

Original Article

YMDD Mutations and Genotypes of Hepatitis B Virus in Northern China

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SUMMARY: The objective of this research was to determine the relationship between YMDD mutations and the genotypes of hepatitis B virus (HBV) during lamivudine treatment. HBV genotypes were determined by nested PCR with 6 pairs of HBV genotype-specific primers (A to F) in serum specimens from 142 hepatitis B patients receiving lamivudine antiviral therapy. YMDD mutations were detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC). Among 142 serum specimens, 13 samples were genotype B (9.2%), 125 samples were genotype C (88%), 4 samples were genotype D (2.8%), and 80 YMDD mutations were found. The YMDD mutation rates were 69.2 and 54.4% in genotype B and genotype C, respectively. There was no significant difference in the YMDD mutation rate between genotypes B and C. Nine genotype B sera with YMDD mutations were found, including 2 YIDD mutations and 7 YVDD (M + V) mutations. Sixty-eight genotype C sera with YMDD mutations were found, including 34 mutations I (M + I) and 17 mutations V (M + V). There was a significant difference in the YMDD mutation types between genotypes B and C. Our results suggested that the YMDD mutation rate was 56.3% in patients treated with lamivudine for 2-4 years. YIDD was the main mutation type. The YMDD mutation rate showed no significant difference between HBV types B and C ($P > 0.05$), while the YMDD mutation types showed a significant difference between HBV types B and C in Northern China ($\chi^2 = 4.6$, $P < 0.05$).

INTRODUCTION

Hepatitis B, caused by the hepatitis B virus (HBV), exists throughout the world, and the prevalence is especially high in China. There are approximately 1.2 billion people infected by HBV. Lamivudine is one of the nucleoside medicines used to treat HBV. It can improve the condition of chronic hepatitis B patients in terms of virology, biochemistry and histology. Lamivudine cuts down the HBV DNA levels in patients' sera and can produce a certain proportion of HBeAg seroconversion (1). Lamivudine has been promoted for hepatitis B treatment, but the patient's condition may deteriorate rapidly after long-term use. The main reason for this is the occurrence of YMDD mutations in the HBV polymerase gene (2). HBV has been classified into eight genotypes (A - H) based on genome sequence divergences. The genotypes of HBV have distinct geographical distributions. Hepatitis B genotypes are associated with virus replication, virus variation, disease prognosis and the choice of drugs (3-7). In this study, the relationship between the YMDD mutations of HBV and HBV genotypes during lamivudine treatment is determined.

MATERIALS AND METHODS

Study population: A total of 142 serum specimens from patients with chronic hepatitis without cirrhosis who were hospitalized or were being seen in the clinic service at the Infectious Disease Department of the Second Affiliated Hospital of Harbin Medical University, Harbin, China from 2003 to 2005. All cases were diagnosed according to the Standard of Chinese Virus Hepatitis in 2000, and cases with liver

complications caused by other aetiologies or other types of hepatitis virus were excluded. The 142 patients included 112 men and 30 women, and their ages ranged from 18 to 78. DNA levels of HBV in serum from all patients were more than 103 copies/ml. All cases received lamivudine treatment orally, 100 mg everyday for 24-48 months, and did not receive other antiviral therapy during the study. All the patients gave written informed consent. This study was approved by the Harbin Medical University Committee on Clinical Investigation.

Laboratory findings: HBeAg levels in patients sera were measured with enzyme-linked immunosorbent assay (ELISA) using the Diagnostic Kit for Hepatitis B e Antigen (PG Biotechnology, Shenzhen, China). The HBV DNA levels were tested by real-time fluorimetry PCR with TaqMan probe using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology).

Nucleic acid extraction: HBV DNA amplification was performed for sequence analysis. HBV DNA was extracted from 100 μ l test sera and 100 μ l of DNA extract I (PG Biotechnology) were oscillated and mixed well, then centrifuged at 13,000 rpm for 10 min, and the supernatant was discarded. Twenty-five microliters of DNA extract II (PG Biotechnology) were added, oscillated and mixed well, and centrifuged at 2,000 rpm for 10 sec. Then, the mixture was placed in a dry bath for 10 min at 100°C and centrifuged at 13,000 rpm for 10 min, and the supernatant was retained in the stock.

Genotyping of HBV by PCR: HBV genotypes were determined by nested PCR with six pairs of HBV genotype-specific primers (A to F). Vide reference (8).

Detection of YMDD mutation: YMDD was detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC) using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology).

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Table 1. HBV genotype distribution and characteristics of the patients

Genotype	Cases	Positive rate	Men/women	Age (mean ± SD)	HBVDNA (10 ⁶ mean ± SD)	HBeAg ⁺ /HBeAg ⁻	Treatment period (month)
Genotype B	13	9.2%	10/3	37.9 ± 11.0	12.9 ± 21.6	9/4	34.2 ± 6.8
Genotype C	125	88.0%	99/26	38.4 ± 11.9	11.3 ± 24.5	95/30	34.9 ± 6.4
Genotype D	4	2.8%	3/1	38.8 ± 11.1	11.7 ± 19.0	3/1	34.8 ± 5.6

Table 2. YMDD mutation rate and YMDD mutation type in 80 HBV sera

Genotype	<i>n</i>	YIDD	YMDD+YIDD	YVDD	YMDD+YVDD	YIDD+YVDD	YMDD mutation rate
Genotype B	9	2 (22.2)	0	6 (66.7)	1 (11.1)	0	69.2% (9/13)
Genotype C	68	30 (44.1)	4 (5.9)	15 (22.1)	2 (2.9)	17 (25.0)	54.4% (68/125)
Genotype D	3	2 (66.7)	0	0	0	1 (33.3)	75.0% (3/4)
Total	80	34 (42.5)	4 (5.0)	21 (26.2)	3 (3.8)	18 (22.5)	56.3% (80/142)

YMDD mutation type was determined after lamivudine treatment for 2 years, and YMDD mutation type was examined every 6 months.

Statistical analysis: Statistical analyses were performed using Adopt SPSS 10.0 software. The rate comparison was made using the chi-square criterion. A difference with a $P < 0.05$ was considered significant. Student's *t* test and nonparametric test were used to compare differences between groups, where appropriate.

RESULTS

Analysis of HBV genotypes and clinical data: A simple and precise genotyping system based on nested PCR with 6 pairs was developed for the determination of genotypes of HBV. All 142 samples were genotyped by nested PCR analysis: the majority was genotype C, which accounted for 88%; genotype B accounted for 9.2%; and genotype D accounted for only 2.8%. No other genotypes were detected (genotypes A, E, F). No statistically significant differences were observed in mean age, male-to-female ratio, mean serum DNA levels, HBeAg positive and treatment period with genotype B and with genotype C. Genotype D was not included in the statistical analysis because of the low number of cases (Table 1).

YMDD mutation rate and YMDD mutation type in 80 HBV sera: FH-PCR-MC in the detection of HBV YMDD mutation has high sensitivity and specificity. It is a convenient and rapid, and may be used in YMDD typing. HBV wild type and mutation type were detected in 62 and 80 specimens, respectively by FH-PCR-MC. The YMDD mutation rate was 56.3% in the 142 specimens. Among the 80 YMDD mutations, 38 YIDD (M + I) mutations (47.5%), 24 YVDD (M + V) mutations (30%) and 18 YIDD + YVDD mutations (22.5%) were found. Among the 80 YMDD mutations, the YIDD mutation rate was higher than the YVDD mutation rate. The mutation type was mainly YIDD. The YMDD mutation rates were 69.2 and 54.4% in genotypes B and C, respectively. Although the YMDD mutation rate was higher in patients with genotype B than in those with genotype C, there was no statistically significant difference between genotypes B and C ($P > 0.05$). Genotype D was not included in the statistical analysis because of the low number of cases (Table 2).

Relation between the HBV YMDD mutation type and genotypes B and C: Among the 68 patients with genotype C, 23 (33.8%) patients were of the mixed mutation type. Among the 9 patients with genotype B, 1 (11.1%) patient

Table 3. Relationship between HBV YMDD mutation type and genotypes B, C

Genotype	<i>n</i>	YIDD (M + I)	YVDD (M + V)
Genotype B	9	2 (22.2)	7 (77.8)
Genotype C	51	34 (66.7)	17 (33.3)

were of the mixed mutation type. Although the YMDD mixed mutation type rate was higher in patients with genotype C than in those with genotype B, there was no statistically significant difference between genotypes B and C ($P > 0.05$). Among the 36 patients with YIDD (M + I) mutation types, 2 patients were genotype B and 34 patients were genotype C. Among the 24 patients with YVDD (M + V) mutation types, 7 patients were genotype B and 17 patients were genotype C. The YMDD mutation types (YIDD and YVDD) showed significant differences between genotypes B and C ($\chi^2 = 4.6$, $P < 0.05$). Genotype D was not included in the statistical analysis due to the low number of cases (Table 3).

DISCUSSION

Lamivudine has been shown to be a potent and nontoxic inhibitor of HBV replication in chronically infected patients. Long-term lamivudine treatment for chronic HBV infection induces the emergence of lamivudine-resistant HBV YMDD mutant strains. In the case of YMDD variants, the methionine (M) is substituted with either isoleucine (I), designated as YIDD, or valine (V), designated as YVDD (9).

It has been reported that the HBV YMDD mutation rate increases along with the duration of lamivudine therapy. The YMDD mutation rates were 16-32%, 47-56% and 69-75% in the groups of patients administered lamivudine for 1, 2 and 3 years, respectively (10). A study from Asia found that the 1-, 2- and 3-year YMDD mutation rates were 15, 38 and 53% after lamivudine therapy, respectively (11). The present 142 chronic hepatitis B patients received lamivudine for 2-4 years. The HBV YMDD mutation rate should be 56.3%, based on the previous reports. The mutation type was mainly YIDD. The presence of the YIDD motif preceded the exclusive presence of the YVDD motif, and we concluded that the YIDD motif could occur as a temporal intermediate (12). It has been reported that the YIDD or YVDD motif alone did not shift to the mixed type (YVDD and YIDD) when they were examined 12 months after the detection of the mutant virus in Japan (13). In our study, the YMDD mutation type was determined every 6 months for 2 years after lamivudine

treatment. Recently we found that 2 patients, who were not included in this study, had shifted to the mixed type (YIDD and YVDD) (unpublished observation), indicating that the shift can occur over a relatively long period. During the past few years, many researchers have investigated the forecasting factors of drug resistance and mutations, but without reaching a conclusion. Some factors related to YMDD mutations could increase the curative effect of lamivudine. Recently, many investigations have concentrated on the time of the YMDD mutation appearance, the mutation type, the YMDD mutation and ALT level, the DNA level, and the rate of HBeAg seroconversion, but no relationship between the mutations of HBV and the HBV genotypes has been reported. The viral genotype that represents the features of natural infection is the result of evolutionary variation of the virus. The HBV genotype has a certain association with the route of virus infection, genetic mutations, the progression of chronic hepatitis B, and the efficacy of antiviral therapies. The genotypes of HBV also have distinct geographical distributions. Genotypes B and C have only been found in individuals who originated from eastern Asia and the Far East, including Taiwan (14). In Japan, the great majority of HBV isolates belong to genotype B or C (15). Genotypes B and C are spread dominantly in China, while genotypes A and D relatively rare. Genotype B is prevalent in South China and genotype C in North China (16). The clinical prognosis after infection with HBV varies according to the genotype. In the previous study, the clearance of HBeAg occurred earlier and more frequently, and the development of cirrhosis was less common in patients with genotype B compared to those with genotype C (15). Genotype C is associated with chronic liver disease and genotype B is mostly associated with acute hepatitis in China (17). The present study analyzed 142 sera of chronic hepatitis B in North China. The results showed that genotype C occupied 88%, genotype B 9.2%, and genotype D 2.8%. Genotype C is predominant in patients with chronic liver disease in North China. A study in Japan reported that among 234 patients with chronic hepatitis B infected with genotype A, B, or C and treated with lamivudine for more than 1 year, the emergence of mutations was not different among genotypes A, B, and C as determined by the Kaplan-Meier method (18). In Japan, it was reported that the emergence rate of lamivudine resistance was independent of the genotype (A, B, or C) after the lamivudine treatment of patients with chronic hepatitis B with various genotypes. In contrast, the emergence rate was significantly higher in the Ba (a stands for Asia) subgroup of HBV than in Bj (j for Japan) subgroup ($P < 0.05$) (19). In the Hong Kong study, there was no difference in the antiviral response and the rate of development of YMDD mutations in 82 patients with chronic hepatitis B with genotypes B and C after 1 year of lamivudine treatment (20). In a report on 87 patients receiving lamivudine in Guiyang, the results showed that, though genotype B was dominant in that region, there was a higher mutation rate of drug resistance among genotype C cases (21). In another report, 135 chronic hepatitis B patients received lamivudine for 1 year, and the YMDD mutation rate in genotype C cases was higher than that in genotype B cases in Guangdong (22). But Guiyang and Guangdong provinces are located in the southern parts of China. In the present study, 9 YMDD mutations were found in genotype B, and 68 YMDD mutations were found in genotype C. The YMDD mutation rates were 69.2 and 54.4% in genotype B and in genotype C, respectively, in North China ($P > 0.05$). It has been reported

that the YIDD mutation occurs more frequently in genotype D, while YVDD is more common in genotype A (23). It is suggested that the YMDD mutation type is related to the genotype. But the present study showed that the YVDD mutation occurred more frequently in genotype B and the YIDD mutation in genotype C, and that the YMDD mutation showed significant differences between the HBV genotypes B and C ($\chi^2 = 4.6$, $P < 0.05$). It has been reported that the replication ability of the YVDD mutant strain might be stronger than that of the YIDD mutant strain (24). The mutation types among different genotypes are not identical, because the genotypes are associated with virus replication and virus variation. Because of the small number of cases of genotype D, only HBV genotypes B and C were examined in this study. Large-scale prospective studies of each genotype should be conducted in the future to confirm these findings.

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