was positive in 40% and anti-HBe in 60% of the patients. YMDD motif mutants were not detected in any of the patients despite the liver enzyme levels and the presence of HBeAg or anti-HBe.

Conclusion: Although the natural occurrence of YMDD motif mutants in lamivudine-untreated patients with chronic hepatitis B has been reported, these mutants were not detected in Iranian lamivudine-untreated chronic hepatitis B patients.

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**Introduction**

Hepatitis B is one of the most common infectious diseases in the world. It is estimated that more than 300 million people worldwide have chronic hepatitis B virus (HBV) infection and that 10% of these patients will die as a direct consequence of persistent viral infection. The global prevalence of chronic HBV infection varies widely, from high (>8%), e.g., in Africa, Asia, and the Western Pacific, to intermediate (2–7%), e.g., in Southern and Eastern Europe, to low (<2%), e.g., in Western Europe, North America, and Australia.

Nucleoside analogue therapy allows the safe, long-term suppression of HBV, and its use marks a major milestone in the treatment of chronic hepatitis B. Lamivudine, the first of these agents approved worldwide, effectively suppresses viral replication, reduces disease activity, improves liver histology, and delays clinical progression. However, the development of lamivudine-resistant mutations occurs in 14–32% of patients after 1 year of therapy. The longer the treatment is continued, the more frequently resistance is seen (65% at 5 years). Lamivudine-resistant HBV is associated with mutations of the YMDD motif in the polymerase gene. The key mutations are the substitutions of methionine at the rtM204 (domain C) to either isoleucine (rtM204I, YIDD variant) or valine (rtM204V, YVDD variant). The rtM204V variant is almost always accompanied by an additional rtL180M mutation in the domain B.

HBV mutants with mutations in the YMDD motif of viral polymerase have been described in patients infected with HBV who have not received lamivudine therapy. Also, cases of early emergence of mutants have been described, suggesting that mutations conferring lamivudine resistance were already present in the liver of these patients before starting therapy. This would mean that these mutant strains circulate among the population, so that a certain proportion of HBV carriers or chronic hepatitis B patients from a given geographical area might have any of them.

The presence of a drug-resistant HBV variant before treatment could be a reason for selecting an alternative drug for therapy. Thus, routine testing for mutations involved in antiviral drug resistance might be useful for candidate patients. To judge whether this activity would be appropriate, however, prior knowledge of the prevalence of these variants among the HBV infected population is required. This study was performed to determine the rate of YMDD motif mutants in lamivudine-untreated chronic hepatitis B patients in Iran.

**Patients and methods**

In this cross-sectional study, 77 chronic hepatitis B patients were tested for the presence of YMDD motif mutants. One or both of the following inclusion criteria had to be met: (1) HBsAg persistence for more than six months with liver enzymes more than 1.5 times the upper limit of normal; (2) HBsAg persistence for more than six months and liver biopsy with signs of chronic hepatitis confirmed by a pathologist according to the Knodell necroinflammatory score system. None of the patients had been treated with antiviral medications including lamivudine.

Blood samples were collected from the patients and the serum stored at −70 °C. All samples were tested by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for detection of YMDD motif mutants. Simultaneously, liver enzymes (ALT and AST) were also determined in all of them. Samples were also tested for hepatitis C antibody (anti-HCV), hepatitis B e antigen (HBeAg), and hepatitis B e antibody (anti-HBe) by ELISA. The commercial ELISA kits used were: anti-HCV, Bio-Rad Laboratories, Segrate, Italy; HBeAg and anti-HBe, Radim, Barcelona, Spain. All assay protocols, cut-offs, and result interpretations were carried out according to the manufacturers’ instructions.

**PCR-RFLP**

HBV variants at M552 (nucleotides 741 or 743) were detected by PCR-RFLP. Three separate PCR assays were used to amplify three fragments around position 552 of the polymerase gene. A 274-bp fragment was amplified using the primer pair P2 (5′-AAA CCT TCG GAC GGA AAC TGC-3′) and P4 (5′-CTG GATCCA GG TGTTTAA TTGATA CCC-3′), and the fragment was digested with FokI restriction enzyme, which cuts the sequence of the wild type YMDD and YVDD variant into 143 bp, 100 bp, and 31 bp and the sequence of the YIDD variant into 243 bp and 31 bp. The 31-bp fragment was not related to the identification of YMDD variants. A 181-bp fragment was amplified using the primer pair P5 (5′-TGGAATCA CCT GTCTCC TACCTC CAT-3′) and P6 (5′-CAGACTTGG CCC CATA CCA CAT GTCGCA-3′), which introduces an Alw441 site only into the YVDD mutant product), and the fragment was digested with Alw441 restriction enzyme, which cuts the sequence of the YVDD variant into 158 bp and 23 bp. A 138-bp fragment was amplified using the primer pair P4 (5′-CTG GATCCA GG TTGAAA TGTTA CCA CCC-3′) and P3 (5′-TTTCCCATCTTGGCTTT CAGTAA TAT-3′), which introduces an SspI site only into the YIDD mutant product), and the fragment was digested with SspI restriction enzyme, which cuts the sequence of the YIDD variant into 109 bp and 29 bp. Restriction enzyme SspI and Alw441 cutting sites were introduced into primers P3 and P6, respectively.

For DNA extraction, 100 μl of each patient’s serum was mixed with 150 μl of TES buffer containing 10% sodium dodecyl sulfate and protease K, and then incubated at 60 °C for 1 hour. After digestion with protease K, DNA was extracted by phenol/chloroform/isooamyl alcohol. Extracted DNA was precipitated with absolute ethanol, then washed with 70% ethanol and dissolved in 30 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The DNA was used for three amplification reactions of PCR. Final concentrations in the three PCR reactions were as follows: 20 mmol Tris (pH 8.3), 0.2 mmol KCl, 1.5 mmol MgCl2, 18 mmol NaCl, 0.2 mmol dNTPS, 0.5 μmol each primer, and 2.5 U Taq polymerase per 50 μl of reaction mixture. The amplification conditions in the three PCR reactions all included initial denaturation at 94 °C for 3 minutes, 35 cycles of amplification with denaturation at 94 °C for 35 seconds, primer annealing at 56 °C for 50 seconds, extension of primer at 72 °C for 50 seconds, followed by a final extension at 72 °C for 5 minutes.

After amplification, aliquots of the product DNA of the three PCR reactions were digested with three enzymes, respectively, according to the manufacturer’s specifications (Shanghai Biology Project Technology Company). The digestion reactions included 15 μl amplified HBV DNA, 10 U
enzyme, 10 x restriction enzyme digestion buffer 2 μl, and 3 μl sterile ddH₂O in a sterile Eppendorf tube. The tube was spun at 500 rpm for 3 seconds. The Spli and Alw441 enzyme reaction mixtures were incubated in a 37 °C water bath for 16 hours. The FokI enzyme reaction mixture was incubated in a 55 °C water bath for 3 hours. The results of PCR-RFLP were analyzed by 8.4% polypropylene acrylamide gel electrophoresis. The detection limit of the assay was 10,000 copies per ml.

Statistical analysis

Chi-square and t² tests were used with the SPSS 11.5 program for statistical analysis. Data are presented as means ± standard deviations or, when indicated, as absolute number/C6 for statistical analysis. Data are presented as means per ml.

Results

A total of 77 patients were enrolled in this study; 73% of them were male and 27% were female and they were aged between 11 and 50 years (mean 27.4 ± 9.3). Chronic hepatitis B was diagnosed in patients based on HBsAg persistence for more than six months with liver enzymes more than 1.5 times the upper limit of normal and/or HBsAg persistence for more than six months with liver biopsy signs of chronic hepatitis confirmed by a pathologist. None of the patients had been treated with antiviral medications including lamivudine. In these patients ALT, AST, HBeAg, anti-HBe, and anti-HCV tests and detection of YMDD motif mutants were done simultaneously.

The patients’ ALT levels were between 28 and 500 IU/l (mean 124.4 ± 73.4) and AST levels were between 22 and 500 IU/l (mean 103.1 ± 81). HBeAg and anti-HBe were positive in 40% and 60% of the patients, respectively. Anti-HCV was negative in all of them.

In 46 patients, the diagnosis of chronic hepatitis B was based on the liver biopsy. The liver biopsy scores were between 2 and 13 (mean 5.9 ± 2.4), grade (activity) was between 1 and 9 (mean 3.6 ± 2.3), and stage (fibrosis) between 0 and 3 (mean 1.2 ± 1.1) according to the Knodell necroinflammatory score system.

PCR was ’not determined’ in 48% (37/77) of the patients and was ’sensitive’ in 52% (40/77) of them. No YMDD motif mutations (0) were detected in chronic hepatitis B patients by RFLP.

Discussion

HBV is one of the most common infectious diseases in the world. HBV genotypes represent naturally occurring strains of HBV that have evolved over the years and reflect the geographical distribution of HBV throughout the world. Genotype D is most often found in Southern Europe, parts of Central Asia, India, Africa, and the Middle East. In Iran, genotype D is the only detected type found in all HBV infected patients.

Lamivudine is an antiviral drug approved for the treatment of chronic hepatitis B. However, the emergence of viral mutants resistant to lamivudine is the main concern with this treatment. The cause of lamivudine-resistant HBV was revealed to be the amino acid substitution from leucine to methionine at codon 180 of the B domain (rtL180M) and amino acid substitutions of the YMDD motif from methionine to valine or isoleucine at codon 204 of the C domain (rtM204V or rtM204I) of the reverse transcriptase (rt) region of the polymerase gene. 20–24

Data supporting the natural occurrence of lamivudine-resistant strains among the population of chronic HBV carriers have been reported recently from Japan and France. Some researchers have found the YMDD mutational strains in the serum of cases with chronic hepatitis B who did not receive lamivudine treatment. 24,25 Yan et al. showed that 19 out of 110 chronic hepatitis B untreated cases had YMDD mutations. The results from a study in Spain indicate that the prevalence of these mutants is about 4% among Spanish chronic HBV carriers. In a study in Japan, mutations were detected in five out of 18 untreated chronic hepatitis B patients. In addition, Zhang et al. found that 26.2% of chronic hepatitis B cases had YMDD mutations by genetic chip determination. Matsuda et al. reported that a few chronic hepatitis B cases not treated with lamivudine had YMDD mutations. Naturally occurring YMDD motif variants were detected at a high rate in a group of lamivudine-untreated inactive HBV carriers in Turkey. Other studies have not reported YMDD mutations in non-lamivudine treated hepatitis B patients. In a study in China, the YMDD mutants were not detected in any of the five patients before lamivudine treatment.

Kobayashi et al. found that anti-HBe was positive in all patients with YMDD mutations, and Ye et al. also found that anti-HBe was positive in most patients with YMDD mutations and considered that YMDD mutations might occur more easily if mutations took place in the pre-c zone. In a study in China, the incidence rate of YMDD mutations was 27.1% in patients with positive HBeAg and 26.7% in patients with negative HBeAg and positive anti-HBe. There was no significant difference between the two groups. Lee et al. detected YMDD mutants in eight of 12 HBeAg-positive patients and eight of 16 HBeAg-negative patients before lamivudine treatment.

In our study, the YMDD motif mutants were not detected in any of the chronic hepatitis B patients before lamivudine treatment despite the liver enzyme levels and the presence of HBeAg or anti-HBe. These results do not accord with some studies but are in agreement with others. This low rate may be explained by epidemiological and geographic variations and study conditions such as the number of patients and the method used for detecting the YMDD motif mutants. YMDD mutants have significantly low replication ability. It is thought that the naturally occurring YMDD mutants make up only a very small proportion of the total HBV, and very sensitive investigative tools are required to detect them. RFLP often leaves out those samples with mixed virus populations and a low-component virus population, and is suited only for mono-infection. Further studies are needed to determine exactly the rate of YMDD motif mutants in lamivudine-untreated chronic hepatitis B patients.

In conclusion, although the natural occurrence of YMDD motif mutants in lamivudine-untreated patients with chronic hepatitis B infection has been reported, these mutants were
not detected in Iranian lamivudine-untreated chronic hepatitis B patients. Further studies with more samples and more sensitive investigative tools should be conducted to determine this rate exactly.

Acknowledgment

The authors are grateful to the Infectious Diseases Research Center, Shaheed Beheshti University of Medical Sciences for the financial support of this study.

Conflict of interest: No conflict of interest to declare.

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