

YMDD mutations in patients with chronic hepatitis B untreated with antiviral medicines

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incidence rate have no significant difference between familial aggregation and non-familial aggregation. It may have no significant relationship between YMDD mutations and pre-c-zone mutations. HBV DNA level may not have a positive correlation with YMDD mutations. LAM is clinically effective for CHB patients with YMDD mutations.

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Key words: Hepatitis B virus; Chronic hepatitis; Genotypes; YMDD mutation; Lamivudine

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Abstract

AIM: To polymerase P region (YMDD) mutations of hepatitis B virus gene (HBV DNA) in patients with chronic hepatitis B (CHB) untreated with antiviral medicines and to explore its correlation with pre-c-zone mutations, HBV genotypes and HBV DNA level, and to observe its curative effect.

METHODS: A total of 104 cases (38 cases in group of familial aggregation and 66 cases in group of non-familial aggregation) were randomly chosen from 226 patients with CHB who did not receive the treatment of lamivudine (LAM) and any other antivirus drugs within the last one year. Their serum YMDD mutations were detected by microcosmic nucleic acid and cross-nucleic acid quantitative determination, HBV genotypes by PCR-microcosmic nucleic acid cross-ELISA, HBV DNA quantitative determination and fluorescence PCR analysis, hepatitis B virus markers (HBVM) by ELISA. LAM was taken by 10 patients with YMDD mutations and its curative effect was observed.

RESULTS: Twenty-eight cases (26.9%) had YMDD mutations, of them 11 cases (28.9%) were in familial aggregation group (38 cases) and 17 cases (25.8%) in non-familial aggregation group (66 cases) with no significant difference between the two groups. Twenty-seven point one percent (16/59) cases were positive for HBeAg YMDD mutations, and 26.7% (12/45) cases were negative for HBeAg and positive for anti-HBe. There was also no significant difference between the two groups. Different YMDD incidence rate existed in different HBV genotypes. HBV DNA level did not have a positive correlation with the incidence of YMDD mutations. LAM was effective for all patients with mutations.

CONCLUSION: Wild mutant strains in HBV and their

INTRODUCTION

To investigate the polymerase P region (YMDD) mutations of hepatitis B virus gene (HBV DNA) in patients with CHB who had not received antiviral treatment, 104 cases were randomly chosen from 226 such cases to detect their serum YMDD mutations. To explore its correlation with HBV genotypes, HBV DNA level and HBV e antigen system, and to investigate its incidence rate between familial aggregation and non-familial aggregation. LAM treatment was given to some patients with YMDD mutations. The therapeutic results were analyzed.

MATERIALS AND METHODS

Patients and materials

A total of 104 patients were from out-patient and in-patient departments of our hospital. Among them 38 cases were in familial aggregation group (2 or more close-contacted family members were infected with HBV but had no direct evidence of blood transmission), including 26 males and 12 females aged 17-69 years (average, 35 years). Sixty-six cases were in non-familial aggregation group, including 45 males and 21 females aged 16-56 years (average, 34.6 years). All the cases met the diagnosis standards made at the Tenth Viral Hepatitis Conference^[1] and had not received LAM treatment or any other antiviral treatment within one year before detection of serum YMDD.

Detection of YMDD mutations

YMDD mutations of HBV DNA were detected by micro-

osmic nucleic acid cross-nucleic acid quantitative determination. The detection of 25 cases was completed at the Biomedicine Diagnosis and Research Center of Basic Medicine Department of the First Military Medical University, and the detection of the other 79 cases was completed under the same conditions at our hospital's central laboratory. For specimen processing, 50 μ L serum to be tested and 50 μ L processed liquid were fully mixed, boiled at 100 °C for 5 min, spun by centrifugation at 12 000 r/min for 5 min, and 8 μ L supernatant was put into a tube containing reaction liquid, spun by centrifugation for 10 s at 10 000 r/min, then put into the processing machine and pre-denatured at 92 °C for 2 min, followed by 35 cycles (denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, extension at 72 °C for 65 s) and a final extension at 72 °C for 3 min. For HBV DNA primer design, HBV W1 (TGGA CGTCGCATG GGAGAACCACCGTGAA) and HBV W2 (GAAAGCTTCTGCGACGCCGTGATTGAG) were taken as primers of YMDD. For hybridization, amplified products were denatured at 98 °C for 10 min and then cooled in an ice-bath for 10 min. Two hundred micro liters of hybridization liquid was added and kept at 55 °C for 6 min, and the step was repeated twice. One hundred micro liters of enzyme anti-body liquid was added and kept at 37 °C for 30 min, discarded gently and completely, then 200 μ L enzyme lotions was added, kept at 37 °C for 5 min, and the step was repeated twice. Color reagents A and B (30 μ L) were added, kept at room temperature for 10 min, then 60 μ L termination liquid was added and the results were observed. The spatial absorption value (A) was detected with an enzyme labeling machine. YMDD was considered to be positive if $P/N \leq 3.5$.

Detection of HBV genotypes

HBV genotypes were detected by PCR-microcosmic nucleic acid cross-ELISA^[2]. Twenty-five cases were completed in Biomedicine Diagnosis and Research Center, Basic Medicine Department of the First Military Medical University, and the other 79 cases were detected in our hospital according to the introduction manual.

Quantitative determination of HBV DNA

Shanghai FX990 micro-fluorometer and HBV-PCR fluoroscopy reagent kits (termination value, 10^3 copy/mL), provided by the Shanghai Fu Xing Company, were used. The procedure was strictly manipulated according to the introduction manual and estimated by experts.

Detection of HBVM

For the detection of HBVM, ELISA was adopted. Reagent kits were provided by Zhongshan Bioengineering Co. Ltd, Guangdong. The manipulations were strictly completed according to the introduction manual.

Treatment

LAM (10 mg/d p.o.) was given to patients with YMDD mutations and their liver functions, HBVM and HBV DNA were detected every 4 wk and observed continuously for 42 wk.

Statistical analysis

The incidence rate of YMDD mutation was compared by

χ^2 test between different genotypes, HBV DNA level, HBV e system, familial aggregation and non-familial aggregation case. The statistical analysis was processed with SAS statistical software. $P < 0.05$ was considered significant.

RESULTS

YMDD mutation data of 104 CHB cases

Twenty-eight cases (26.9%) out of 104 had YMDD mutations. The incidence rate of YMDD mutations was 25.8% (17/66) in non-familial aggregation cases, and 28.9% (11/38) in familial aggregation cases. No significant difference could be found between the two groups. ($\chi^2 = 0.124$, $P > 0.05$).

Relationship between HBeAg system and YMDD mutations

Of the 59 cases who were positive for HBeAg, 16 had YMDD mutations and the incidence rate was 27.1%. Of the 45 cases who were negative for HBeAg and positive for anti-HBe, 12 cases had YMDD mutations and the incidence rate was 26.7%. There was no significant difference between the two groups ($\chi^2 = 0.0027$, $P > 0.05$).

YMDD mutations in different HBV genotypes

Genotypes of all these cases included D, C, B, non-classified types and mixed forms of CD, CB and DB. YMDD mutations of different genotypes are shown in Table 1. It demonstrated that the mutations mostly occurred in genotype C and its mixed genotypes, accounting for 71.4% (20/28).

Table 1 YMDD mutations in different HBV genotypes

Patterns of genotypes	<i>n</i>	Case number of YMDD mutations	Incidence rate (%)
CD	25	8	32.0
CB	16	6	37.5
C	15	6	40.0
D	9	2	22.2
B	9	-	-
DB	12	-	-
Non-classified types	18	6	33.3
Total count	104	28	26.9

Comparison of mutations in different groups ($\chi^2 = 0.9334$, $P > 0.05$).

Relationship between HBV DNA level and YMDD mutations

There was no significant difference between high and low HBV DNA level groups, suggesting that HBV DNA level might not have a positive correlation with YMDD mutations (Table 2).

Table 2 Comparison of YMDD mutations in different HBV DNA levels

HBVDNA (copy/mL)	<i>n</i>	YMDD mutations	
		Positive	Positive rate (%)
$\leq 10^5$	56	16	28.6
$> 10^5$	48	12	25.0

Comparison between the two groups ($\chi^2 = 0.1676$, $P > 0.05$).

Observation of curative effect

Of the 28 patients with YMDD mutations, 18 gave up treatments because of financial difficulty, the other 10 had abnormal ALT level and were positive for HBeAg at the same time. In the 10 cases, HBV DNA level decreased by different degrees 4 wk after LAM treatment. At the end of 42 wk, HBV DNA level in 8 cases (80%) was lower than 10^3 /mL, and ALT was returned to normal and baseline level of ALT did not rebound. HBeAg converted to negative in 4 (40%) cases and 1 (10%) case had HBeAg/anti-HBe serum conversion. It was suggested that LAM had a good curative effect in the patients with wild YMDD mutations.

DISCUSSION

HBV belongs to DNA viral species and its duplication course is similar to that of reversed transcription viruses. HBV DNA polymerase has an activity of reversed transcription and a highly conservative YMDD order locating in the polymerase structural region C area, which is the combing and functioning site of LAM (nucleoside antiviral medicine). A great number of studies in recent years showed that long-term (longer than 6 mo) LAM treatment could contribute to YMDD mutations^[3-12] and disease recurrence. Some patients even went worse, and eventually died^[13-17]. But it has been seldom reported whether YMDD mutations have natural existence. Some scholars^[18,19] found that YMDD mutational strains were positive in the serum of the cases infected with CHB who did not receive LAM treatment. Yan *et al.*^[20] showed that 19 cases had YMDD mutations out of 110 CHB cases untreated with LAM. Fontaine *et al.*^[21] reported that 5 had YMDD mutations out of 18 cases of asymptomatic HBV carriers, and so they considered that the natural existence of YMDD mutational strains was associated with a great amount of HBVs existing in CHB patients and its mutations. Fontaine *et al.*^[21] revealed that YMDD mutations also occurred in HBV-infected cases who underwent kidney transplantation and dialysis therapy. In addition, Zhang *et al.*^[22] found that 32 (26.2%) cases (including wild YMDD genotypes and mutant genotypes) had YMDD mutations in 122 CHB cases by genetic chip determination. Matsuda *et al.*^[23] reported that a few CHB cases not treated with LAM had YMDD mutations. Of the 104 CHB cases in this study who had not received LAM and any other Chinese traditional or Western antiviral treatment, 28 (26.9%) had YMDD mutations and the detection rate of mutational strains was in accordance with that reported by Zhang *et al.*^[22], showing that wild YMDD mutational strains were in existence in HBV DNA. However, the spontaneous incidence rate of YMDD mutations had no correlation with familial aggregation.

Kobayashi *et al.*^[19] found that anti-HBe was positive in all patients with YMDD mutations, and 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 pre-c-zone. In this study, we found that the incidence rate of YMDD mutations was 27.1% in patients with negative HBeAg while 26.7% in patients with negative HBeAg and positive anti-HBe. There was no significant difference between the two groups. These results do not accord with those of Kobayashi

et al.^[19] and Ye *et al.*^[24] but accord with the studies of Da Silva *et al.*^[25] and Yan *et al.*^[20]. Therefore, YMDD mutations might not have a relationship with pre-c-zone mutations.

We found that there was a difference in the incidence rate of HBV YMDD wild mutational strains between HBV genotypes C, D, non-classified types and mixed genotypes of CD and CB. Most of the mutations (71.4%) occurred in genotype C and its mixed forms partly because genotype C occupied a great proportion in CHB cases. Though there was no significant difference among these genotypes, the relation between YMDD mutations and HBV genotypes could not be excluded as the cases were comparatively small in our study.

Some scholars^[26,27] proposed that a high HBV DNA level of serum had a positive correlation with the incidence of mutations. After HBV DNA was detected in 104 CHB patients, we found 12 YMDD mutations (25.0%) in 48 cases with a higher level of HBV DNA ($>10^5$ copies/mL), while 16 (28.6%) mutations were found in 26 cases with a lower HBV DNA level ($\leq 10^5$ copies/mL) and there was no significant difference between the two groups, suggesting that HBV DNA level might not be positively correlated with YMDD mutations. Further studies with more samples should be conducted.

LAM has been acknowledged and identified as one of the first-line medicines for CHB at World Gastroenterology Conference, and by Asian-Pacific Region Hepatology Association and European-American Hepatology Association^[28,29]. In our study, 10 patients with YMDD mutations were treated with LAM. We found that HBV DNA level decreased by different degrees 4 wk after treatment, and at the end of 42 wk HBV DNA level in 8 cases was lower than 10^3 copies/mL, and their ALT recovered to normal and baseline level of ALT did not rebound, HBeAg became negative in 4 cases and 1 case had HBeAg/anti-HBe conversion. These indicated that LAM had a short-term curative effect on CHB with YMDD mutations. The results might be associated with the fact that wild viral strains were dominant while YMDD mutational strains were minor and had a lower duplication activity and weaker pathogenicity. The samples were small and the course of treatment was not long enough in our study to assess. To know whether and how the curative effect of LAM was affected by YMDD mutational strains, a contoured study with a non-YMDD mutation group and larger sample size is needed. Some scholars^[30-34] considered that LAM combined with other antiviral medicines in the treatment of CHB patients would partly improve the curative effect and could delay or decrease the occurrence of drug-tolerant YMDD mutation strains. Thus, it is worthwhile to explore antiviral medicines for the treatment of patients with natural YMDD mutations.

In conclusion, YMDD wild mutational strains exist in HBV and its incidence rate has no relation to familial aggregation. LAM has a good short-term curative effect in CHB patients with YMDD mutations. But some issues still need further research, such as the factors that contribute to mutations, the relationship between mutations and the development of hepatic diseases, the correlation of pre-c-zone mutations with genotypes, the relationship between HBV DNA level and the incidence of mutations.

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