Lamivudine resistance mutations in patients infected with hepatitis B virus genotype D

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AIM: To determine the distribution of viral genotypes for primary or acquired lamivudine resistance.

METHODS: A total of 283 patients with chronic hepatitis B virus (HBV) infection (245 patients with chronic hepatitis B and 38 inactive hepatitis B surface antigen carriers) were included in the study. The HBV genotype was determined by using quantitative real-time polymerase chain reaction and sequence analysis, and tyrosine-methionine-aspartate-aspartate (YMDD) motif mutations were determined using the reverse transcriptase hybridization method.

RESULTS: Lamivudine resistance was determined in a total of 25 (10.7%) chronic hepatitis B patients. Eight subjects (4%) had primary resistance to lamivudine, and 17 (53.1%) had secondary resistance to lamivudine. Genotype D, which was isolated from 267 of the patients with chronic HBV infection, was the dominant genotype in Turkey.

CONCLUSION: Identification of YMDD motif mutations should have a positive impact on the selection of proper antiviral medication for patients, even for those who are nucleoside naïve.

Key words: Hepatitis B virus; Genotype; Resistance; Lamivudine; Tyrosine-methionine-aspartate-aspartate mutation

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important cause of morbidity and mortality worldwide[1]. The objective in the treatment of this disease is to cease viral replication and provide improvement in liver histopathology; thereby preventing complications such as cirrhosis and hepatocellular carcinoma and the associated mortality.[2]

Lamivudine is a synthetic nucleoside analogue that suppresses viral replication by inhibiting viral polymerase activity[3]. Although lamivudine is an effective and well-tolerated medication, duration of treatment is quite long, and resistance is an important issue[4]. Resistance to lamivudine is associated with mutations in the HBV polymerase gene. The most frequent mutation that develops with lamivudine is the tyrosine-methionine-aspartate-aspartate (YMDD) mutation of the HBV polymerase gene. It has been shown that all lamivudine resistant patients had a HBV YMDD mutation[5]. Among them, 41.3% of the patients had the rtL180M/M204I mutation, 25.7% had the rtL180M/M204I mutation and 33% had the rtM204I mutation. The frequency of mutation increases proportionally with the duration of lamivudine treatment, and the five-year post-treatment frequency is about 65%-70%[6-7]. On the other hand, resistance to lamivudine might also be seen in nucleoside naïve chronic hepatitis B patients or inactive HBsAg carriers. This resistance is associated with structural changes in the DNA polymerase enzyme gene[8-9]. The recommended methods of treatment aimed at preventing resistance include the use of more potent agents, changing the treatment in patients in whom the disease progressed during treatment, and the use of combined antiviral agents.

There are eight HBV genotypes (A–H), and the distribution of these genotypes varies by geographic region. Genotype A and C are prevalent in the American continent, whereas the prevalent genotypes are B and C in Asian countries, D in Europe, and E in Africa[10-13].

The aims of the present study were to determine the dominant viral genotype in chronic HBV infection in Turkey, and determine the prevalence of primary or acquired lamivudine resistance by genotype.

MATERIALS AND METHODS

This is a multi-center study including 17 centers from Turkey between January and September 2007. Patients between the ages of 18 and 65 years who were inactive HBsAg carriers or who had chronic hepatitis B (regardless of antiviral treatment status) were included in this study. Patients with co-infections with hepatitis C virus, hepatitis D virus or human immunodeficiency virus, along with patients with primary or secondary causes of liver disease other than hepatitis B (e.g., autoimmune hepatitis, steatohepatitis, hemochromatosis, Wilson’s disease, alpha-1 antitrypsin deficiency, and severe cardiopulmonary disease) were excluded from the study. This study was approved by the Local Ethics Committee of the Department of Medicine at the Erciyes University in Turkey. A written informed consent was obtained from all participants before laboratory tests were performed.

Laboratory tests

The complete blood count was determined using an automated cell counter (Coulter LH750). Liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in serum using standard commercial kits (Boehringer, Mannheim, Germany). All samples were screened to detect HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc (total), and anti-HBc IgM, by using third-generation microparticle enzyme immunoassays (Abbott Laboratories, Chicago, IL). HBV DNA was investigated by real-time polymerase chain reaction (PCR) (COBAS TaqMan 48 analyzer, Roche Diagnostics) according to manufacturer’s instructions.

HBV genotypes were determined by DNA sequencing. HBV DNA was extracted from serum samples. HBV DNA polymerase gene region was amplified by nested-PCR using specific primers. PCR products were analyzed on UV transilluminator and purified from agarose gel. The purified products were sequenced by the Visible Genetics OpenGene system (Visible Genetics, Toronto, Ontario, Canada) and the Cy5.5 dye terminator cycle sequencing kit (Amersham, Pharmacia Biotech, Piscataway, NJ, United States). YMDD mutation in the HBV DNA polymerase gene was determined with RT hybridization (INNO-LiPA HBV DR-Innogenetics, Ghent-Belgium), according to manufacturer’s instructions. A part of HBV domains B and C of the pol gene was amplified. The biotinylated PCR fragments were reverse hybridized using typing-membrane-based INNO-LIPA HBV DR strips. After hybridization, streptavidin labeled with alkaline phosphatase were added and bound to the previously formed biotinylated hybrids. Incubation with the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium chromogen resulted in a purple-brown color development.

A percutaneous liver biopsy was performed at each center under local anesthesia (optional). A biopsy specimen of more than 1 cm in length with five to six portal tracts was collected. Chronic hepatic disease activity was classified according to the Knodell histological activity index (HAI)[14].

Diagnostic criteria

Chronic hepatitis B: (1) HBsAg positive for at least 6 mo; (2) serum HBV DNA > 20,000 IU/mL (> 10^5 copies/mL), often ranges between 2,000-20,000 IU/mL in HBeAg-negative patients; (3) persistent or intermittent elevation of ALT/AST levels; and (4) moderate or severe necro-inflammation. Inactive HBsAg carriers: (1) HBsAg positive for at least 6 mo; (2) HBeAg-negative, anti-HBe-positive; (3) serum HBV DNA < 2,000 IU/mL (< 10^5 copies/mL); (4) persistently normal ALT/AST; and (5) no signs of hepatitis in liver biopsy.

Demographic features of the patients, possible routes of transmission of HBV, and the antiviral treatments
administered and their durations were all recorded. The decision to start antiviral therapy was based on the serum HBV DNA and aminotransferase levels, histological grade and stage at each center[15,16]. Lamivudine (100 mg/d) was administered orally in all eligible chronic hepatitis B patients. Resistance status was determined for patients receiving and not receiving lamivudine. The relationship between resistance and sex, body mass index, genotype, HBV DNA levels, ALT/AST levels, HBeAg positivity or negativity, and necro-inflammation and fibrosis in the liver were investigated in subjects with primary resistance.

Statistical analysis

“SPSS 12.0 for Windows” was used for statistical analyses. For continuous variables, descriptive statistics (mean, median, standard deviation, minimum and maximum) were calculated. For comparisons of categorized variables, the Chi-square test was used, and for continuous variables, the Student’s t test and Mann-Whitney U test were used. P < 0.05 was considered statistically significant.

RESULTS

A total of 283 patients with chronic HBV infection composed of 245 patients with chronic hepatitis B and 38 inactive HBsAg carriers were included in the study; 193 were males, and 90 were females. No differences were observed between the two groups except for the levels of HBV DNA and ALT/AST (Table 1). The histopathological findings of chronic hepatitis B patients who underwent optional liver biopsy are presented in Table 1. The median Knodell fibrosis score was 2 (0-4), and the median HAI was 7 (1-18).

Genotype determination with sequence analysis revealed that the HBV isolated from 267 patients were all of genotype D. Comparison of subgroups of chronic hepatitis B patients in terms of the “e” antigen revealed that the levels of ALT were higher in patients who were positive for HBeAg compared to those who were negative (121.6 U/L and 81.9 U/L, respectively, P = 0.012). However, there was no statistically significant difference between the levels of HBV DNA (1.7 × 10^8 copies/mL and 1 × 10^7 copies/mL, respectively, P = 0.150, Table 2).

HBV drug resistance

HBV was isolated from a total of 267 patients with chronic HBV infection and analyzed for YMDD resistance using INNO-LiPA HBV DR. Of these patients, 104 (39%) had a history of previous treatment with an antiviral, and 32 (30.8%) of these patients had received lamivudine. The mean duration of lamivudine treatment was 18.5 ± 14.5 mo in these subjects, and 17 of these (53.1%) developed secondary resistance. Primary resistance to lamivudine was determined in 8 (4%) out of 202 lamivudine naïve patients. The frequency of YMDD

Table 1  Demographic features of study patients

| No. of patients, n (%) | Chronic hepatitis B | Inactive HBsAg carriers | P value |
|-----------------------|---------------------|-------------------------|---------|
| Sex (male/female)     | 245 (86.6)          | 38 (13.4)               |         |
| BMI (mean ± SD, kg/m²) | 25.9 ± 4.3          | 25.7 ± 3.6              | 0.914   |
| Hemoglobin (mean ± SD, g/dL) | 14.9 ± 1.5      | 14.9 ± 1.6              | 0.949   |
| White cell count (mean ± SD, /mm³) | 6577 ± 1783      | 6615 ± 1599             | 0.999   |
| Thrombocyte count (mean ± SD, 1000/mm³) | 200 482 ± 57 762 | 219 727 ± 63 743 | 0.088   |
| AST (mean ± SD, IU/L)  | 56.2 ± 51.3        | 25.2 ± 11.1             | 0.000   |
| ALT (mean ± SD, IU/L)  | 91.6 ± 92.6        | 25.3 ± 13.2             | 0.000   |
| HBeAg positivity, n (%) | 57 (23.2)          | 0                       |         |
| HBV DNA (mean ± SD copies/mL) | 4.9 × 10^9 ± 5.9 × 10^9 | 2.0 × 10^7 ± 2.6 × 10^7 | 0.000   |

Histopathological findings, n=143

| Total HAI score, median (range) | 7 (1-18) |
| FN and BN, median (range) | 2 (0-7) |
| ID and FN, median (range) | 1 (0-4) |
| PI, median (range) | 2 (0-4) |
| Fibrosis, median (range) | 2 (0-4) |

1Number of patients who underwent liver biopsy. BMI: Body mass index; HAI: Histological activity index; PN: Piecemeal necrosis; BN: Bridging necrosis; ID: Intralobular degeneration; FN: Focal necrosis; PI: Portal inflammation; HBV: Hepatitis B virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2  Levels of hepatitis B virus DNA and alanine aminotransferase in chronic hepatitis B by HBeAg positivity

| HBeAg-positive (n = 57) | HBeAg-negative (n = 210) | P value |
|-------------------------|--------------------------|---------|
| ALT (IU/L)              | 17 ± 20                  |         |
| HBV DNA (copies/mL)     | 1 × 10^4 ± 9.3 × 10^9     | 7 ± 20  |
|                        | 121.6 ± 120.3            | 423 ± 81.9 ± 77.4 | 0.012 |

ALT: Alanine aminotransferase; HBV: Hepatitis B virus.
mutation in patients with chronic hepatitis B is shown in Table 3. Wild-type HBV was determined in 241 (90.3%) patients. YMDD mutation was determined in 26 (9.7%) patients, and only one of these subjects (1/33, 3%) was an inactive HBsAg carrier.

No relationship was established between YMDD motif mutation and viral load, body mass index, liver function tests, HBeAg positivity and liver histopathology. Similarly, there was no difference in those same parameters between chronic hepatitis B patients with primary and secondary resistance to lamivudine.

**DISCUSSION**

Lamivudine is the first nucleoside analogue approved for the treatment of chronic hepatitis B infection. It has been used successfully for years in the treatment of chronic hepatitis B patients due to its rapid suppression of HBV DNA levels and perfect side effects profile. It is often received as a single daily 100 mg dosage and is generally well-tolerated [17]. Although lamivudine is a potent medication against HBV, the duration of the treatment is quite long, and resistance is an important issue [4]. It has been shown that YMDD mutations increase with the duration of lamivudine treatment in chronic hepatitis B patients treated with lamivudine for long periods of time [18,19].

In the study by Alvarado-Esquivel et al [20], the prevalence of wild-type HBV was found to be 94.9%, and the prevalence of genotypic resistance to lamivudine was reported as 2.6%. Sun et al [8], on the other hand, observed the YMDD mutation in 17% (42/247) of patients treated with lamivudine. These patients had used lamivudine for a mean duration of 533 d, and the cumulative incidence of YMDD mutation was 41.3%. The YMDD motif mutation has been investigated in the polymerase gene in 267 of 283 chronic hepatitis B patients. Among the subjects, 90.3% were infected with wild-type HBV. The frequency of the YMDD mutation was 10.7%, and the majority of these were chronic hepatitis B patients. However, the prevalence of YMDD mutation was lower in inactive HBsAg carriers (3%). This patient group was infected with viruses that replicate less compared to wild-type viruses. The cause of YMDD mutations and the effects of these mutant serotypes on hepatic activation are not known. The findings of the present study are in line with the literature, as mutations were observed in 53.1% of the total 32 patients whose lamivudine therapy duration was 18.5 mo (secondary resistance).

Sensitive diagnostic tests allow for the identification of rtM552I/V mutant HBV in patients receiving lamivudine at the third month of treatment. The wild-type virus re-emerges upon termination of treatment with lamivudine, and resistant serotypes emerge immediately upon re-initiation of treatment. However, lamivudine resistance has also been reported in chronic hepatitis B patients with no history of lamivudine use, and inactive HBsAg carriers (primary resistance). The frequency of primary resistance, which is associated with structural changes in the DNA polymerase enzyme gene, ranges between 0% and 27.7% [9,20-24]. Five (27.7%) out of 18 nucleoside naïve patients, and 4 out of 36 (11.1%) patients were found to have the YMDD mutation in studies by Kobayashi et al [20] and Kirishima et al [21], respectively. Both studies revealed that all patients who were positive for mutation were also anti-HBe positive. Heo et al [23] observed mutations in 3 (7.5%) out of 40 lamivudine naïve patients and reported HBeAg positivity in one of these subjects. However, no YMDD mutation was observed in the first study by Matsuda et al [23] performed on nucleoside naïve chronic hepatitis B patients, whereas 2 (2.8%) out of 71 subjects were found to have the mutation in their second study.

Akarsu et al [24] reported from Turkey that 13 (18.3%) out of 71 asymptomatic carriers of hepatitis B with no history of antiviral treatment were infected with lamivudine-resistant HBV. In another study from Turkey, 6 (7.8%) of 77 nucleoside naïve patients were reported to have the YMDD motif mutation [9]. In the present study, YMDD motif mutations were found in 8 (4%) out of 202 patients with no history of antiviral treatment. The reason for the marked difference between these studies might be due to differences in geographical location, mean age, age at infection, HBeAg positivity, HBV genotype and levels of viral load.

Some studies have shown that YMDD mutation is a feature of patients with low viral load [20,23]. However, no relationship was demonstrated in other studies between viral load and mutation [22-24]. In this study, no relationship was found between YMDD motif mutation and viral load, body mass index, liver function tests, HBeAg positivity and liver histopathology.

Studies from Turkey performed by Tunçbilek et al [25], Senturker et al [26] and Bozdayı et al [27] have reported that all examined patients (77, 23 and 41 patients, respectively) were infected with HBV genotype D. However, the results from the previous studies may not be generalizable to the larger population of Turkey due to the inadequate number of patients and limited geographic locations from which the data in these studies were obtained.

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**Table 3. Rates of tyrosine-methionine-aspartate-aspartate mutation in patients with chronic hepatitis B virus infection *n (%)***

| Chronic hepatitis B | Inactive HBsAg carriers | Total |
|---------------------|-------------------------|-------|
| Previous lamivudine treatment | Yes | No | Total |
| YMDD + YIDD positive | 17 (53.1) | 8 (4) | 25 (10.7) | 1 (3) | 26 (9.7) |
| YMDD negative | 15 (46.9) | 194 (96) | 209 (89.3) | 32 (97) | 241 (90.3) |
| Total | 32 | 202 | 234 | 33 | 267 |

*Patients who are receiving or received lamivudine previously. YMDD: Tyrosine-methionine-aspartate-aspartate; YIDD: Tyrosine-isoleucine-aspartate-aspartate; YMDD: Tyrosine-valine-aspartate-aspartate.*
In the present study, all 267 Turkish chronic hepatitis B patients were infected with HBV genotype D according to the genotype analysis performed with the sequence analysis method. This multi-center and comprehensive study was the first to show the dominance of the genotype D in Turkey.

In conclusion, lamivudine therapy has been associated consistently with secondary resistance in chronic hepatitis B patients. The assessment of genotypic resistance prior to re-treatment of chronic hepatitis B patients with lamivudine or a different antiviral agent might increase the efficacy of treatment and prevent complications. In this study, the frequency of primary resistance to lamivudine was found to be 4% in nucleoside naïve patients with chronic hepatitis B. In addition, the identification of YMDD motif mutations prior to nucleoside treatment should have a positive impact on the selection of proper antiviral medication for patients, even for those who are nucleoside naïve.

**COMMENTS**

**Background**

Hepatitis B virus (HBV) infection is a worldwide problem, with potentially 400 million individuals with active HBV infection. HBV is a significant cause of morbidity and mortality from cirrhosis, liver failure, and hepatocellular carcinoma in these populations. Lamivudine is still used widely to treat HBV infection in Turkey. However, lamivudine resistance is a major concern.

**Research frontiers**

Eight genotypes of HBV (A-H) have been identified, and increasing evidence suggests that certain genotypes may have a significant impact on both the choice of treatment and clinical outcome, and is also affected by the patterns of resistance manifested by local strains. The authors studied the genotype profiles and lamivudine resistance patterns of HBV isolates among Turkish patients.

**Innovations and breakthroughs**

The tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation has been investigated in the polymerase gene in chronic hepatitis B patients. The findings of the present study are in line with the literature. A significant proportion of patients with lamivudine resistance would not be able to receive proper antiviral medication and may run the risk of disease progression in Asia. Although it is expected that HBV genotype D to be dominant in Turkey, it was surprising that no other genotypes were detected.

**Applications**

Identification of YMDD motif mutations prior to nucleoside treatment should help in decisions regarding selection of antiviral therapy, and expected responses to treatment.

**Peer review**

In this manuscript, the authors have reported that the HBV genotype is prevalent in Turkey. The frequency of YMDD mutants was also determined. The methodology employed in this study was very much standard. The results are of significant local importance.

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S-Editor Tian L  L-Editor Rutherford A  E-Editor Li JY