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Clinical features of chronic hepatitis B patients with YMDD mutation after lamivudine therapy

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Abstract: Objective: To study the clinical features of chronic hepatitis B (CHB) patients with tyrosine-methionine-aspartate-aspartate (YMDD) mutation after lamivudine therapy. Methods: This investigation was a retrospective study of 63 CHB patients with YMDD mutation during lamivudine therapy. Clinical data, including period and types of YMDD mutation; hepatitis B virus (HBV) DNA levels and alanine aminotransferase (ALT) levels before and after YMDD mutation were measured. YMDD mutation in the HBV DNA polymerase gene was determined using polymerase chain reaction (PCR) and direct sequencing. HBV DNA quantification was determined using real-time PCR. Relevant serum markers of HBV were measured. The follow-up period was 12 months after YMDD mutation. Results: YMDD mutation occurred 7~44 months (median, 21.5 months) after the start of lamivudine therapy. The majority of the cases (42/63, 66.6%) had YMDD mutants detected between 12 and 24 months. Four types of YMDD mutation were observed in this study, rtL180M/M204V mutation was the predominant type (26/63, 41.3%). A proportion of patients (16/63, 25.4%; 12/63, 19.1%) had higher HBV DNA levels and ALT levels (after mutation vs before mutation), respectively. Conclusion: The majority of patients with YMDD mutants had similar or lower HBV DNA levels and ALT levels compared with baseline values. This subset of patients might have benefited from the continued lamivudine therapy. The patients with increased ALT and HBV DNA levels (breakthrough hepatitis) should benefit from the addition of a newer nucleotide analogue (e.g. adefovir).

Key words: Chronic hepatitis B (CHB), Tyrosine-methionine-aspartate-aspartate (YMDD) mutation, Lamivudine
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INTRODUCTION

Chronic hepatitis B (CHB) remains a major public health problem, affecting more than 350 million people worldwide. Cirrhosis, liver failure, or hepatocellular carcinoma will develop in approximately 15 to 40 percent of infected patients (Ganem and Prince, 2004). Lamivudine, an oral nucleoside analogue, inhibits HBV replication (de Clercq, 2001; 2004; Lai *et al*., 1997; 1998; Marcellin *et al*., 2004). It can markedly reduce serum HBV DNA levels and normalize alanine aminotransferase (ALT) levels in association with decreased liver necroinflammatory activity in a majority of patients (Lai *et al*., 1998). Continuous treatment with lamivudine may delay clinical progression in patients with CHB and advanced fibrosis or cirrhosis by significantly reducing the incidence of hepatic decompensation and the risk of hepatocellular carcinoma (Liaw *et al*., 2004; Wands, 2004). However, the greatest drawback with lamivudine treatment is the emergence of drug-resistant HBV mutants with concomitant rise in ALT, DNA and worsening histology in some patients. This mutation of the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the C domain of the HBV DNA polymerase gene has also been associated with flares of liver disease (Dienstag *et al*., 1999). The aim of this study was to report the clinical features of CHB patients with YMDD mutation after lamivudine therapy.

MATERIALS AND METHODS

Patients

This investigation was a retrospective study of 63 patients with CHB. These patients were assessed in an inpatient and outpatient setting for the presence of the YMDD mutation during lamivudine therapy in our hospital from April 1999 to May 2005. They were 49 men and 14 women, aged from 7~68 years. Among these patients, 27, 21 and 15 patients, respectively, had CHB history for less than 5 years, 6~10 years and more than 11 years. In addition, 49 cases were HBeAg positive and 14 were HBeAg negative, HBV DNA levels varied from 10^5~10^8. Each patient was treated with a single oral dose of 100 mg of lamivudine every day. After YMDD mutation, all patients were followed up monthly; the follow-up period was 12 months. Among these patients, 6 patients discontinued lamivudine therapy; instead, they were treated with Chinese herbs at their own request, 57 patients continued lamivudine therapy. After the emergence of breakthrough hepatitis (ALT becoming abnormal after a period of ALT normalization and re-elevation of HBV-DNA levels after YMDD mutation), 27 patients received lamivudine (100 mg/d) and adefovir dipivoxil (10 mg/d) in combination for 12 months and 6 patients received lamivudine (100 mg/d) and Chinese herbs in combination. Another 24 patients without breakthrough hepatitis continued lamivudine monotherapy (Fig.1).

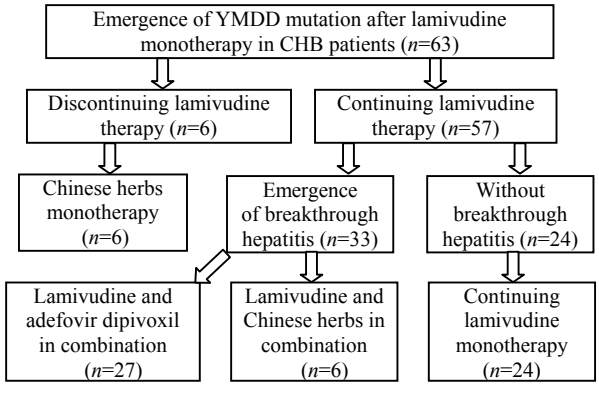

Fig.1 Clinical course of 63 CHB patients with YMDD mutation after lamivudine monotherapy

Polymerase chain reactions and sequencing

Mutation in the HBV DNA polymerase gene was determined using polymerase chain reaction (PCR) and direct sequencing. Briefly, DNA was extracted from sera using a QIA Amp DNA blood kit (Qiagene, Inc. Hilden, Germany) and then amplified using PCR. The amplification reactions were performed as follows: the amplification reaction contained 1 μl each of 25 μmol/L specific primers, 1 μl 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 4 μl 25 mmol $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega, USA) and 5 μl 10×PCR buffer solution. The total volume was brought to 50 μl using ddH_2O. The PCR amplifications were performed in a PTC-200 peltier thermal cycler (MJ Research, USA) under the following conditions: after an initial denaturation for 5 min at 94 °C, samples were subjected to 35 cycles of amplification (94 °C 45 s, 55 °C 45 s, 72 °C 1 min), followed by a final extension of 5 min at 72 °C. The forward primer was (5′ CTCCAATCACTCACCAAC 3′) and the reverse primer was (5′ GGGTTTAAATGTATACCCA 3′). The purification of PCR products was performed by QIAquick PCR purification kit according to manufacturer's instructions (QIAGEN, USA). Sequence analysis of the PCR products was performed by DYEnamic^{TM} ET dye terminator cycle sequencing Kit (AmershamBioscience) in a MegaBACE^{TM} 500 according to manufacturer's instructions. The three primers for sequencing were the forward primer and reverse primer as mentioned above, and the sequencing primer (5′ GTAATTCCCATCCC 3′). Sequence analysis software was used to analyze the results.

Real-time fluorescence PCR

HBV DNA quantification was determined using real-time PCR. Briefly, HBV DNA was extracted from sera using a QIA Amp DNA blood kit (Qiagene, Inc. Hilden, Germany) and then quantified using real-time PCR. Sense primer was (5′ ATCCTGCTGCTATGCCTCATCTT 3′) and reverse primer was (5′ ACAGTGGGGGAAAGCCCTACGAA 3′). A fluorogenic probe (5′ R-TGGCTAGTTTACAGTGCCATTTG-Q 3′) located between the primers was synthesized by Biosearch Inc. Amplification and detection were done with FTC-2000 detection system (Funglyn Biotech). The 50 ml reaction mixture contained 5 ml of 10×Taqman buffer, 200 nmol/L each dATP, dTTP, dGTP dCTP, 0.5 μmol/L each primer, 0.25 μmol/L of Taqman probe, 0.5 U uracil

N-glycosylase (UNG), 1.5 U hotstar *Taq* DNA polymerase (QIAGEN, USA). Following activation of UNG (2 min at 37 °C) and hotstar *Taq* DNA polymerase (15 min at 95 °C), the 35 cycles amplification procedure consisted of heating at 93 °C for 10 s and 62 °C for 35 s. Fluorescence values of each tube were measured at the end of 62 °C step.

Relevant serum markers of HBV and biochemical detection

HBeAg was determined by radioimmunoassay (RIA) diagnostic kits (Wei Fang 3V Biotechnology, Wei Fang, China). ALT measurements were carried at the Centre of Clinical Laboratory, on a computerised automatic multi-analyser Hitachi 7600 (Hitachi Limited, Japan).

RESULTS

Occurrence of YMDD mutation

YMDD mutant occurred 8~44 months (median, 21.5 months) after the commencement of lamivudine therapy. Among them, 8 cases (8/63, 12.7%) had YMDD mutant less than 12 months; 42 cases (42/63, 66.6%) 12~24 months and 13 cases (13/63, 20.7%) more than 24 months.

Types of YMDD mutation

Among the 63 patients, 26 patients (26/63, 41.3%) had rtL180M/M204V mutation, 14/63 (22.2%) had rtL180M/M204I mutation and 18/63 (28.6%) had rtM204I mutation, respectively. There were also 5/63 (7.9%) who had only the rtL180M mutation. However, no YMDD mutants were found in these patients before the start of lamivudine therapy.

HBV DNA levels before lamivudine therapy and after YMDD mutation

Before lamivudine therapy, the HBV DNA levels of the 63 patients were as follows: (10^5, 12 cases, 19.1%); (10^6, 30 cases, 47.6%); (10^7, 15 cases, 23.8%); (10^8, 6 cases, 9.5%); (10^9, 0 case, 0.0%).

After YMDD mutation, the HBV DNA levels of the 63 patients were as follows: (10^5, 26 cases, 41.3%); (10^6, 22 cases, 34.9%); (10^7, 12 cases, 19.0%); (10^8, 2 cases, 3.2%); (10^9, 1 case, 1.6%) (Fig.2).

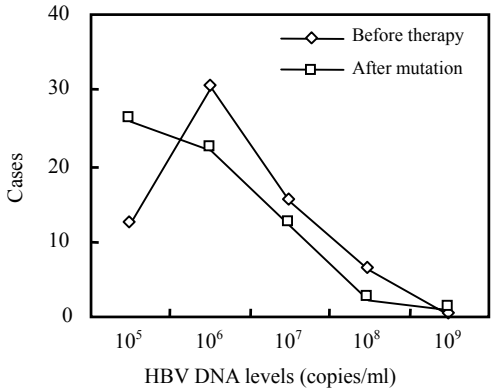

Fig.2 HBV DNA levels before lamivudine therapy and after YMDD mutation

Among the 63 patients, 17 patients (17/63, 27.0%) had similar HBV DNA levels (after mutation vs before therapy); 30 patients (30/63, 47.6%) had lower HBV DNA levels (after mutation vs before therapy) and 16 patients (16/63, 25.4%) had higher HBV DNA levels (after mutation vs before therapy), respectively (Fig.3).

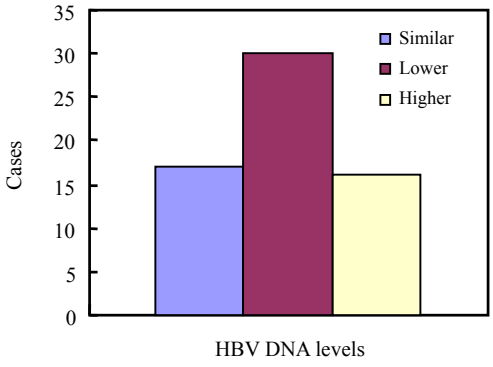

Fig.3 Comparison of DNA levels (after YMDD mutation vs before lamivudine therapy)

ALT levels before lamivudine therapy and after YMDD mutation

Before lamivudine therapy, the ALT levels of the 63 patients were as follows: (<2 upper limit of the normal range (ULN), 17 cases, 27.0%); (2~5 ULN, 41 cases, 65.1%); (6~10 ULN, 5 cases, 7.9%); (>10 ULN, 0 case, 0.0%).

After YMDD mutation, the ALT levels of the 63 patients were as follows: (<2 ULN, 30 cases, 47.6%); (2~5 ULN, 21 cases, 33.4%); (6~10 ULN, 6 cases, 9.5%); (>10 ULN, 6 cases, 9.5%) (Fig.4).

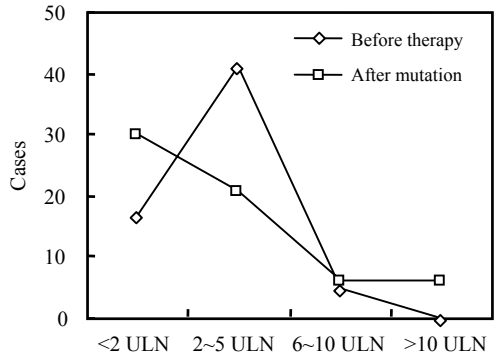

Fig.4 ALT levels before lamivudine therapy and after YMDD mutation

Among the 63 patients, 30 patients (30/63; 47.6%) had similar ALT levels (after mutation vs before therapy); 21 patients (21/63; 33.3%) had lower ALT levels (after mutation vs before therapy) and 12 patients (12/63; 19.1%) had higher ALT levels (after mutation vs before therapy), respectively (Fig.5).

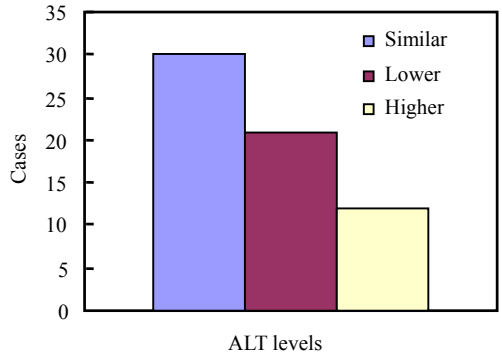

Fig.5 Comparison of ALT levels (after YMDD mutation vs before lamivudine therapy)

Results after retreatment

Twenty-seven patients received lamivudine (100 mg/d) and adefovir dipivoxil (10 mg/d) in combination for 12 months after the emergence of breakthrough hepatitis during continuous lamivudine therapy. The mean viral load had decreased by 2 log at least, ALT levels were all less than 2 ULN. There were no toxic side effects due to adefovir treatment. However, there were no significant changes in the levels of HBV DNA and ALT observed in other 57 patients including 6 patients treated with lamivudine and Chinese herbs in combination, 24 patients continuing lamivudine monotherapy and 6 patients treated with Chinese herbs only.

DISCUSSION

CHB can be treated with a nucleoside analogue, lamivudine (2′3′-dideoxy-3′-thiacytidine). In the short term, substantial inhibition of HBV replication can be achieved. The risk of developing lamivudine resistance increases with the duration of therapy. In a study from Asia, genotypic resistance increased from 14% in year 1 to 38%, 49%, 66%, and 69% after 2, 3, 4, and 5 years, respectively, of treatment (Guan *et al*., 2001). Resistance is associated with mutations in the highly conserved YMDD motif (codons 203~206 of the reverse transcriptase (rt)), which is part of the catalytic site of the HBV polymerase (Allen *et al*., 1998; Chayama *et al*., 1998). These resistant HBV strains revealed isoleucine (I) or valine (V) substitutions instead of methionine (M) in the YMDD motif of the RNA-dependent DNA polymerase. The same changes were also observed in lamivudine-resistant human immunodeficiency virus (HIV). Recently, methionine to serine (M204S) in YMDD motif was reported (Bozdayi *et al*., 2004). These changes are associated frequently with other changes in the amino acid 180 (leucine to methionine) (Melegari *et al*., 1998). The clinical course of hepatitis B in patients with lamivudine-resistant mutants is variable and the long-term outcome remains to be determined. In some patients, emergence of lamivudine-resistant mutants may be accompanied by acute exacerbations of liver disease and rarely hepatic decompensation (Bartholomew *et al*., 1997; Dienstag *et al*., 2003; Liaw *et al*., 1999; Tipples *et al*., 1996).

In this study, our results indicated that YMDD mutants occurred 8~44 months (median, 21.5 months) after the commencement of lamivudine therapy. Most of the cases (42/63, 66.6%) developed YMDD mutants between 12 and 24 months.

Our results also indicated that four types of YMDD mutation occurred in this study, L180M/M204V mutation was the predominant type (26/63, 41.3%). The results were in accordance with other research group's reports (Pillay *et al*., 1998).

HBV DNA and ALT levels determined after YMDD mutations were similar or lower than before these mutations. A minority of patients had higher

HBV DNA levels (16/63, 25.4%) or ALT levels (12/63, 19.1%) after mutation. This result indicated that despite resistance to lamivudine, some patients with YMDD mutants had significantly lower HBV DNA and ALT levels compared with baseline values. This might have resulted from the decreased replication efficiency of the mutants (Liaw *et al*., 2000; Melegari *et al*., 1998; Ono-Nita *et al*., 1999).

For patients with confirmed lamivudine resistance, the options from AASLD Practice Guideline include continuing lamivudine treatment as long as it benefits the patient (based on clinical assessment, ALT, and HBV DNA levels) is maintained; discontinuing treatment and monitoring for hepatitis flares; or switching to other antiviral agents such as adefovir, that are effective in suppressing lamivudine-resistant HBV (Lok and McMahon, 2004). Recently, a study (Chen *et al*., 2004) showed that patients who discontinued lamivudine therapy had an increased frequency of flare-ups and higher ALT peaks than those who continued therapy for 4 months post breakthrough. Therefore, it is recommended that patients should be followed up carefully once YMDD mutants appear, especially for those with hepatic decompensation at the onset of biochemical breakthrough of YMDD mutants. They could benefit from newer nucleotide analogue (e.g. adefovir) to combat HBV mutants. Our follow-up study also demonstrated the role adefovir plays in treating patients with YMDD mutation. More samples are needed for further study.

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