

## Frequency of YMDD mutations in patients with chronic hepatitis B untreated with antiviral medicines

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### Abstract

**Background:** Investigators were suspicious of tyrosine-methionine-aspartate-aspartate (YMDD) mutations occurred only in patients who were treated by lamivudine. However, YMDD mutations of hepatitis B virus gene (HBV DNA) in patients with chronic hepatitis B (CHB) untreated with antiviral medicines was reported in some studies. The aim of this study was to evaluate YMDD mutations in Iranian Patients with chronic hepatitis B (CHB) untreated with antiviral medicines.

**Methods:** In a cross sectional study, 151 adult patients with positive Hepatitis B surface antigen (HBsAg) (78 asymptomatic hepatitis B virus carriers, 73 active chronic hepatitis B patients or cirrhosis patients) were evaluated for YMDD mutants. The patients who were treated with interferon and Lamivudine or Adfovier in one year prior to the study were excluded. YMDD mutations of HBV DNA were detected by PCR-RFLP (PCR Restriction Fragment Length Polymorphism) in a single laboratory.

**Results:** The mean ( $\pm$ SD) age of patients was  $37\pm 4$  years. Eighty one (54%) cases were male and 70 (46%) were female. Eight cases (5.3%) out of 151 had YMDD mutations. The type of mutation in all of these patients was YSD. There was no significant relationship between YMDD mutation and viral load and HDV Ab ( $p>0.05$ ).

**Conclusions:** The mutant strains of the YMDD motif of HBV polymerase can be found in some patients without lamivudine treatment. However, in view of rather clinically insignificant YMDD mutation frequency, routine testing for YMDD mutations prior to antiviral therapy is not recommended in these patients.

**Keywords:** Hepatitis B virus, YMDD mutation, Lamivudine.

### Introduction

Hepatitis B virus (HBV) is one of the oldest and the most common infectious diseases in the world. More than 300 million people worldwide are estimated to have chronic HBV infection. The highest population prevalence of chronic HBV infection is found in the oldest area of the world, in

Asia. Ten percent of these patients will die as a direct consequence of persistent viral infection [1]. Nucleoside analogue therapy allows safe, long-term suppression of HBV and is a major milestone in the treatment of chronic hepatitis B. Lamivudine, the first of these agents approved worldwide, effectively suppresses viral replication, reduces disease activity, improves liver histology, and

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delays clinical progression [2-4]. However, the development of lamivudine resistant mutations occurs in 14%-32% of patients after 1 year of therapy [5,6]. The longer the treatment is continued, the more frequently resistance is seen (65% at 5 years) [7]. Lamivudine-resistant HBV is associated with mutations of the YMDD motif in the polymerase gene [8]. The key mutations are the substitutions of methionine at the rtM204 (domain C) to either Isoleucine (rtM204I, YIDD variant) or Valine (rtM204V, YVDD variant) [8,9]. The rtM204V variant is almost always accompanied by an additional rtL180M mutation in the domain B [10,11]. The YSDD variant has also recently been described by Bozdayi et al., 2003, and Niesters et al., 2002 [12,13].

Interestingly, the YMDD mutant HBV strains are also present in untreated HBsAg positive persons [14]. To better clarify the evolution of YMDD mutation in untreated infected patients we evaluated HBV strains in sera of treatment naïve patients.

### Methods

In a cross sectional study we evaluated consecutively 151 adult patients who had hepatitis B surface antigen (HBsAg) referred by a primary consultation center special for patients with viral hepatitis to gastroenterology department of Imam Khomeini hospital in 2008. Informed consent was obtained from all subjects according to the local ethical committee.

### Patients

Studied group were asymptomatic hepatitis B virus carriers, active chronic hepatitis B patients and liver cirrhosis patients. The patients who were treated with  $\alpha$ -interferon in recent one year and history of treatment by Lamivudine or Adfovier prior to the study were excluded. Asymptomatic carriers were identified by normal liver enzymes, normal serum albumin, normal prothrombin time, and normal liver ultrasound in the recent year. Active chronic hepatitis B patients were identified by elevation of liver enzymes; and liver cirrhosis were identified by

presence of portal hypertension, elevated prothrombin time, small coarse liver by ultrasound, or histopathologic documents. The diagnosis of cirrhosis was confirmed histologically and/or clinically by standard stigmata of cirrhosis. Familial aggregation (2 or more close-contacted family members infected with HBV without direct evidence of blood transmission) was asked from patients.

### Methods

Blood samples were collected from the patients and the serum stored at  $-70^{\circ}\text{C}$ . All samples were tested by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for detection of YMDD motif mutants. Serum samples were also tested for hepatitis C antibody (anti-HCV), hepatitis B e antigen (HBeAg), and hepatitis B e antibody (anti-HBe) by ELISA. The commercial ELISA kits used were: anti-HCV, HBsAg (Diasorin, Spain); HBeAg and anti-HBe (Radim, Barcelona, Spain). The anti-HDV enzyme-immunoassay was performed as issued in the methodology supplied by the manufacturer (Radim, Italy). The viral load of the patients was measured with Cobas Amplicor, Roche, New Jersey [15]. All assay protocols, cut-offs, and result interpretations were carried out according to the manufacturers' instructions.

### PCR-RFLP analysis

HBV variants at M552 (nucleotides 741 or 743) were detected by PCR-RFLP. Four separate PCR assays were used to amplify four fragments around position 552 of the polymerase gene. A 274-bp fragment was amplified using the primer pair P3 (5'-aaaccttcggacggaaactgc-3') and P5 (5'-ctg gat ccagggtttaaagtata ccc-3'), and the fragment was digested with FokI restriction enzyme, as described previously. A 181-bp fragment was amplified using the primer pair P6 (5'-tgg aattcaaatgtattccatcc cat-3') and P7 (5'-cag act tgg ccc ccaatacca cat cgtgca-3'), and the fragment was digested with Alw441 restriction enzyme. A 138-bp fragment was amplified using the primer pair P5 (5'-ctg gat ccagggtttaaagtata ccc-3') and

Table 1. Demographic specifications and lab findings of patients with YMDD mutations.

Patient	Gender	Age	Familial aggregation	<i>HBeAg</i>	<i>HBeAb</i>	Viral load (copy/mL)	<i>HCV Ab</i>	<i>HDV Ab</i>	Status of liver disease
1	Male	19	-	-	+	3200	-	-	Chronic hepatitis
2	Male	21	+	-	+	N/A	-	-	Inactive carrier
3	Female	23	-	-	+	7400	-	-	Inactive carrier
4	Female	25	-	-	+	5100	-	-	Inactive carrier
5	Male	32	+	-	+	437	-	-	Inactive carrier
6	Female	43	+	-	+	1200	-	-	Inactive carrier
7	Male	69	+	-	+	1000	-	-	Inactive carrier
8	Male	71	-	-	+	10500	-	-	Cirrhosis

Table 2. Primers used in the study.

Primers	Nucleotide Sequence	Direction
P3	5'-aaacctcggacggaaactgc-3'	Sence
P4	5'-ctg gat ccagggttaaatgtata ccc-3'	Sence
P5	5'-ctg gat ccagggttaaatgtata ccc-3	Antisence
P6	5'-tgg aattcaaatgtattccatcc cat-3'	sence
P7	5'-cag act tgg ccc ccaatacca cat cgtgca-3'	Antisence
P8	5'-cactgtytgctagc tat-3'	Sence
P9	5'-gtt yaa atg tat acc caa ag 3'	Antisence

P4 (5'-ttt ccc ccaactgtttgcttt cag taa tat-3'), and the fragment was digested with *SspI* restriction enzyme. A 120-bp fragment was amplified using the primer pair P8 (5'-cactgtytgctagctat-3') and P9 (5'-gttyaaatgtatacccaaag-3') and the fragment was digested with *SfcI* restriction enzyme, as described previously [12] (Table 2). Restriction enzyme *SspI*, *Alw441* and *SfcI* were introduced into primers P4, P7 and P9, respectively [12,16]. The results of PCR-RFLP were analyzed by 8.4% polypropylene acidemide gel electrophoresis. The detection limit of the assay was 200 copies per ml.

For DNA extraction, 100 µl of each patient's serum was mixed with 150 µl of TES buffer containing 10% sodium dodecyl sulfate and proteinase K, and then incubated at 60.8°C for 1 hour. Extracted DNA was precipitated with absolute ethanol, then washed with 70% ethanol and dissolved in 30 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM

EDTA). The DNA was used for four amplification reactions of PCR. Final concentrations in all four PCR reactions were as follows: 20 mmol Tris (Ph 8.3), 0.2 mmol KCl, 1.5 mmol MgCl<sub>2</sub>, 18 mmol NaCl, 0.2 mmol dNTPS, 0.5 µmol each primer, and 2.5 U Taq polymerase per 50 µl of reaction mixture. The amplification conditions in the four PCR reactions all included initial denaturation at 94.8°C for 3 minutes, 35 cycles of amplification with denaturation at 94.8°C for 35 seconds, primer annealing at 56.8°C for 50 seconds, extension of primer at 72.8°C for 50 seconds, followed by a final extension at 72.8°C for 5 minutes.

After amplification, aliquots of the product DNA of the four PCR reactions were digested with four enzymes, respectively. The digestion reactions included 15 µl amplified HBV DNA, 10 U enzyme, 10× restriction enzyme digestion buffer 2 µl, and 3 µl sterile ddH<sub>2</sub>O in a sterile Eppendorf tube. The tube

Table 3. Overview of the studies related to detection of YMDD variants in lamivudine-untreated HBV infected populations.

Author	No of patients	Detection method	Percentage of variants
Kobayashi et al. <sup>14</sup>	18	PCR-ELMA, sequencing	27.7%
Kirishima et al. <sup>27</sup>	36	PCR-PNA with RFLP, conventional RFLP	11.1%
Matsuda et al. <sup>31</sup>	40	PCR-ELMA, PCR-RFLP	0%
Matsuda et al. <sup>32</sup>	71	PCR-ELMA, PCR-RFLP, PCR-PNA with RFLP	2.8%
Heo et al. <sup>33</sup>	40	Oligonucleotide chip assay, RFLP, sequencing	7.5%

PCR-ELMA, SMITEST HBV-YMDD mutation detection kit (Genome Science, Tokyo, Japan); PNA, peptide nucleic acid; RFLP, restriction fragment length polymorphism.

was spun at 500 rpm for 3 seconds. The SspI, Alw44I and SfcI enzyme reaction mixtures were incubated in a 37.8°C water bath for 6 hours. The FokI enzyme reaction mixture was incubated in a 55.8°C water bath for 3 hours.

#### Statistical analysis

The incidence rate of YMDD mutation was compared by Chi-square test between different genotypes. The statistical analysis was processed with SPSS 11.5 statistical software. A p-value of <0.05 was considered significant.

#### Results

Study involved total of 151 patients. Eighty one (54%) cases were male and 70 (46%) were female. Seventy eight (51.6%) were asymptomatic carriers, 59 (39.1%) had active chronic hepatitis B and 14 (9.3%) had liver cirrhosis. The mean ( $\pm$  SD) age of patients was 37 $\pm$ 4 years.

Eight cases (5.3%) out of 151 had YMDD mutations. The type of mutation in all of the patients was YSDD (Fig. 1). The characteristics of these patients are shown in table 1. The mean age of patients with YMDD mutation were 37.8  $\pm$  21.23 years versus 35.4  $\pm$  13.73 years in the other group. The incidence rate of YMDD mutations was 6.17% (5/81) in male, and 4.28% (3/70) in female population. (p= not significant). YMDD mutations were found in 7.7% (6/78) of asymptomatic carriers and 2.7% (2/73) of active chronic hepatitis and liver cirrhosis cases. Seven percent (8/112) of patients with positive HBe Antibody had YMDD mutations

and this ratio in patients with negative HBe Antibody was 0% (0.39) (p=0.74). None of the patients with positive HDV antibody had YMDD mutation, and this ratio in patients with negative HDV antibody was 6.8% (8/119). In YMDD mutation group 4 cases (50.0%) had familial aggregation with no significant difference compared to the group without familial aggregation (p=NS). All cases with YMDD mutation had negative HBe Ag, and showed a statistically significant difference as compared to patients without YMDD mutations. There were 75% carrier status and 25% chronic hepatitis and cirrhosis status among YMDD positive group with statistically significant difference (p<0.05). There was no HCV or HDV co-infection in YMDD mutation group as compared to 4.5% and 7.5% co-infection, respectively, in the group with negative result for YMDD mutation.

Patients were sorted into three groups ac-

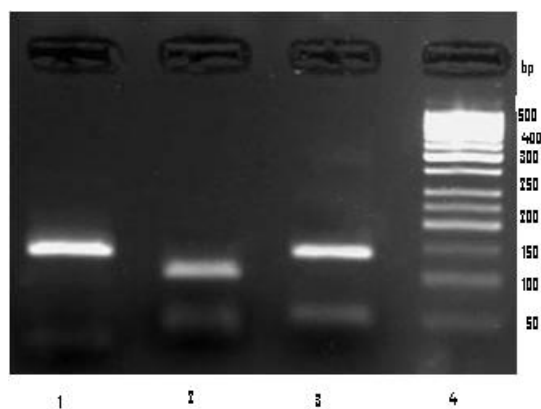


Fig. 1: Lane 1: Undigested PCR product (120 bp). Lane 2: YSDD variant (105 bp after *SfcI* digestion). Lane 3: Wild-type (120 bp after *SfcI* digestion). Lane 4: DNA marker from bottom to up: 50, 100, 150, 200, 250, 300, 400, 500 bp.

ording to their viral loads. HBV viral loads between 104 and 105 log copies/ml in Group 1, between 103 and 104 log copies/ml in Group 2 and either less than 103 log copies/ml or undetectable by the quantitative PCR in Group 3. Eight of 106 patients in Group 2 and 3 showed YMDD mutations while none of the 45 patients in Group 1 showed YMDD mutations ( $p=0.44$ ) (Table 1). There was no significant difference between high and low HBV DNA level groups, suggesting that HBV DNA level might not have a correlation with YMDD mutations. In this study, primary detection of YMDD mutation had clinical implication in 2 of 73 cases (2.7%) with active chronic hepatitis B or liver cirrhosis.

### Discussions

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. This motif localized in the polymerase structural region C area, which is the combining and functioning site of Lamivudine (a nucleoside antiviral medicine). Lamivudine-resistant HBV strains with YMDD mutations are an important factor for the failure of chronic hepatitis B treatment [17]. A great number of studies in recent years showed that long term (longer than 6 mo) lamivudine treatment could contribute to YMDD mutations and disease recurrence [10,18-27]. Some patients with mutation even went worse, and eventually died [19-23]. However, it has been rarely reported whether YMDD mutations have natural existence. Some scholars [14,28] found that YMDD mutational strains were positive in the serum of the cases infected with CHB (Chronic Hepatitis B) who did not receive Lamivudine treatment. Yan et al [29] showed that 19 cases had YMDD mutations out of 110 CHB cases untreated with Lamivudine. Fontaine et al [30] 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 [30] revealed that YMDD mutations also occurred in HBV-infected cases who underwent kidney transplantation and dialysis therapy. In addition, Zhang et al [31] 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 [32] reported that a few CHB cases not treated with Lamivudine had YMDD mutations. In our study, 8 out of 151 CHB cases (5.3%) who had not received lamivudine and any other antiviral treatment had YMDD mutations and these finding shows that naive YMDD mutational strains were existent in HBV DNA.

The reported rate of wild YMDD variants shows a wide range, changing between 0% to 27.7% for asymptomatic carriers and 0% to 26.9% for chronic HBV infected patients, in existence or without the wild-type HBV DNA strains (Table 3). These studies are mostly from Asian countries such as Japan, China and Korea. The most common route of the HBV infection in these areas would be vertical and most of these patients got the infection from childhood. In view of lack of enough literature from Iran, no definite speculation can be made, however, differences could be due to the relatively small number of subjects in some studies, detection methods or the unknown characteristics of study groups such as the duration of the infection, viral load, genotype of the virus and other amino acid variations of the viral genome.

However, the spontaneous YMDD mutations reports showed some controversy correlation with type or stage of disease. Kobayashi et al [14] found that anti-HBe was positive in all patients with YMDD mutations which is in concordance with results of our study. Our results show that the incidence of YMDD mutations was 6.4% in patients with negative HBe Ag as compared to 0% in patients with positive HBeAg, however, the result was not statistically significant. These results do not accord with those of Koba-

yashi et al [14] and Ye et al [35] but accord with the studies of Da Silva et al. [36] and Yan et al [29]. Therefore, YMDD mutations might not have a relationship with pre-c-zone mutations. We found that there were no differences in the incidence of HBV YMDD naive mutational strains between positive and negative HDV Antibody and these findings are in concordance with the results of the previous studies [12, 25]. Some authors [37,38] proposed that a high HBV DNA level of serum had a positive correlation with the incidence of mutations but our study did not support that.

Type of YMDD mutation observed in our study was YSDD. This mutation was for the first time reported by Nister et al in 2002 [12]. In this mutation, serine replaces methionine at codon 204 in C region related to DNA polymerase (rtM204S). In another study, Bozdayı et al showed that a YSDD variant of HBV polymerase, in combination with the rtL180M change conferred lamivudine resistance in vivo and caused biochemical and virological breakthrough, that restored replication activity [13]. To the best of our knowledge, this is the first time when YSDD mutation is reported in treatment naïve patients. The types of YMDD mutation reported in other studies include YIDD and YVDD.

One limitation of our study was the lack of cloning and sequencing of the samples that carried YMDD variants in order to evaluate the dominance of the different quasi-species and to reconfirm the PCR-RFLP findings. Detailed evaluation of the quasispecies was not one of the aims of the study, and the reliability of the findings was ensured by using a well-evaluated commercial assay and determining the repeatability of the results by re-running the assay on samples with unusual band patterns.

We have to know more about the clinical relevance of the naturally existing variants at the YMDD region, especially in specific clinical situations like pre-transplantation status, influence of HBV genotype on YMDD mutations (pre or post-treatment), effect of YMDD mutations on the envelope

proteins.

In conclusion, the mutant strains of the YMDD motif of HBV polymerase can be found in HBsAg positive and treatment naïve patients. However, in view of rather clinically non-significant YMDD mutation frequency, routine testing for YMDD mutations prior to beginning of antiviral medication in these patients is not recommended. Since numbers of cases in our study were comparatively small, we recommend further studies on larger groups that can help in final judgment about best management and appropriate anti-viral therapy in these patients.

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