Lamivudine resistance in untreated chronic hepatitis B patients in Turkey

Türkiye'deki tedavi görmemiş kronik hepatit B'li hastalarda lamivudin direnci

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Background/aims: *Hepatitis B virus infection is an epidemiological problem throughout the world, including in Turkey. Lamivudine is one of the hepatitis B virus reverse-transcriptase inhibitors used for the treatment of chronic hepatitis B virus infection. Lamivudine resistance can develop not only following treatment; it can also be seen in untreated patients. This resistance is related with structural changes in the tyrosine-methionine-aspartate-aspartate motif of the polymerase enzyme gene. Our objective was to evaluate the prevalence of lamivudine resistance in Turkish chronic hepatitis B virus-infected patients with D genotype before antiviral treatment.* ***Methods:*** *Seventy-seven patients with chronic hepatitis B virus infection were evaluated for viral loads, HBeAg, anti-HBe antibody, ALT levels, histological activity index, and tyrosine-methionine-aspartate-aspartate mutations.* ***Results:*** *Tyrosine-methionine-aspartate-aspartate motif mutations were determined in 3 of 24 HBeAg positive and 3 of 53 anti-HBe positive patients with a rate of 7.8%. Two of the mutations were YIDD and 4 were YVDD. Median ALT value in patients with mutations was 88 IU/L (range 55-276) and histological activity index was 9 (range 6-10); these values in patients without mutations were 58 (range 19-176) and 10 (range 2-18), respectively. Knodell fibrosis scores of patients were as follows: 0: 13.2%, 1: 28.9%, 2: 21.1%, 3: 34.2%, and 4: 2.6%. There were no significant differences between the patients regarding Knodell fibrosis scores. One patient was diagnosed as cirrhosis.* ***Conclusions:*** *Evaluation of chronic hepatitis B virus patients for lamivudine resistance and planning the treatment accordingly may prevent complications and can increase the effectiveness of the treatment.*

Key words: Hepatitis B, YMDD, genotype D, lamivudine, untreated

Amaç: *Hepatit B virus infeksiyonu tüm dünyada ve Türkiye'de epidemiyolojik bir problemdir. Lamivudin hepatit B virus revers transkriptaz inhibitörlerinden biridir ve kronik hepatit B virus infeksiyonunda kullanılmaktadır. Lamivudin direnci ilaç kullanımından sonra oluşabileceği gibi tedavi almamış hastalarda da görülebilmektedir. Bu direnç polimeraz enzim genindeki tirozin-metionin-aspartat-aspartat motifindeki yapısal değişiklik ile ilişkilidir. Bu çalışmada Türkiye'deki D genotip'li kronik hepatit B'li hastalarda antiviral tedavi öncesi lamivudin direncinin prevalansını belirlemeyi amaçladık.* ***Yöntem:*** *Kronik hepatit B virus infeksiyonlu yetmiş yedi hastanın viral yükleri, HBeAg, antiHBe antikorları ve tirozin-metionin-aspartat-aspartat mutasyonları değerlendirildi.* ***Bulgular:*** *Tirozin-metionin-aspartat-aspartat motif mutasyonu 24 HBeAg pozitif hastanın 3'ünde, 53 antiHBe pozitif hastanın 3'ünde %7.8 oranında belirlendi. Mutasyonların ikisi YIDD, dördü YVDD olarak belirlendi.* ***Sonuç:*** *Kronik hepatit B virus hastalarında lamivudin direncinin değerlendirilmesi ve tedaviyi buna göre planlamak komplikasyonları önleyecek ve tedavinin etkinliğini artıracaktır.*

Anahtar kelimeler: Hepatit B, YMDD, genotip D, lamivudin, tedavisiz

INTRODUCTION

Lamivudine (dideoxy-2',3'-thiacytidine) is the first hepatitis B virus (HBV) reverse-transcriptase (RT) inhibitor to be approved for the treatment of chronic hepatitis B (1, 2). Lamivudine effectively suppresses viral replication, reduces disease activity, improves liver histology, and delays clinical progression (2-4). The most important mechanism of anti-polymerase/RT activity of lamivudine and

other nucleoside analogues is inhibiting elongation of the HBV DNA minus strand through competition with the natural polymerase substrate dCTP and by acting as a chain terminator through its incorporation in the nascent DNA strand (5, 6). HBV DNA polymerase is a highly conservative YMDD order locating in the polymerase structural region C area, which is the combining and functioning site of lamivudine (7).

Drug resistance can develop during treatment with lamivudine, occurring in 14%-32% of patients after one year of therapy (8,9). The rate of resistance increases with increased duration of treatment, 38% after two years, 53% to 76% after three years (10) and 65% to 70% after five years (11,12). However, lamivudine resistant mutants are also seen in untreated asymptomatic carriers or chronic hepatitis B patients (13-15). This resistance is related with structural changes in gene of polymerase enzyme. Lamivudine resistance increases the potential of cross-resistance to other L-nucleoside and nucleotide analogues and limits sensitivity to entecavir, thereby limiting treatment options (16).

Testing for drug-resistant mutations in selected patients might be useful for determination of the need for alternative drug therapy. In order to decide whether this would be appropriate, the prevalence of these variants among the HBV- infected population needs to be ascertained. The aim of this study was to evaluate the prevalence of lamivudine resistance in naive Turkish HBV-infected patients with D genotype.

MATERIALS AND METHODS

Serum samples of 77 patients aged between 3 – 63 years who were diagnosed as chronic hepatitis B with liver biopsy and/or biochemical and molecular tests, with genotype D, and who did not receive antiviral treatment were evaluated in this study. After approval of the experimental protocol by the local human ethics committee, informed consent was obtained from each subject or the subject's guardian. Patients with chronic hepatitis due to other reasons, who were infected with human immunodeficiency virus (HIV) or hepatitis C virus, and who had any other serious diseases were not included in this study.

The viral load of the patients was measured with real-time polymerase chain reaction (PCR), "Roche Taq Man", HBeAg and anti-HBe antibody with ELISA. YMDD mutation analysis was done by real-time PCR and confirmed by DNA sequence analysis, and genotype was also detected by DNA sequence analysis.

Real-time PCR

The real-time PCR reactions were carried out in a total volume of 10 µl with 0.5 µM of each primer, 0.2µM of Cy-5 labeled probe (17) and 4mM MgCl2 using FastStart DNA master SYBR Green I kit (Roche Applied Sciences, Germany). The cycling parameters were 10 min at 95 °C for activating hot start Taq polymerase, 50 cycles of 10 s at 95 °C, 10 s at 55 °C and 72 °C for 12 s for amplification, and were followed by a melting program of 40 to 75 °C at 0.2 °C/s with continuous monitoring of the fluorescence.

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 (18). The purified products were sequenced by Visible Genetics Open-Gene system using Cy5.5 dye terminator kit (Amersham Biosciences, USA) according to the manufacturer's protocol.

Histological activity index (HAI)

Liver biopsy specimens were evaluated as defined by Knodell et al. (19) by an independent pathologist.

Statistical Method

Viral load values regarding mutation group were described using mean, median, standard deviation, and minimum and maximum values. The difference between median viral load values was evaluated by Mann-Whitney U test because of nonnormal distribution. For all tests, a two-tailed P-value of 0.05 was considered as indicating significance level. The analysis was performed by SPSS software (version 15.0, SPSS Inc. Chicago, USA). Power analysis was performed based on descriptive statistical results and with PASS 2005 (Hintze J, 2006, Kaysville, USA).

RESULTS

The mean age of the 77 (22 [28.6%] female) chronic hepatitis B patients was 31.6±13.8 years (range 3 – 63 years). YMDD motif mutations were determined in 3 of 24 HBeAg positive patients and 3 of 53 anti-HBe positive patients. Total mutation

rate was 7.8%; 2 of the mutations were YIDD (tyrosine, isoleucine, aspartate, aspartate) and 4 were YVDD (tyrosine, valine, aspartate, aspartate) (Table 1). There was full concordance between sequencing and real-time PCR results. The mutation ratio was the same with both methods. The detection limit of real-time PCR was 1000 copies per milliliter of mutants.

The median viral load of the YMDD motif group (202.679.711 copies/ml) was lower than in the other mutation group (317.976.423 copies/ml), but the difference between them was not statistically significant (p= 0.482) (Table 2).

The median alanine aminotransferase (ALT) value in patients with mutations was 88 IU/L (range 55-276) and in patients without mutations was 58 (range 19-176). HAI of patients with mutations was 9 (range 6-10) and of patients without mutations was 10 (range 2-18). Due to insufficiency of data, no statistical comparisons of ALT and HAI were made.

Knodell fibrosis scores of the patients were 0 in 13.2%, 1 in 28.9%, 2 in 21.1%, 3 in 34.2%, and 4 in 2.6%. There were no significant differences between the patients regarding Knodell fibrosis scores. Only one patient was diagnosed as cirrhosis.

DISCUSSION

Lamivudine was the first nucleoside analogue licensed for the treatment of chronic HBV. Lamivudine is a cytosine analogue and inhibits RT by competing for incorporation into growing DNA chains causing chain termination. Lamivudine can be taken orally in a dosage of 100 mg daily, is generally well tolerated and has an excellent safety profile (20).

Lamivudine resistance can be seen among patients who received treatment or not. Moreover, no patient-related factors such as age, gender and disease status affect the rate of lamivudine-resistant mutations. However, the YMDD mutation rates change between 7.5% - 29.5% (21-23) among

different populations. It may be suggested that this difference is related with the genotype of HBV infecting that population, as it has been reported that mutations mostly occurred in genotype C and its mixed genotypes (23).This may explain the difference in YMDD mutation rates among the different series and the 7.8% mutation rate seen in our patients, all of whom were infected with D genotype.

Another factor related with lamivudine-resistant mutations may be the status of HBe antigen. Although we could not find a relationship between anti-HBe antibody and the rates of mutations, Kobayashi et al. (13) showed a YMDD mutation rate of 27.7% in asymptomatic carriers who never received treatment and all of whom were anti-HBe positive. Lamivudine resistance is reported to be 26.9% in untreated chronic hepatitis patients, 42.8% of whom were anti-HBe positive (14). Ye et al. (24) 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. However, there are studies showing that there is no difference in mutation rates between patients who have anti-HBe antibody or not in accordance with the results of this study (21, 23, 25). Therefore, YMDD mutations might not have a relationship with pre-c-zone mutations.

HBV DNA level has been reported as not demonstrating a positive correlation with the incidence of YMDD mutations (14, 23, 25). We also showed similar viral loads in patients with or without YMDD mutations.

Lamivudine has potent inhibitory effects on HBV replication. Prolonged therapy is required for sustained suppression. HBeAg seroconversion occurs in 16 to 22% of patients by one year compared with 4 to 13% of untreated controls (2, 26, 27). Higher cumulative HBeAg seroconversion rates were observed with increased duration of lamivudine treatment, with 29% at two years, 40% at three years, and 47% at four years of therapy (27-29). Re-

Table 1. The mutations, HBeAg/anti-HBe status and viral loads of the patients with mutations

Age	Gender	HBeAg/anti-HBe	Viral load copies/ml	Mutation	Genotype
41	Male	Negative/Positive	13.870	YIDD	D
12	Male	Positive/Negative	3858×10^5	YVDD	D
8	Male	Positive/Negative	64×10^7	YIDD	D
7	Female	Positive/Negative	64×10^7	YVDD	D
30	Male	Negative/Positive	1.42^7	YVDD	D
45	Male	Negative/Positive	11.730	YVDD	D

Table 2. Power Analysis: Group sample sizes of 61 and 6 achieve 99% power to detect a difference of 190.754.925 between the YMDD motif group (mean±SD of 86.882.913±117.564) and YMDD mutation group (mean±SD of 277.637.838±192.906.935) with a significance level (alpha) of 0.482 using a two-sided Mann-Whitney test.

	N	Mean	Median	Standard Deviation	Minimum	Maximum
YMDD motif group	61	86.882.913	202.679.711	117.564	913	64x10⁷
YMDD mutation group	6	277.637.838	317.976.423	192.906.935	1.427	64x10⁷
Total	67	103.965.444	219.125.747	117.564	913	64x10⁷

Viral load (copies/ml)

duction of serum HBV DNA occurs in 98% of patients (10). Prolonged duration of ongoing lamivudine therapy after HBeAg seroconversion and low pretreatment HBV DNA seem to be associated with decreased relapse rates and prevention of adverse clinical outcomes in patients with advanced liver disease (bridging fibrosis or cirrhosis) (30). Disease progression, defined as a two-point increase in Child-Turcotte-Pugh score, and development of hepatocellular cancer were found to be significantly decreased in lamivudine-treated patients compared with untreated controls (31). The major drawback of lamivudine, which significantly limits its use as first-line therapy, is the high rate of occurrence of viral resistance. Resistance to lamivudine may emerge after 9-10 months of therapy, with an incidence of 38% and 67% after two and four years of lamivudine therapy, respectively. Patients who develop YMDD mutant during lamivudine therapy for HBV infection exhibit various clinical courses. The emergence of lamivudine-resistant mutants is usually associated with an increase in serum HBV DNA and ALT, and selection of YMDD variants has been associated with worsening of liver histology (31-33). Although viral clearance with or without emergence of distinct lamivudine-resistant mutants may occur after such exacerbations, 20% of the exacerbations are complicated with decompensation or even fatality. The exacerbations appear to be more severe than those that occur during the natural course of wild-type HBV chronic infection (34).

Lamivudine resistance, at a rate of one in every 10 chronic HBV-infected patients as shown in this study, is not a value that can be underestimated. Thus, evaluation of chronic HBV patients for lamivudine resistance and planning the treatment accordingly may prevent complications and can increase the effectiveness of the treatment.

REFERENCES

1. Dienstag JL, Perrillo RP, Schiff ER, et al. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995; 333: 1657-61.

2. Liaw YF, Sung JJ, Chow WC, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004; 351: 1521-31.

3. Dienstag JL, Schiff ER, Wright TL, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999; 341: 1256-63.

4. Lok AS, Lai CL, Leung N, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003; 125: 1714-22.

5. Severini A, Liu XY, Wilson JS, et al. Mechanism of inhibition of duck hepatitis B virus polymerase by (-)-beta-L-2',3'-dideoxy-3'-thiacytidine. Antimicrob Agents Chemother 1995; 39: 1430-5.

6. Zoulim F, Dannaoui E, Borel C, et al. 2',3'-dideoxy-beta-L-5-fluorocytidine inhibits duck hepatitis B virus reverse transcription and suppresses viral DNA synthesis in hepatocytes, both in vitro and in vivo. Antimicrob Agents Chemother 1996; 40: 448-53.

7. Marcellin P, Lau GK, Bonino F, et al. Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. N Engl J Med 2004; 351: 1206-17.

8. Heathcote J. Treatment of HBe antigen-positive chronic hepatitis B. Semin Liver Dis 2003; 23: 69-80.

9. Hadziyannis SJ, Papatheodoridis GV, Vassilopoulos D. Treatment of HBeAg-negative chronic hepatitis B. Semin Liver Dis 2003; 23: 81-8.

10. Lai CL, Dienstag J, Schiff E, et al. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 2003; 36: 687-96.

11. Wright TL. Clinical trial results and treatment resistance with lamivudine in hepatitis B. Semin Liver Dis 2004; 24 Suppl 1: 31-6.

12. Liaw YF. The current management of HBV drug resistance. J Clin Virol 2005; 34 Suppl 1: 143-6.

13. Kobayashi S, Ide T, Sata M. Detection of YMDD motif mutations in some lamivudine-untreated asymptomatic hepatitis B virus carriers. J Hepatol 2001; 34: 584-6.

14. Huang ZM, Huang QW, Qin YQ, et al. YMDD mutations in patients with chronic hepatitis B untreated with antiviral medicines. World J Gastroenterol 2005; 11: 867-70.

15. Matsuda M, Suzuki F, Suzuki Y, et al. Low rate of YMDD motif mutations in polymerase gene of hepatitis B virus in chronically infected patients not treated with lamivudine. J Gastroenterol 2004; 39: 34-40.

16. Locardini S. Molecular virology and the development of resistant mutants: implications for therapy. Semin Liver Dis 2005; 25 Suppl 1: 9-19.

17. Cane PA, Cook P, Ratcliffe D, et al. Use of real-time PCR and fluorimetry to detect lamivudine resistance-associated mutations in hepatitis B virus. Antimicrob Agents Chemother 1999; 43: 1600-8.

18. Stuyver L, Van Geyt C, De Gendt S, et al. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. J Clin Microbiol 2000; 38: 702-7.

19. Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981; 1: 431-5.

20. Buster EH, Janssen HL. Antiviral treatment for chronic hepatitis B virus infection--immune modulation or viral suppression? Neth J Med 2006; 64: 175-85.

21. Horgan M, Brannigan E, Crowley B, et al. Hepatitis B genotype and YMDD profiles in an untreated Irish population. J Clin Virol 2006; 35: 203-4.

22. Shin YM, Heo J, Kim GH, et al. Natural YMDD motif mutations of HBV polymerase in the chronic hepatitis B virus infected patients. Taehan Kan Hakhoe Chi 2003; 9: 1-9.

23. Huang ZM, Huang QW, Qin YQ, et al. Clinical characteristics and distribution of hepatitis B virus genotypes in Guangxi Zhuang population. World J Gastroenterol 2005; 11: 6525-9.

24. Ye XG, Wang RL, Guo HB. Detection and analysis of YMDD mutate genes in patients of chronic hepatitis B before being treated. Zhonghua Jianyan Yixue Zazhi 2002; 25: 248.

25. Lee C-Z, Lee H-S, Huang G-T, et al. Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment. World J Gastroenterol 2006; 12: 5301-5.

26. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61-8.

27. Leung NW, Lai CL, Chang TT, et al. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001; 33: 1527-32.

28. Liaw YF, Leung NW, Chang TT, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000; 119: 172-80.

29. Chang TT, Lai CL, Chien RN, et al. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J Gastroenterol Hepatol 2004; 19: 1276-82.

30. Song BC, Suh DJ, Lee HC, et al. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000; 32: 803-6.

31. Liaw YF, Chien RN, Yeh CT, et al. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999; 30: 567-72.

32. Rizzetto M, Tassopoulos NC, Goldin RD, et al. Extended lamivudine treatment in patients with HBeAg-negative chronic hepatitis B. J Hepatol 2005; 42: 173-9.

33. Suzuki F, Akuta N, Suzuki Y, et al. Clinical and virological features of non-breakthrough and severe exacerbation due to lamivudine-resistant hepatitis B virus mutants. J Med Virol 2006; 78: 341-52.

34. Liaw YF. Impact of YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. Antivir Chem Chemother 2001; 12 Suppl 1: 67-71.