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Original Article

YMDD motif mutations in chronic hepatitis B antiviral treatment naïve patients: a multi-center study

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Objective: This study aimed to determine the natural prevalence of variants of tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif in patients with chronic hepatitis B (CHB), and to explore its relation with demographic and clinical features, hepatitis B virus (HBV) genotypes, and HBV DNA levels.

Methods: A total of 1,042 antiviral treatment naïve CHB patients (including with lamivudine [LAM]) in the past year were recruited from outpatient and inpatient departments of six centers from December 2008 to June 2010. YMDD variants were analyzed using the HBV drug resistance line probe assay (Inno-Lipa HBV-DR). HBV genotypes were detected with polymerase chain reaction (PCR) microcosmic nucleic acid cross-ELISA, and HBV deoxyribonucleic acid (DNA) was quantitated with real-time PCR. All serum samples underwent tests for HBV, HCV, and HDV with ELISA.

Results: YMDD variants were detected in 23.3% (243/1042) of CHB patients. YMDD mutation was accompanied by L180M mutation in 154 (76.9%) patients. Both wild-type HBV and YMDD variant HBV were present in 231 of 243 patients. Interestingly, 12 patients had only YIDD and/or YVDD variants without wild YMDD motif. In addition, 27.2% (98/359) of HbeAg-positive patients had YMDD mutations, which was higher than that in HbeAg-negative patients (21.2%, 145/683). The incidence of YMDD varied among patients with different HBV genotypes, but the difference was not significant. Moreover, the incidence of YMDD in patients with high HBV DNA level was significantly higher than that in those with low HBV DNA level.

Conclusion: Mutation of YMDD motif was detectable at a high rate in CHB patients in this study. The incidence of YMDD may be correlated with HBeAg and HBV DNA level.

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Introduction

Hepatitis B virus (HBV) is a leading cause of liver diseases worldwide, and China has a high prevalence of HBV infection. HBV can replicate through reverse transcription using viral ribonucleic acid (RNA) dependent deoxyribonucleic acid (DNA) polymerase, which has been a target of most current therapeutic approaches for inhibiting HBV replication. Several nucleos(t)ide analogues (NA) such as lamivudine, adefovir, and entecavir have been developed for the treatment of chronic hepatitis B (CHB). However, prolonged treatment with antiviral drugs is often associated with a high incidence of viral resistance. Drug-resistance associated with tyrosine-methionine-aspartic acid-aspartic acid (YMDD) mutation has become the most important cause of treatment failure using NA.¹

Gene mutations resulting in lamivudine resistance occur particularly in the highly conserved YMDD motif of the catalytic domain (C domain) of DNA polymerase.² Substitutions of methionine at codon 204 to either isoleucine (rtM204I, YIDD variant) or valine (rtM204V, YVDD variant) are the predominant mutations causing lamivudine resistance.^{3,4} The YVDD variant is almost always accompanied by an additional rtL180M mutation of the B domain.⁵

Although the presence of drug-resistant strains is closely related to the duration of lamivudine treatment, and the mutations of YMDD motif are thought to be secondary to lamivudine use, it has become increasingly evident that YMDD mutations occur naturally and exist in HBV patients who did not receive antiviral therapy, particularly in those with antibody against hepatitis B e antigen (anti-HBe) in serum.⁶⁻¹³ It is unknown, however, whether or not pretreatment YMDD mutations are selected by lamivudine during treatment.

A test system using the line probe assay (LIPA) has been developed for the detection of resistance mutations in the HBV polymerase gene.¹⁴ LIPA is a useful tool for rapid and accurate detection of mutations, which are present in as little as 10% of the HBV population.¹⁵⁻¹⁷ The present study aimed to determine the natural prevalence of YMDD mutation in chronic HBV patients in China.

Patients and methods

Patients

A total of 1,042 patients were recruited from outpatient and inpatient departments of six centers from December 2008 to June 2010 (628 patients from The No. 3 People's Hospital of Zhenjiang, 215 patients from The No. 2 Hospital of Nanjin, 89 patients from The First Affiliated Hospital of Suzhou University, 56 patients from The Affiliated Hospital of Yangzhou University, 29 patients from The People's Hospital of Wujiang, 25 patients from The People's Hospital of Danyang). Demographic and clinical features of these patients are shown in Table 1. Of these patients, 359 were positive for HBeAg, and the remaining were HBeAg negative. The diagnosis of CHB was made according to the criteria of the National Program for Prevention and Treatment of Viral Hepatitis.¹⁸ Patients had not received antiviral treatment

in the past year. All patients were negative for HCV and HDV. These patients were excluded from the study as no further analysis of the viral DNA could have been performed. Blood was collected twice from each patient: the first blood sample was used for biochemical tests including alanine aminotransferase (ALT) aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (GGT), total protein, albumin, total bilirubin and platelet count; the second was stored at -80°C and used for HBV drug resistance LIPA and detection of HBV DNA by polymerase chain reaction (PCR). All patients were also evaluated for hepatosteatosi s by abdominal ultrasonography.

HBV/HCV/HDV serology

Serum samples were tested for HBV, HCV and HDV using ELISA (Roche – Shanghai, China).

HBV drug resistance LIPA (Inno-Lipa HBV DR)

HBV DNA was isolated from 200 μL of serum using High Pure Viral Nucleic Acid Kit (Roche). The Inno-Lipa HBV DR v1 assay (Inno-Lipa; Innogenetics, Beijing, Beijing Pason Pharmaceuticals Inc.) was performed according to the manufacturer's instructions using Hot Start Taq DNA polymerase (Invitrogen – USA). The assay was based on amplification of a part of viral polymerase gene by the pre-designed primers and reverse hybridization by the probes coated on a strip, and detected the HBV polymerase wild-type mutations and known drug-induced mutations associated with lamivudine and famciclovir resistance (codons 180, 204, and 207).

Measurement of serum HBV DNA by real-time PCR

Serum and HBV DNA were measured quantitatively by real-time PCR (Model 5700, ABI Company - USA) with a lower limit of detection of 1×10^3 HBV DNA copies/mL and 1×10^3 copies/ μg total DNA.

Detection of HBV genotypes

HBV genotypes were detected by PCR-microcosmic nucleic acid cross-ELISA. There were 25 patients whose HBV genotyping was performed at the Biomedicine Diagnosis and Research Center, Basic Medicine Department of the First Military Medical University, and for 1,017 patients HBV genotyping was performed in our hospital.

Statistical analysis

All quantitative data were presented as mean \pm standard deviation (SD). Demographic data were analyzed using descriptive statistical tests. Mann-Whitney's U-test, chi-squared test, and Fisher's exact test were used for comparison of groups. The factors included patient age, gender, ALT, AST, ALP, GGT level, HBeAg status, HBV DNA level, alcohol consumption, family history of hepatosteatosi s, and HBV genotype. Initially, univariate analyses were conducted. Then, all factors found to be at least marginally associated with the incidence of YMDD mutation ($p < 0.15$) were tested by multivariate analysis using a stepwise logistic model. A p -value < 0.05 was considered statistically significant. Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS Inc. – Chicago, IL, USA) for Windows was used for statistical analyses.

Results

YMDD mutation data of 1042 CHB cases

YMDD variants were found in 23.3% (243/1042) of HBV patients. The demographic and clinical features of the study group are summarized in Table 1. Of 243 patients with YMDD mutations, 154 (63.4%) had concomitant L180M mutation, and two had a mutation at codon 207. Both wild-type and YMDD variant carrying HBV were present in 231 of 243 patients. Mixtures of YMDD+YVDD, YMDD+YIDD, and YMDD+YVDD+YIDD were found in 48, 78, and 118 patients, respectively. In 12 patients, only YIDD and/or YVDD variants were observed, and YIDD + YVDD, YIDD, and YVDD variants were shown in five, four, and three patients, respectively.

Relationship between hepatosteatosis and YMDD mutations

There were 124 patients (11.9%) who had hepatosteatosis diagnosed ultrasonographically. In the hepatosteatosis group, there were 15 patients whose body mass index (BMI) was greater than 30. In addition, there were six patients who had a history of alcohol consumption. Only two patients with

hepatosteatosis had a history of alcohol consumption and BMI over 30. Twenty-six of 124 patients had YMDD variants, but there was no significant difference between HBV patients with hepatosteatosis and HBV patients with no hepatosteatosis ($\chi^2 = 1.837$, $p = 0.054$).

Relationship between HBeAg status and YMDD mutations

Of the 359 patients positive for HBeAg, 98 (27.3%) had YMDD mutations. Of the 683 patients negative for HBeAg but positive for anti-HBe, 145 (21.2%) had YMDD mutations. There was a significant difference between these two groups in the positivity rate of YMDD mutation ($\chi^2 = 4.846$, $p = 0.028$).

YMDD mutations in patients with different HBV genotypes

Genotypes of these patients included D, C, B, non-classified types and mixed forms of CD, CB and DB. YMDD mutations in patients with different genotypes are shown in Table 2. Results demonstrated that the YMDD mutations mostly occurred in patients with genotype C or its mixed genotypes, accounting for 51.4% (536/1042).

Table 1 - Demographic and clinical features of CHB patients

Characteristics	Total (n = 1042)	YMDD variants (n = 243, 23.3%)	Without YMDD variants (n = 799, 76.7%)	p-value
Age (years)	42 ± 13	42 ± 14	43 ± 12	0.783
Male	763 (73.2)	173 (71.2)	590 (73.9)	0.414
Female	279 (26.8)	70 (28.8)	209 (26.1)	0.414
Alcohol consumption	125 (12.0)	26 (10.7)	99 (12.4)	0.472
Family history [†]	475 (45.6)	105 (43.2)	370 (46.3)	0.325
Hepatosteatosis [‡]	124 (11.9)	26 (7.4)	98 (12.2)	0.054
AST (IU/L)	53 ± 25	56 ± 24	54 ± 23	0.963
ALT (IU/L)	67 ± 26	64 ± 33	69 ± 31	0.955
ALP (IU/L)	102 ± 45	106 ± 46	112 ± 54	0.067
GGT (IU/L)	26 ± 4	25 ± 3	26 ± 3	0.852

CHB, chronic hepatitis B; YMDD, tyrosinemethionine-aspartic acid-aspartic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ -glutamyl transpeptidase; SD, standard deviation.

Data are expressed as mean ± SD or as n (%).

[†]Family history of positive HBsAg including third-generation relatives.

[‡]Determined by ultrasonography.

Table 2 - YMDD mutations in patients with different HBV genotype

Genotypes	n	Patients with YMDD mutations	Positive rate (%)
CD	225	51	22.7
CB	156	36	23.1
C	195	47	24.1
D	47	10	21.2
B	109	24	22.1
DB	128	27	21.3
Non-classified types	182	48	26.4
Total	1042	243	23.3

YMDD, tyrosinemethionine-aspartic acid-aspartic acid; HBV, hepatitis B virus.

$\chi^2 = 2.413$, $p = 0.878$

Relationship between HBV DNA level and YMDD mutations

HBV-DNA levels of $\leq 10^3$, $> 10^3 \sim \leq 10^5$, and $> 10^5$ were found in 16.0% (41/257), 22.9% (131/573) and 29.2% (71/212) of patients with YMDD mutations, respectively. There was a significant difference in the positivity rate of YMDD mutations between patients with HBV DNA level of $\leq 10^3$ and those with HBV DNA level of $> 10^5$ among different groups (Table 3, $\chi^2 = 20.130$, $p = 0.000$), suggesting that the HBV DNA level was positively correlated with YMDD mutations. Moreover, the HBV-DNA level in HBeAg positive patients and HBeAg negative patients were $3.47 \pm 0.45 \times 10^7$ copies/mL and $6.44 \pm 1.25 \times 10^5$ copies/mL, respectively, showing marked difference ($t = 4.29$, $p = 0.03$).

Analysis of factors associated with YMDD mutations

Univariate analysis of individual factors showed that the HBV DNA level and the HBeAg status were associated with YMDD mutations. There was no association with the other factors: patients' age, gender, ALT, AST, ALP, GGT level, HBeAg status, HBV DNA level, alcohol consumption, family history of hepatosteatosi, and HBV genotype. Multivariate analysis

revealed that HBV DNA level ($p = 0.025$) and HBeAg status ($p = 0.043$) were also associated with YMDD mutations (Table 4).

Relationship between HBV DNA level and HBeAg status

HBV DNA level was associated with HBeAg status by univariate analysis (Table 5).

Discussion

In this study, the results showed that YMDD variants naturally existed in 23.3% of untreated CHB patients, most of whom also had wild-type HBV. Previous reports showed that the incidence of YMDD variants ranged from 0%~27.7% for asymptomatic HBV carriers and 0%~26.9% for CHB patients. These studies were mostly from Asian countries including Japan, China, and Korea. The discrepancy among these studies may be attributed to the relatively small sample sizes, the different methods for detection, and the distinct characteristics of subjects such as the duration of infection, viral load, viral genotype, and other amino acid variations of viral genome. In this study, a well-characterized commercial assay was used, which can detect variants that comprise as little as 10% of the viral population.¹⁵ A recent report investigated the Inno-Lipa HBV DR assay in monitoring HBV-infected patients receiving NA treatment, and showed that the LIPA test had convincing diagnostic sensitivity and accuracy. Moreover, in the present study, a large number of subjects were recruited, which makes it possible to obtain a more accurate prevalence of YMDD variants existing naturally in Chinese patients.

In the present study, the majority of YMDD-positive patients had both wild-type HBV and YMDD variant carrying HBV, which is consistent with other studies.⁸ Interestingly, there were 12 patients presenting hybridization only with

Table 3 - YMDD mutations in patients with different HBV DNA levels

HBV DNA (copies/mL)	n	YMDD mutations (n, %)
$\leq 10^3$	257	41 (16.0)
$>10^3 \sim \leq 10^5$	573	131 (22.9)
$>10^5$	212	71 (29.2)

YMDD, tyrosinemethionine-aspartic acid-aspartic acid; HBV, hepatitis B virus; DNA, deoxyribonucleic acid; $\chi^2 = 20.130$, $p < 0.001$.

Table 4 - Multivariate analysis of factors associated with YMDD mutation

Factors	Category	Odds ratio	95% CI	p-value
HBV DNA levels (copies/mL)	1: $\leq 10^3$	1		
	2: $> 10^3 \sim \leq 10^5$	2.563	0.146-1.386	0.048
	3: $> 10^5$	4.376	0.375-3.287	0.025
HBeAg status	1: negative	1		
	2: positive	0.763	0.076-1.438	0.043

CI, confidence interval; p-value by logistic regression analysis; YMDD, tyrosinemethionine-aspartic acid-aspartic acid; HBV, hepatitis B virus; DNA, deoxyribonucleic acid.

Table 5 - Analysis of the relationship between HBV DNA levels and HBeAg status

HBV DNA levels (copies/mL)	HBeAg negative (n = 683)	HBeAg positive (n = 359)	Odds ratio	95% CI	p-value
$\leq 10^3$	136	3	1		
$> 10^3 \sim \leq 10^5$	349	140	6.387	2.176-23.153	0.012
$> 10^5$	198	216	9.267	3.175-33.176	0.001

CI, confidence interval; p-value by logistic regression analysis; YMDD, tyrosinemethionine-aspartic acid-aspartic acid; HBV, hepatitis B virus; DNA, deoxyribonucleic acid.

YVDD and/or YIDD variants. LIPA assay indicated that wild-type virus was either absent or represented only a minor population in these patients. One of the possibilities for the presence of mutant strains may be the acquisition of samples from lamivudine-resistant subjects. Medical records of patients showing hybridization only with YVDD and/or YIDD variants were carefully re-examined, but no further information related to the source or the duration of infection could be identified. They were known to be the only HBV-infected member of their families.

Kobayashi et al.¹⁹ and Da Silva et al.²⁰ found that anti-HBe was positive in all patients with YMDD mutations, and Ye et al.²¹ noted that anti-HBe was positive in most patients with YMDD mutations and considered that YMDD mutations might occur more easily if mutations take place in the pre-c-zone. In this study, the findings revealed that the incidence of YMDD mutations was 21.2% in patients negative for HBeAg and 27.2% in patients positive for HBeAg showing significant difference. Moreover, multivariate analysis revealed that the HBeAg status ($p = 0.043$) was associated with YMDD mutations. These findings were not in accordance with those of Kobayashi et al.⁵ and Ye et al.²¹

Some researchers^{19,22} reported a positive correlation between high serum HBV DNA level and incidence of YMDD mutations. The present results were consistent with these studies that, in terms of incidence of YMDD mutations, a significant difference was found among patients with different serum HBV DNA levels, suggesting that the HBV DNA level might have a positive correlation with YMDD mutations. Moreover, the serum HBV-DNA level in HBeAg positive patients ($3.47 \pm 0.45 \times 10^7$ copies/mL) was dramatically different from that in HBeAg negative patients ($6.44 \pm 1.25 \times 10^5$ copies/mL). Univariate analysis of the relationship between HBV DNA level and HBeAg status revealed that HBV DNA level was associated with HBeAg status. Thus, it is possible that the difference of YMDD mutations of HBeAg system may be correlated with viral loads of HBV.

The correlation between HBV genotypes and YMDD mutations (pre or post-treatment) remains unclear and conflicting data have been reported.²³⁻²⁶ The present results revealed a difference in the incidence 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 (56.4%) occurred in patients with genotype C or its mixed forms, partially because patients with genotype C account for the majority of CHB patients in China. Although there was no significant difference among these genotypes, the relationship between YMDD mutations and HBV genotypes could not be ruled out, as the sample size was relatively small.

One limitation of the present study was the lack of cloning and sequencing of samples carrying YMDD variants, in order to evaluate the dominance of different quasispecies, and to reconfirm the LIPA findings. Detailed evaluation of quasispecies was not one of the aims of this study. The findings were validated by using a well-evaluated commercial assay and repeatedly testing the samples that had unusual results.^{27,28}

In conclusion, naturally occurring YMDD variants are detectable in the majority of CHB patients without antiviral therapy. Incidence of YMDD mutation may be correlated with HBeAg and HBV DNA level.

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Conflict of interest

All authors declare to have no conflict of interest.

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