Clinical significance of periodic detection of hepatitis B virus YVDD mutation by ultrasensitive real-time amplification refractory mutation system quantitative PCR during lamivudine treatment in patients with chronic hepatitis B

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Monitoring hepatitis B virus (HBV) mutants periodically during nucleoside analogue treatment is of great clinical significance, particularly in persistently HBV DNA-positive patients. However, few studies have investigated the dynamic changes of HBV YMDD (Tyr–Met–Asp–Asp) and YVDD (Tyr–Val–Asp–Asp) populations in chronic hepatitis B (CHB) patients whilst undergoing lamivudine (LMV) treatment. In this study, we sought to investigate the dynamic changes of HBV YMDD and YVDD variants by ultrasensitive real-time amplification refractory mutation system quantitative PCR (RT-ARMS-qPCR) and evaluate its significance for changes in the treatment of CHB patients. RT-ARMS-qPCR was established and evaluated with standard recombinant plasmids. Fifteen CHB patients receiving LMV (100 mg daily) were consecutively recruited and followed up for 60 weeks. Serum samples were obtained from each patient at baseline and every 12 weeks. The total HBV DNA, HBV YMDD DNA and YVDD DNA levels were measured using RT-ARMS-qPCR at all given time points after treatment. Routine liver biochemistry parameters, including aspartate aminotransferase and alanine aminotransferase, were also measured every 12 weeks. The low detection limit was 1×10^4 copies ml^-1. After 60 weeks of LMV treatment, nine patients experienced virological breakthrough. The YVDD variant could be detected 12–48 weeks before virological breakthrough. The YVDD variant was detected as the predominant population (range 69.4–100 %) in patients by the time virological breakthrough appeared. We concluded that RT-ARMS-qPCR was sensitive for the detection and quantification of low levels of HBV mutation. Periodic detection of HBV YM(V)DD every 12 weeks during LMV treatment is helpful for therapeutic decision making.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem affecting >350 million people worldwide according to the World Health Organization. Chronic HBV infection can lead to chronic hepatitis B (CHB), cirrhosis and even hepatocellular carcinoma (Zoulim & Locarnini, 2009). The overall goal of therapy in patients with CHB is to achieve sustained suppression of HBV replication and remission of liver disease (Lok & McMahon, 2007). However, due to the presence of covalently closed circular
DNA in hepatocytes, full eradication of HBV is rarely achieved. Currently, approved treatments for CHB include nucleoside analogues and IFN-α (Zoulim & Locarnini, 2009). One well-documented nucleoside analogue, lamivudine (LMV), is widely used as a primary therapeutic drug in practice in China due to financial reasons (Liu et al., 2014). Unfortunately, long-term use of LMV is associated with a high drug resistance rate. Recent data indicated the incidence of LMV resistance was >80% after 48 months of treatment (Zoulim & Locarnini, 2009).

HBV drug resistance does not happen overnight. Instead, it is characterized by a dynamic stepwise process. Under the dual pressures of nucleoside analogues and host immunity, in some patients the HBV population gradually changes from wild to mutant strains and the mutant strains may even fully replace all the wild strains to become the predominant population (Zeng et al., 2014). As a consequence, the switch reduces the sensitivity of HBV to nucleoside analogues and finally confers clinical resistance.

Although certain features of the development of antiviral resistance during LMV therapy have been studied (Pallier et al., 2006; Feng et al., 2008), many important questions regarding the dynamic development of the mutant population are still unanswered. For example, when will the resistance mutants appear? How quickly will the minor variants become dominant? What mutant/WT ratio may cause treatment failure? How can we seize the opportunity to change medication according to the changing mutant strains? What is their clinical significance?

To address these issues, it is necessary to explore the dynamic regularity of the genotypic resistance of HBV based on its quasi-species features and describe the changing characteristics of HBV during nucleoside analogue antiviral therapy. In the present study, we observed the dynamic changes in the HBV YMDD (Tyr–Met–Asp–Asp) and YVDD (Tyr–Val–Asp–Asp) populations every 12 weeks by real-time amplification refractory mutation system quantitative PCR (RT-ARMS-qPCR), an ultrasensitive technique allowing us to quantify absolutely both variant and WT HBV DNA in CHB patients during LMV treatment. We also analysed the evolution of HBV YVDD resistance mutations and evaluated the potential value of periodic monitoring of HBV mutants.

**METHODS**

**Patients.** Fifteen CHB patients receiving LMV (100 mg daily) monotherapy were consecutively recruited and followed up for 60 weeks from the Center of Liver Diseases of The First Affiliated Hospital of Fujian Medical University, between January 2011 and July 2013. Sera were collected at baseline and every 12 weeks (weeks 12, 24, 36, 48 and 60).

Further inclusion criteria were as follows. (1) All patients conformed to the diagnostic standard of CHB in Guideline of Prevention and Treatment for Chronic Hepatitis B (2010 Version), enacted by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases, Chinese Medical Association. (2) Age >16 years. (3) HBsAg-positive for at least 6 months before enrolment. (4) HBV DNA ≥2 × 10⁴ IU ml⁻¹ (≥1 × 10⁵ copies ml⁻¹) (measured by a Sansure Diagnostics kit with a linear range from 5 × 10² to 5 × 10⁶ IU ml⁻¹ before enrolment). (5) Alanine aminotransferase (ALT) level greater than or equal to two times the upper limit of normal.

Exclusion criteria were as follows. (1) Previous use of antiviral drugs such as IFN, entecavir, telbivudine and adefovir dipivoxil. (2) Evidence of autoimmune hepatitis or markers of hepatitis C virus (HCV), hepatitis D virus (HDV) or co-infection with human immunodeficiency virus. (3) Evidence of liver decompensation such as drug-induced liver disease or alcoholic hepatitis. (4) Current use of immune-modulating, systemic corticosteroids or chemotherapeutic agents. (5) Pregnant and nursing women. (6) Patients with poor compliance.

The study was performed in accordance with the approval of the ethics committee of The First Affiliated Hospital of Fujian Medical University and the ethical principles of the 1975 Declaration of Helsinki. Written informed consent was also obtained from each patient.

**HBV DNA extraction.** HBV DNA was extracted from serum samples using a commercially available virus DNA extraction kit (Tiangen Biotech) according to the manufacturer’s instructions and stored at −20 °C until assayed.

**Quantitative analysis of WT and mutant DNA by RT-ARMS-qPCR.**

Primers for RT-ARMS-qPCR. RT-ARMS-qPCR, an in-house real-time PCR assay for HBV YMDD/YVDD DNA quantitative detection, is a technique patented by our laboratory. Specifically, the forward primer, KF, was at nt 396–416 for the amplification of both YMDD and YVDD. The reverse primers, MR and VR, were designed to detect YMDD and YVDD, respectively. The introduction of an additional mismatched nucleotide immediately 3′-end terminal to the mutation site greatly increased the binding specificity of the allele-specific modified primers towards either the WT or the mutant templates (Table 1). The primers were synthesized by Sangon Biotech.

**Preparation of YMDD and YVDD plasmids for calibration curves.** YMDD and YVDD plasmids were constructed using the pMD18-T vector (TaKaRa), and PCR products amplified from clinical templates using primer pairs KF/BR and KF/VR, respectively. Plasmids were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The corresponding copy numbers were calculated based on the size and molecular mass of the plasmid DNA, and then 10-fold serially diluted from 1 × 10⁹ to 1 × 10⁴ copies ml⁻¹ using Easy Dilution Buffer (TaKaRa) to generate standard concentrations for calibration curves.

**Absolute quantification of HBV YMDD and YVDD DNA by RT-ARMS-qPCR.** The RT-ARMS-qPCR assay was performed on a StepOne Plus Real-Time PCR system (Life Technologies). The final volume of 25 μl PCR amplification reaction mixture contained 12.5 μl 2 × SYBR Premix Ex Taq (TaKaRa), 0.4 μl of each primer (20 μM), 9.7 μl double-distilled H₂O and 2.0 μl DNA template. The thermal cycling conditions were: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 61 °C for 30 s. Negative controls were included in each assay. Post-amplification melting curve analysis was performed to identify non-specific products, such as primer-dimers, that had Tₘ,s distinct from the specific product of interest.

**Proportions of HBV YMDD and YVDD DNA analysed by RT-ARMS-qPCR.** Two separate PCR systems including amplification plots and standard curves of RT-ARMS-qPCR for pMD-18-YMDD (Fig. S1, available in the online Supplementary Material) and pMD-18-YVDD (Fig. S2) were included in each run. There was an excellent linear correlation between cycle number and HBV DNA copy number from
Periodic detection of HBV YMDD

Table 1. Primers for RT-ARMS-qPCR

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence (5’→3’)†</th>
<th>Position (nt)</th>
<th>Length (bp)</th>
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<tr>
<td>KF</td>
<td>TCATMTTCCCTKCATCCTGC</td>
<td>396–416</td>
<td>21</td>
</tr>
<tr>
<td>MR</td>
<td>CCCAAWACACATCATCCCAT</td>
<td>739–758</td>
<td>20</td>
</tr>
<tr>
<td>VR</td>
<td>CCCAAWACACATCATCGCG</td>
<td>739–758</td>
<td>20</td>
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*KF, common forward primer for all reactions; MR/VR, specific RT-ARMS-qPCR reverse primers.
†M, A/C; K, G/T; W, A/T. The underlined base indicates the additional mismatch. Bases in bold indicate allele-specific nucleotides.

a concentration of $1 \times 10^{12}$ to $1 \times 10^5$ copies ml$^{-1}$ with correlation coefficients of 0.996 and 0.993 for the WT and mutant target sequences, respectively. The low detection limit of the assay was $1 \times 10^3$ copies ml$^{-1}$. The mean intra- and inter-run coefficient of variation for a concentration of $1 \times 10^6$ copies ml$^{-1}$ was 1.71 and 2.29 %, respectively. For a concentration of $1 \times 10^4$ copies ml$^{-1}$, the mean intra- and inter-run coefficient of variation was 15.5 and 17.3 %, respectively. It should be noted that only concentrations between $1 \times 10^2$ and $1 \times 10^5$ copies ml$^{-1}$ could be accurately and quantitatively measured, whilst $1 \times 10^4$ copies ml$^{-1}$ could not be accurately and quantitatively measured. The sensitivity of the assay for the detection of mutant DNA was 1 % (data not shown).

The proportion of HBV YMDD and YVDD DNA was calculated from the absolute quantification of WT and mutant sequences.

Serological measurements and direct sequencing. Serological levels, including HBsAg, HBeAg, anti-HBe, anti-HBc, anti-HDV antibodies and anti-HCV antibodies, were measured via an Architect-i2000 system with its commercial reagents (Abbott Laboratories). Routine liver biochemistry parameters, including AST (aspartate aminotransferase) and ALT levels, were determined using an automated analyser (Olympus AU2700).

HBV DNA fragments containing the polymerase reverse transcriptase domain were amplified using a HBV sequencing kit (Shenyou). Purified positive PCR products were directly sequenced with an ABI 3130 Genetic Analyzer (Life Technologies). HBV genotypes and sequences were then analysed based on the sequencing results.

Statistical analysis. HBV DNA concentration was log$_{10}$ transformed for analysis. The statistical analysis and graphing were performed using SPSS 16.0 (SPSS) and Prism 5.0 (GraphPad). One-sample Kolmogorov–Smirnov tests were used to determine the distribution of each group of data. Data following a normal distribution are expressed as mean ± SD. Data following a skewed distribution are presented as medians (quartile intervals).

RESULTS

Baseline characteristics of patients enrolled in this study

Among the 15 enrolled patients, nine experienced virological breakthrough, defined as $>1$ log serum HBV DNA increase from the nadir (lowest value) on therapy (Chang et al., 2010; European Association for the Study of the Liver, 2012). Six patients did not experience virological breakthrough. The baseline characteristics of the patients are shown in Table 2.

There were no significant differences in age, gender, genotype, ALT level or HBV DNA level between the virological breakthrough and non-virological breakthrough groups at baseline (Table 2).

Total HBV DNA, ALT levels and the proportions of HBV YMDD or YVDD DNA in CHB patients without virological breakthrough

As shown in Fig. S3, HBV DNA and ALT levels declined rapidly in six patients (A–F) during the first 12 weeks of LMV therapy. No virological or biochemical breakthrough (elevation in serum ALT level whilst on treatment after achieving normalization) was observed in any of the patients following continued LMV therapy. HBV YVDD variants were not detected in patients A–E at any time point. However, a minority of HBV YVDD variants that comprised 5.5 % of the total population were already present in patient F and this proportion progressively increased to 31 % after 24 weeks of therapy. Interestingly, this patient did not experience breakthrough at the end of follow-up.

Total HBV DNA, ALT levels and the proportions of HBV YMDD or YVDD DNA in CHB patients with virological breakthrough

Virological breakthrough occurred in patients G–O. Similar to the non-virological breakthrough group, the early period of LMV administration induced a rapid decline in HBV DNA and ALT levels. However, virological breakthrough was observed after several months of sustained response to LMV. Biochemical breakthrough soon followed, except for patient M.

As shown in Fig. S4, the proportions of HBV YVDD variants increased gradually and finally became predominant, representing ~100 % of the viral population by the time breakthrough occurred. YVDD-resistant variants could be detected at baseline (patient K) or at 12–48 weeks after medication (patients G–J and L–O). The time from when YVDD was first detectable to virological breakthrough could be considerable (12–48 weeks) (Table 3).

DISCUSSION

Drug resistance poses a growing problem in the treatment of CHB in regard to its association with virological or biochemical breakthrough. Undoubtedly, preventing HBV
antiviral drug resistance to nucleoside analogues and taking appropriate action when or before resistance occurs has become a major focus in the management of CHB (Keeffe et al., 2008). It is necessary to understand the evolution of HBV drug-resistant mutation by dynamic observations of the clinical changes in HBV variants, because of the dynamic stepwise process of HBV change. Previous research documented that minor pre-existing mutants could be gradually selected to become the dominant species and finally precede the occurrence of virological or biochemical breakthrough (Pallier et al., 2006). However, further studies, especially on the clinical significance of dynamic changes in HBV YVDD during LMV treatment in patients with CHB, are still needed.

In the present study, we analysed the changing characteristics of HBV YMDD and YVDD DNA by RT-ARMS-qPCR in 15 CHB patients receiving standardized LMV treatment. Consistent with other observations, the results presented here revealed that the early administration of LMV induced a considerable decline in HBV DNA and ALT levels in all patients who exhibited a high level of HBV DNA prior to antiviral therapy (Chen et al., 2009). During the ongoing therapy, the patients’ HBV DNA remained at a low level for some time. However, nine patients (G–O) developed virological and biochemical rebound after 12–48 weeks of treatment with LMV. In this group, the proportion of YVDD variants was below the limit of identification at baseline; however, over the duration of therapy, the proportion of HBV YVDD increased gradually and finally overtook the YMDD population, ranging from 69.4 to 100 % at the time virological breakthrough occurred. Our findings suggest that HBV YMDD mutation could be detected 12–48 weeks before virological rebound. However, presumably due to the different detection methods used, a slight difference was found from a previous study documenting that virological breakthrough was preceded by 2–4 months (i.e. 8–16 weeks) by the detection of quasi-species variants bearing amino acid substitutions within the YMDD motif and that were known to confer LMV resistance (Pallier et al., 2006). This was, no doubt, a critical period for diagnosing HBV resistance, because most of the quasi-species variants were already both resistant and highly fit (Pallier et al., 2006). As variants could be detectable 12–48 weeks before virological breakthrough, it may be possible to lower the risk of treatment failure if we can change the antiretroviral regimen according to mutation detection before breakthrough.

The current preliminary study indicated the clinical significance of periodic (12-weekly) detection of HBV YVDD during LMV treatment in patients with CHB. However, some limitations of this study are worth noting. (1) The number of patients enrolled was relatively small, but the preliminary dynamic analysis provided a general view of mutant evolution. (2) Only one mutation site (rtM204V) was included. Despite these limitations, there

<table>
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<th>Table 2. Baseline characteristics of patients</th>
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<tr>
<td>Variable</td>
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<td>---------------------------------------------</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Gender (male/female)</td>
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<tr>
<td>Genotype (B/C)</td>
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<tr>
<td>ALT (U ml⁻¹)</td>
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<tr>
<td>HBV DNA [log₁₀(copies ml⁻¹)]</td>
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YVDD mutation was combined with other mutations. In the non-virological breakthrough group, WT HBV DNA was dominant through LMV therapy when the analysis of HBV YMDD and YVDD was performed. Some research has indicated that 10–30 % of CHB patients may have YMDD mutations naturally (Kobayashi et al., 2001; Akarsu et al., 2006; Zhao et al., 2012). Similarly, we found that minor detectable YMDD mutations were pre-existent in patients F and K before LMV therapy. This finding can be explained by the pre-existing pool of circulating viral quasispecies. However, due to their lower replication ability, the quantity of mutants was much lower than that of the WT.

It has been shown that YMDD mutations are more likely to be infected with genotype C HBV (Hsieh et al., 2009; Wu et al., 2012); however, reports differ (Buti et al., 2002; Akuta et al., 2003) and some even proposed the opposite opinion. In our present study, there seemed to be a tendency for patients with HBV genotype B to have a higher YVDD detection rate than those with genotype C (although the study only included a small number of samples).

Antiviral medication is a long-term process. Previous reports indicated that during the course of LMV therapy, HBV DNA in patient serum often reduces to an undetectable level—a situation which lasted for a long time and then reversed to the pre-treatment level (Li et al., 2005; Pallier et al., 2006). During this process, resistant variants were generated which finally replaced all WT YMDD strains and then the pathological effects appeared. Obviously, virological breakthrough came too late for diagnosis of HBV resistance, because most of the quasi-species variants were already both resistant and highly fit (Pallier et al., 2006). As variants could be detectable 12–48 weeks before virological breakthrough, it may be possible to lower the risk of treatment failure if we can change the antiretroviral regimen according to mutation detection before breakthrough.
are several innovations in the present study that should be noted. (1) An ultrasensitive assay (RT-ARMS-qPCR) was developed for the quantification of HBV YVDD variants. The in-house assay had a much higher analytical sensitivity for detecting minor variants than direct sequencing. This assay was a SYBR Green-based quantitative real-time PCR, which is an inexpensive and easily extended method. Moreover, instead of the ΔΔCt calculation method, the assay used standard curves in each run for absolute quantification of the amounts of WT and mutant DNA; therefore, the accuracy of the assay was increased. For the first time, to our knowledge, not only the assay methodology but also its application were included in our present study. (2) The evolution regularity of HBV YVDD was preliminarily revealed via the dynamic observations of virological and biochemical characteristics of 15 CHB patients receiving LMV treatment. Although validation data were restricted to rtM204V, the present study might provide a valuable reference for the dynamic changes in other single or combined variants in future studies if other suitable RT-ARMS-qPCR techniques are established. Therefore, if we consider the dynamic monitoring of HBV YVDD as an important development, establishing an assay that can detect two or more resistance-associated mutations and analyse the data in larger sample size studies, we can gain a better understanding of the natural history of antiviral resistance during the clinical treatment of CHB. In our future studies, larger numbers of patients and other LMV-resistant mutations, such as rtM204I and rtL180M, will be investigated.

In conclusion, we report a sensitive assay (RT-ARMS-qPCR) that allowed the detection and quantification of low levels of HBV mutation. We also explored the dynamic changes in HBV YVDD variants and their significance for changes in treatment timing. The study may provide useful information for understanding the evolution of HBV YVDD variants, help clinicians to detect drug resistance as early as possible before HBV quasi-species variants become highly fit and contribute to the management of therapy or clinical decision making.

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