Resistant mutations and quasispecies complexity of hepatitis B virus during telbivudine treatment

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Ultra-deep pyrosequencing (UDPS) was used to analyse the dynamics of quasispecies and resistant mutations during telbivudine (LDT) treatment of hepatitis B patients. Twenty-six HBeAg-positive chronic hepatitis B patients were treated with LDT for a period of 104 weeks and were characterized as 16 responders, six partial responders and four viral breakthrough patients based on hepatitis B virus (HBV) DNA levels. The plasma samples were subjected to UDPS of the reverse transcriptase (RT) region of HBV. Mutations rtM204I, rtL80I and rtL80V were detected in at least three of the four viral breakthrough patients, indicating the significant roles of the mutations in resistance to LDT. The degree of complexity of viral quasispecies remained in a steady state in the absence of selection pressure, but increased after the LDT treatment. The complexity in the responder group at week 12 was significantly higher than that in the group comprising partial responders and viral breakthrough patients. In vitro replication efficiency analyses showed that the RT mutations had different impacts on HBV replication, with a tendency of rtM204I > rtL80V > rtL80I. Furthermore, double mutations rtL80I/M204I and rtL80V/M204V had replication efficiency similar to that of rtL80I and rtL80V, respectively. Consistent with previous studies, mutation rtM204I was found to be highly resistant to LDT. However, in contrast with their sensitivity to lamivudine, rtL80I and rtL80V were moderately resistant to LDT. Our results indicated that rtL80I and rtL80V may not only serve as replication complementary mutations to rtM204I, but also directly contribute to the LDT resistance.

INTRODUCTION

Hepatitis B virus (HBV; family Hepadnaviridae) contains circular, partially double-stranded DNA. Despite substantial progress in treatment over the past decade, more than 350 million people are chronically infected with the virus, accounting for one million deaths per year (Lavanchy, 2004). Currently, five nucleoside/nucleotide analogues (NAs) are approved for the treatment of chronic hepatitis B, including lamivudine (LAM), telbivudine (LDT), entecavir, adefovir (ADV) and tenofovir. NAs primarily function by inhibiting the activity of the reverse transcriptase (RT) of HBV polymerase (Kwon & Lok, 2011; Stein & Loomba, 2009). The aim of the treatment is to reduce serum HBV DNA to low or undetectable levels and attempt to alleviate liver cirrhosis, hepatic failure and hepatocellular carcinoma (Dienstag, 2008). NAs are well tolerated and are widely used in practice. A large number of treated patients normally exhibit very low or undetectable serum HBV DNA, normal serum alanine aminotransferase (ALT) level and histological improvement. A major limitation of NA therapy, however, is the development of drug resistance during long-term treatment (Locarnini, 2008).

HBV exists as a genetically diverse pool of closely related variants called quasispecies, and the role of quasispecies in the development of drug resistance has been revealed (Ngui & Teo, 1997). The high genetic heterogeneity of HBV is mainly caused by error-prone RT, high virion production rate and short generation time. Quasispecies are commonly present in RNA viruses and the biological implications of quasispecies have been demonstrated in hepatitis C virus (Forns et al., 1999), human immunodeficiency virus-1 (Wain-Hobson, 1992), vesicular stomatitis virus (Steinhauer et al., 1989), foot-and-mouth disease virus (Domingo et al., 1992) and poliovirus (Vignuzzi

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Four supplementary tables are available with the online Supplementary Material.
et al., 2006). Various studies have concluded that a viral quasispecies is a group of variants with different fitness levels (Domingo & Gomez, 2007; de la Torre & Holland, 1990; Vignuzzi et al., 2006). Variations among components of quasispecies contain drug-resistant mutations, which gradually accumulate during their evolution and contribute to viral adaptability, persistence, drug resistance and progression of disease (Clavel & Hance, 2004; Domingo & Gomez, 2007; Fishman & Branch, 2009).

Attempting to determine the complexity of viral quasispecies has been challenging when using traditional genetic testing methods such as direct PCR sequencing and clonal sequencing (Fishman & Branch, 2009). However, the recent development of ultra-deep pyrosequencing (UDPS) has provided a technology powerful enough to investigate this question (Hedskog et al., 2010; Margeridon-Thermet et al., 2009). The advantages of UDPS in characterizing HBV quasispecies in the RT and the overlapping hepatitis B surface protein (S) regions have been well demonstrated (Ciftci et al., 2014; Margeridon-Thermet et al., 2009; Ramirez et al., 2013; Rodriguez et al., 2013; Solmone et al., 2009). However, the significance of HBV quasispecies in treatment response and drug resistance during prolonged therapy has yet to be considered. In this study, we used UDPS to analyse the quasispecies of HBV RT in HBeAg-positive chronic hepatitis patients undergoing LDT therapy for 104 weeks. The results demonstrated that quasispecies complexity increased after LDT treatment, while the viral quasispecies remained in a steady state before drug administration. As mutations rtM204I, rtL80I and rtL80V were detected in at least three out of the four viral breakthrough patients, in vitro replication efficiency and LDT sensitivity assays were conducted on the RT mutations. Our results indicated that rtL80I and rtL80V may not only serve as replication complementary mutations to rtM204I, but also directly contribute to the LDT resistance.

RESULTS

Patients and responses

Twenty-six HBeAg-positive chronic hepatitis patients (A–Z) receiving LDT therapy for a period of 104 weeks were the subjects in this study. The serum HBV DNA levels were checked at 0, 12, 24, 36, 52, 64, 76, 88 and 104 weeks. The viral loads and other information concerning the patients are shown in Table 1. By week 104, according to the definition of responses, the 26 patients were classified as 16 responders (11 genotype B and four genotype C), six partial responders (all genotype B) and four viral breakthrough patients (three genotype B and one genotype C).

UDPS samples and data

UDPS was performed on a total of 76 plasma samples from 26 patients. The samples included plasma from all 26 patients at baseline, from 20 patients at week 12 (six samples were excluded because of low levels of HBV DNA), and 23 samples from the four viral breakthrough patients at weeks 24, 36, 52, 64, 76, 88 and 104. Seven plasma samples collected 4 weeks before baseline (week −4) were also included to illustrate quasispecies dynamics in the absence of antiviral selection pressures. Details of the samples are provided in Table 1.

After data cleaning, a total of 2,917,032 sequence reads were collected. A mean of 9,231 reads were obtained per amplicon, ranging from 1,669 to 18,451. The mean read length was 378 bp. Based on statistical analyses of the mismatch error rate of the plasmid controls, the exclusionary cut-off of mutation rate was set as <1.0 %.

Dynamics of NA-resistant mutations during treatment

No NA resistance mutations were found in the baseline samples. Among all the reported mutations that induce resistance to NAs (Lok et al., 2007; Sheldon & Soriano, 2008; Sloan et al., 2008), only rtL80I, rtL80V, rtA181T and rtM204I were detected. Mutations rtL80I, rtL80V and rtM204I were present in all of the three genotype B viral breakthrough patients (patients J, O and Y), and only rtM204I was observed in patient C, who was the only viral breakthrough patient infected with genotype C HBV. Mutation rtA181T was detected in patients with different treatment responses.

Mutation rtM204I is the primary cause of resistance to LDT (Nash, 2009), and patients J and O exhibited similar profiles related to this mutation (Fig. 1a, b). The mutation was initially detected at low prevalence at week 52 (20 % for patient J and 2.2 % for patient O) and increased steadily until it became dominant at week 76 (99 % for both patients). Viral breakthrough was detected in both patients at week 64, while the prevalence of rtM204I was 76.1 % in patient J and 41.6 % in patient O. After adding ADV at week 80, the prevalence of rtM204I decreased; for patient J it dropped to 86 % at week 104, while for patient O it dropped to an undetectable level at week 104. Patient Y, who developed viral breakthrough at week 104, showed a different pattern of rtM204I compared with patients J and O (Fig. 1c). Mutation rtM204I progressed rapidly from an undetectable level at week 88 to dominance (99 %) at week 104, leading to viral breakthrough at week 104. Patient C was the only viral breakthrough patient infected with HBV genotype C. Mutation rtM204I was detected with very low abundance (1.1 %) at the same time point as viral breakthrough at week 104 (Fig. 1d).

Mutants rtL80I and rtL80V have been reported in LDT-resistant patients previously (Lai et al., 2007; Matthews, 2007; Standring et al., 2006). These mutants were found in three out of the four viral breakthrough patients (Fig. 1). Two different patterns of rtL80I/V appeared, gradually becoming dominant and fluctuating. The
Table 1. Samples used for UDPS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Group</th>
<th>Treatment response</th>
<th>Samples selected for UDPS analysis (viral load)</th>
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<tr>
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<td></td>
<td>Week 04 Baseline</td>
<td>Week 12 Week 24 Week 36 Week 52 Week 64 Week 76 Week 88 Week 104</td>
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<tr>
<td>A</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>1.14x10^6 1.54x10^6 1.91x10^6 2.25x10^6 1.22x10^6 —† — 1.71x10^7 — —</td>
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<td>B</td>
<td>C</td>
<td>LDT</td>
<td>Responder</td>
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<td>C</td>
<td>C</td>
<td>LDT</td>
<td>Viral breakthrough</td>
<td>1.44x10^6 2.32x10^7 5.79x10^8 7.33x10^9 5.02x10^9 1.08x10^10 — — 2.9x10^11</td>
</tr>
<tr>
<td>D</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>7.97x10^6 6.58x10^7 5.17x10^8 — — — — — —</td>
</tr>
<tr>
<td>E</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>6.23x10^7 1.19x10^8 1.39x10^9 1.96x10^10 1.26x10^11 5.66x10^11 1.87x10^11 3.9x10^11 1.47x10^12 2.65x10^12</td>
</tr>
<tr>
<td>F</td>
<td>C</td>
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<td>Responder</td>
<td>5.36x10^8 5.94x10^9 9.89x10^10 — — — — —</td>
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<td>G</td>
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<td>Responder</td>
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</tr>
<tr>
<td>I</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>6.58x10^8 1.18x10^9 1.61x10^10 1.66x10^10 1.59x10^10 1.25x10^10 1.5x10^10 3.08x10^10 1.8x10^10 2.7x10^10</td>
</tr>
<tr>
<td>J</td>
<td>B</td>
<td>LDT/ADV</td>
<td>Viral breakthrough</td>
<td>5.09x10^7 3.75x10^7 8.96x10^7 3.26x10^8 2.14x10^9 2.57x10^9 2.8x10^9 1.47x10^9 8.15x10^9 4.73x10^9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.09x10^7 3.75x10^7 8.96x10^7 3.26x10^8 2.14x10^9 2.57x10^9 2.8x10^9 1.47x10^9 8.15x10^9 4.73x10^9</td>
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<tr>
<td>K</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
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</tr>
<tr>
<td>L</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
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</tr>
<tr>
<td>M</td>
<td>C</td>
<td>LDT</td>
<td>Responder</td>
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</tr>
<tr>
<td>N</td>
<td>C</td>
<td>LDT</td>
<td>Responder</td>
<td>4.41x10^8 4.69x10^9 1.09x10^10 — — — — — — — —</td>
</tr>
<tr>
<td>O</td>
<td>B</td>
<td>LDT/ADV</td>
<td>Viral breakthrough</td>
<td>1.9x10^8 1.59x10^8 1.64x10^8 1.28x10^9 3.65x10^9 1.32x10^9 6.11x10^9 1.11x10^10 1.9x10^10 —</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9x10^8 1.59x10^8 1.64x10^8 1.28x10^9 3.65x10^9 1.32x10^9 6.11x10^9 1.11x10^10 1.9x10^10 —</td>
</tr>
<tr>
<td>P</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>4.82x10^8 4.4x10^9 3.58x10^9 8.73x10^9 2.9x10^10 2.74x10^10 1.69x10^10 1.4x10^10 1.67x10^10 1.48x10^11</td>
</tr>
<tr>
<td>Q</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>6.23x10^8 2.08x10^9 4.69x10^9 5.49x10^10 1.19x10^11 1.79x10^11 1.32x10^11 1.98x10^11 2.85x10^11 2.9x10^12</td>
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<tr>
<td>R</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>9.66x10^8 9.6x10^8 1.24x10^9 — — — — — — — —</td>
</tr>
<tr>
<td>S</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>6.58x10^8 8.38x10^9 2.32x10^10 1.06x10^11 1.94x10^11 1.59x10^11 2.43x10^11 4.46x10^11 1.38x10^11 2.86x10^11</td>
</tr>
<tr>
<td>T</td>
<td>B</td>
<td>LDT</td>
<td>Partial responder</td>
<td>4.64x10^8 2.77x10^10 2.74x10^10 7.04x10^11 1.24x10^11 1.61x10^11 3.65x10^11 2.75x10^11 2.09x10^11 1.74x10^11</td>
</tr>
<tr>
<td>U</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>3.95x10^8 3.05x10^9 6.81x10^10 1.12x10^11 5.38x10^11 2.74x10^11 1.36x10^11 9.2x10^11 — —</td>
</tr>
<tr>
<td>V</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>2.37x10^8 2.99x10^8 1.64x10^9 — — — — — — — —</td>
</tr>
<tr>
<td>W</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>6.05x10^7 7.04x10^7 2.76x10^8 9.84x10^8 1.14x10^9 8.79x10^9 1.76x10^9 9.25x10^9 — —</td>
</tr>
<tr>
<td>X</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>1.09x10^8 5.3x10^8 4.96x10^9 6.23x10^9 1.35x10^10 — — — — — —</td>
</tr>
<tr>
<td>Y</td>
<td>B</td>
<td>LDT</td>
<td>Viral breakthrough</td>
<td>3.09x10^8 1.1x10^9 8.38x10^9 1.38x10^10 4.24x10^10 4.8x10^10 2.5x10^10 5.13x10^10 8.73x10^10 2.89x10^10</td>
</tr>
<tr>
<td>Z</td>
<td>B</td>
<td>LDT</td>
<td>Responder</td>
<td>3.08x10^8 3.64x10^8 3.32x10^9 2.37x10^9 1.9x10^9 2.69x10^9 — — — —</td>
</tr>
</tbody>
</table>

*Samples were selected for UDPS.
†The level of HBV DNA was undetectable.
prevalence of the rtL80I mutant in patient J increased from undetectable at week 36 to dominant at week 76 (83 %), and remained unchanged afterwards (Fig. 1a). The levels of the rtL80I/V mutant in patients J, O and Y were fluctuating, and this mutant did not become dominant during the treatment (Fig. 1).

Mutation rtA181T was detected at low prevalence, ranging from 1.2 to 5.8 % in 11 plasma samples from 9 patients (Table S1 available in the online Supplementary Material). Among these samples, nine were collected at week 12, comprising 45 % of the samples at the time point (20 samples in total). Interestingly, no rt181T mutation was detected in these patients at baseline. The other two samples were collected from patient Y at weeks 36 and 64.

**Viral quasispecies remained in a steady state in the absence of selection pressure, but complexity increased after the LDT treatment**

To investigate the dynamics of viral quasispecies without NA treatment, representative samples of responders, partial responders and viral breakthrough patients were selected, and their variants’ spectrum and complexity at week −4 and baseline were compared. As shown in Table S2, most of the variants in all of the seven patients at week −4 were consistent with those at baseline. Only a few variants differed between the two time points in the same patient, and the prevalence of these variants was less than 15 %. The complexity of viral quasispecies of the samples was calculated as outlined in Methods and the results are shown in Table S3. No significant difference between week −4 and baseline was observed either at the nt level (P=0.29) or at the aa level (P=0.69, Fig. 2). These results illustrated that the quasispecies of HBV were in a relative steady state without selection pressure.

HBV viral complexity between baseline and week 12 was compared in 20 patients, with 10 responders, six partial responders and four viral breakthrough patients at the nt and aa levels. Samples collected at other time points were not compared because the number of samples was too low for statistical analysis. Except for patient W, who exhibited a minor decrease in quasispecies complexity at the nt level (0.34 at baseline versus 0.32 at week 12), the complexity at week 12 was higher than baseline in all individual patients (Table S3). Statistical analysis also revealed that, for both the responder and the non-responder groups, the complexity at week 12 was significantly higher than at baseline at both nt and aa levels (the four corresponding P-values are all less than 0.05, Fig. 3a, b). These results suggested that HBV viral quasispecies become more

![Fig. 1. Dynamics of drug resistance mutations rtM204I, rtL80I and rtL80V, and HBV DNA levels during 104 weeks of therapy in viral breakthrough patients J (a), O (b), Y (c) and C (d). Patients were treated with LDT during therapy and ADV was added as complementary treatment after viral breakthrough was confirmed. Samples were collected at about 3 month intervals and the exact time points of sampling are shown on the x-axis. Asterisks indicate time of viral breakthrough. Arrows represent time of the addition of ADV.](http://jgv.microbiologyresearch.org)
The complexity of responders as a group was compared with that of non-responders. As shown in Fig. 3(a, b), at baseline, the quasispecies complexity of the responder group was significantly higher than that of the non-responder at the aa level ($P$=0.04), but indistinguishable at the nt level ($P$=0.74). However, at both nt and aa levels, the complexity in the responder group at week 12 was significantly higher than that of the non-responders ($P$=0.02 and $P$=0.04 respectively).

**Dynamics of HBV quasispecies complexity in viral breakthrough patients**

When the dynamics of quasispecies complexity of the viral breakthrough patients were analysed, distinct patterns were revealed (Fig. 4). Patient J and patient O had similar patterns during LDT therapy, where the viral complexity increased significantly during the first 24 weeks and then remained relatively steady, but dropped at week 64, when viral breakthrough was detected. The complexity declined to a low level at week 76, when rtM204I was dominant in both patients. After ADV was administered, the complexity of HBV increased to a profile similar to that observed at the beginning of LDT monotherapy (Fig. 4a, b). In contrast, patient Y showed a multifaceted pattern of complex dynamics. In this patient, the complexity increased during the first 24 weeks, but there was a linear decline at week 36, indicating dominance of certain variants. However, the complexity was restored at week 52 and continued to increase until week 64, indicating that the transient domination at week 36 was not stable under LDT selection pressure. A sharp decline of complexity was observed during weeks 76 and 104, accompanied by a transient increase of rtL80V/I at week 76 and a rapidly achieved dominance of rtM204I between weeks 88 and 104 (Fig. 4c). In patient C, a dramatic increase in complexity was observed between weeks 12 and 24, and the complexity remained at a similar level between weeks 24 and 36. The complexity could not be calculated from weeks 36 to 88 because of low viral load. Viral breakthrough was observed at week 104, and the complexity was consistent with the level observed at week 24 (Fig. 4d).

**Influence of rtL80I, rtL80V and rtM204I mutations on HBV replication and LDT resistance**

*In vitro* assays were conducted to evaluate the effects of the mutations rtL80I, rtL80V and rtM204I on HBV replication competence and susceptibility to LDT. HBV replication-competent plasmids with single mutation rtL80I, rtL80V or M204I, and with double mutations rtL80I/M204I and rtL80V/M204I, were constructed as described in Methods. Huh7 cells were transfected with the plasmids and treated or not with serially diluted LDT. The parental plasmid was used as the wild type (WT) control. The released HBV DNA was measured by quantitative real-time PCR (Q-PCR) analysis.

The replication efficiencies of different mutants without LDT were compared with that of the WT, and the relative replication efficiencies were determined (Table 2). For single-mutation plasmids, rtL80I reduced replication efficiency at a non-significant level ($0.88 \pm 0.08$, $P$=0.0789), rtL80V reduced the replication level to about 42 % of that of the WT ($0.42 \pm 0.09$, $P$=0.0054), and rtM204I significantly affected the replication efficiency ($0.15 \pm 0.01$, $P$=0.0003), a result consistent with previous publications (Ling & Harrison, 1999; Warner et al., 2007). For
double-mutation plasmids, the replication efficiency of rtL80I/M204I (0.89 ± 0.05) was similar to that of rtL80I (P=0.42) and the replication efficiency of rtL80V/M204I (0.38 ± 0.05) was similar to that of rtL80V (P=0.29). These results indicate that (1) the mutations have different impacts on HBV replication, with a tendency of rtM204I > rtL80V > rtL80I, and (2) the double mutations rtL80I/M204I and rtL80V/M204V could partially complement the replication deficiency of rtM204I (Table 2).

The resistances of the WT and mutants to LDT were tested under LDT concentrations of 0.01, 0.1, 1, 10 and 100 μM. The results showed that the WT was sensitive to LDT, with a 50% effective concentration (EC50) of 0.90 ± 2.25 μM (Table 2), consistent with a previous publication (Warner et al., 2007). Mutations rtL80I and rtL80V showed moderate sensitivity to LDT, with EC50 values of 31.93 ± 17.43 and 52.56 ± 21.17 μM, respectively. Mutations rtM204I, rtL80I/M204I and rtL80V/M204I were highly resistant to LDT, as no dose-dependent inhibition of viral replication was observed at LDT concentrations up to 100 μM (Fig. 5).

Fig. 5 shows the relative HBV DNA yields in the Huh7 cells transfected with the WT and the mutants in the absence or presence of serially diluted LDT. As mentioned above, the relative HBV DNA yields of rtM204I, rtL80I/M204I and rtL80V/M204I remained constant at different LDT concentrations. In contrast, rtM80I, rtM80V and WT showed dose-dependent decrease of HBV DNA yields during LDT treatment. The HBV DNA yields of rtL80I/M204I were significantly higher than that of the WT in a wide range of LDT concentrations (0.1, 10 and 100 μM, P<0.05), indicating the selective advantage of the mutant over the WT virus during LDT treatment. At LDT concentrations of 100 μM, the HBV DNA yield of rtL80V/M204I was significantly higher than that of the WT (P=0.0201). The replication yield of rtL80I was significantly higher than that of the WT at an LDT concentration of 0.01 μM (P=0.0394). Although the HBV DNA yield of rtM204I was significantly lower than that of the WT at low LDT concentrations (0.01 and 0.1 μM, P<0.05), it became significantly higher than that of the WT at 100 μM (P=0.0168). The HBV DNA yields of rtL80V and rtL80V/M204I at an LDT concentration of 0.01 μM were significantly lower than that of the WT, with P=0.0138 and P=0.0482, respectively. Apart from the above-mentioned cases, the samples did not show significant difference in HBV DNA yield compared with the WT during LDT treatment.

**DISCUSSION**

It has been reported that development of drug resistance during LDT therapy is primarily due to the rtM204I mutation (Nash, 2009). In three out of the four viral breakthrough patients, a good correlation between the detection
of rtM204I and viral breakthrough was found. In patient C, viral breakthrough happened at week 104; however, rtM204I was only detected at a level of 1%. No other obvious mutation specific to this time point in comparison with other time points was found, suggesting that this may not be a true viral breakthrough patient.

In contrast to rtM204I, which eventually became dominant in viral breakthrough patients during LDT treatment, rtL80I and rtL80V exhibited two dynamic patterns during therapy: an increase to dominance (e.g. rtL80I in patient J), and fluctuating (e.g. rtL80I in patients O and Y, and rtL80V in patients J, O and Y). Mutation rtL80I/V was reported to be significantly associated with LAM resistance, and almost half of the mutations rtM204I/V were co-selected with rtL80I/V during LAM therapy (Rhee et al., 2010; Warner et al., 2007). It is a complementary mutation, which does not affect the sensitivity of HBV to LAM resistance but compensates partially for the loss of replication efficiency caused by the acquisition of rtM204I/V (Warner et al., 2007). The detection of rtL80I/V in LDT resistance patients has also been reported previously but was not found to be as widespread as during LAM therapy (Lai et al., 2007; Matthews, 2007; Standring et al., 2006). In this study, mutations rtL80I and rtL80V were detected in three out of four viral breakthrough patients, emphasizing the importance of rtL80I and rtL80V in the development of LDT resistance. It was generally believed that the complementary mutations were co-selected or sub-selected with primary resistance mutations and the complementary mutations were maintained after development (Allen et al., 1998; Delaney et al., 2003; Locarnini, 2008; Ono et al., 2001; Pallier et al., 2006; Tenney et al., 2004; Warner et al., 2007; Zoulim & Locarnini, 2009). Interestingly, in this study, we found that rtL80I and rtL80V were detected earlier than (patients O and Y) or at the same time as (patient J) the primary mutation rtM204I, and rtL80I and rtL80V mutation might or might not be maintained after the dominance of rtM204I (Fig. 1). The data suggest that rtL80I/V is likely to be selected under the pressure of LDT even before the primary mutation rtM204I.
were moderately resistant to LDT, with an EC50 about
Interestingly, our results showed that rtL80I and rtL80V (Warner
report only mutation rtL80I and not rtL80V was studied
rtL80V has not been reported before, as in the previous
development of LDT resistance, single and double RT
mutations were made, and their replication efficiency
and sensitivity to LDT were tested
experiments were performed in triplicate, and error bars show SD.
Fig. 5. The relative HBV DNA yields in Huh7 cells transfected
with WT and mutants in the absence or presence of LDT. Huh7
cells were transfected with the WT and mutated HBV replication-
competent plasmids, and cultured for 72 h in the absence or
presence of the indicated LDT concentration. Supernatants were
harvested and the secreted HBV DNA yields were analysed using
Q-PCR. The amount of replication in each sample was normalized
to the mean value for the uninhibited WT (100 %). The experiments were performed in triplicate, and error bars show SD.
To understand the roles of rtL80I, rtL80V and rtL204V in the
development of LDT resistance, single and double RT
mutations were made, and their replication efficiency
and sensitivity to LDT were tested in vitro. Our results showed
that the RT mutations impacted differently on HBV replication, with an impact tendency of rtM204I
vs. rtM204I (Table 2). Also, the double mutations
rtL80I/M204I and rtL80V/M204I could compensate for the
loss of replication efficiency of rtM204I to the levels in
rtL80I and rtL80V, respectively (Table 2). To our knowledge,
the difference in replication efficiency between rtL80I and
rtL80V has not been reported before, as in the previous
report only mutation rtL80I and not rtL80V was studied
(Warner et al., 2007).
Interestingly, our results showed that rtL80I and rtL80V
were moderately resistant to LDT, with an EC50 about
30–50-fold increased in comparison with that of the WT
(Table 2). This is different from the result for LAM,
where rtL80I was shown to be more sensitive to LAM
than the WT (Warner et al., 2007). Although LDT is structurally similar to LAM (Matthews, 2007), the difference
between these two drugs must contribute to the sensitivity
of the mutations. Our results indicate that rtL80I and
rtL80V may not only serve as replication complementary
mutations to rtM204I, but also contribute to the LDT
resistance directly.
The difference in replication efficiency between rtL80I and
rtL80V also resulted in a difference in replication competence
between rtL80I/M204I and rtL80V/M204I. In our in vitro assay, the HBV DNA yield of rtL80I/M204I was
always significantly higher than that of rtL80V/M204I
(Fig. 5). We predict that rtL80I/M204I is more likely to
be selected than rtL80V/M204I under long-term LDT
treatment. Future investigation of more clinical samples
will confirm whether or not this is true.
Our results showed that the complexity of HBV quasispecies
remained consistent without drug treatment (Fig. 2).
By administering LDT, the complexity at 12 weeks
increased in most patients (Fig. 3). One of the causes of
the increased heterogeneity may be the adaptive increase
of the mutation rate in response to drug treatment
(Schmidt-Martin et al., 2012). The quasispecies complexity
dynamics of the four viral breakthrough patients showed
dramatic increase until week 24 (Fig. 4). During weeks
24–52, mutation–selection equilibrium was achieved in
patients J and O until variants with resistance were selected
and became dominant, leading to a decrease in complexity
(Fig. 4a, b). The complexity became lowest at week 72 in
these two patients, and rtM204I consistently reached its
highest level at this time point. After ADV was administered,
an increase in complexity was observed in both patients,
indicating again that, under new drug pressure,
the complexity of quasispecies increases.
Many studies have sought to predict the outcome of
therapy based on quasispecies parameters at pre-treatment
or in the early stages of treatment. Studies of HBV quasispecies during treatment with either LAM or entecavir produced similar results indicating that quasispecies complexity was statistically lower in responders than in partial responders at week 4, although the complexity was comparable at baseline (Chen et al., 2009; Liu et al., 2011). In our study, quasispecies complexity at baseline was comparable for both groups at the nt level but was higher for the responder group at aa level, and at week 12, the complexity of the responder group was significantly higher than that of the non-responder group (Fig. 3). These results could be interpreted as the production of more variants in the quasispecies in the responders during the early therapeutic pressure compared with non-responders. The reasons why we obtained different patterns of quasispecies evolution from the previous studies (Chen et al., 2009; Liu et al., 2011) is possibly due to: (1) the method of calculation. In the previous studies, the quasispecies were detected by direct PCR sequencing and the variants in complexity were defined based on the whole length of the reads (Chen et al., 2009; Liu et al., 2011). However, in our study, the complexity was measured by the mean Shannon entropy (Sn) at each nt or aa position following the methods reported previously (Nishijima et al., 2012); (2) the time point of sampling. The quasispecies may show different patterns of evolution at different stages of therapy; (3) different drugs used in the therapy.

In conclusion, our study revealed the dynamics of LDT-resistant mutations during the 104 weeks of therapy in viral breakthrough patients and found that the mutations were related to the dynamics of HBV quasispecies complexity. We found that quasispecies complexity remained relatively stable without drug treatment, but appeared to increase to adapt to the drug treatment until a resistant
mutant evolved. Once the resistant mutant became dominant, the complexity would drop to a lower level. Our UDPs data and in vitro sensitivity assay supported the idea that rtM204I plays a critical role in LDT resistance. In addition, our results indicated that rtL80I and rtL80V may not only serve as replication complementary mutations to rtM204I, but also contribute to the LDT resistance directly. These results provide insight into the quasispecies–therapy response interaction, which may have important implications in research and clinical practice.

METHODS

Patients and samples. Twenty-six HBeAg-positive chronic hepatitis patients who received LDT therapy at Tongji hospital during the Efficacy Optimization of Response to Telbivudine (EFFORT) clinical trial (NCT00962533) were included in this study (Sun et al., 2014). The study was performed according to the World Medical Association Declaration of Helsinki and the procedures were approved by the ethics committee of Tongji Medical College, Wuhan. Patients were selected on the basis of the following criteria: aged 18–65 years, plasma HBV DNA levels $>10^3$ copies ml$^{-1}$, elevated levels of ALT ranging from 2 to 10 times the normal upper limit, presence of hepatitis B surface antigen (HBsAg) for at least 6 months, presence of HBeAg and absence of anti-HBe, no history of NA treatment at any time, no treatment with interferon (IFN) within 1 year, and no accompanying infection of human immunodeficiency virus, hepatitis C virus or hepatitis D virus. Patients with poor compliance during treatment were excluded. Sequences revealed 21 patients were infected with genotype B and 5 were infected with genotype C.

Patients were treated with 600 mg LDT day$^{-1}$ and followed up at weeks 0, 12, 24, 36, 52, 64, 76, 88 and 104. The HBV DNA levels and HBV serological markers were measured using the COBAS Taq-Man HBV test (Roche Diagnostics) and ARCHITECT i2000SR (Abbott). Viral response was defined as an undetectable HBV DNA level at week 104, partial response was defined as HBV DNA level $>300$ copies ml$^{-1}$ at week 104, while viral breakthrough was defined as either HBV DNA elevation to more than 10-fold the level at the lowest point or when HBV DNA level reached a measurable level after falling below the threshold of detection. For comparison, the patients were grouped into responder group (containing responders) and non-responder group (containing partial responders and viral breakthrough patients). For viral breakthrough patients, ADV was added at 10 mg day$^{-1}$ as the complementary treatment after viral breakthrough was confirmed. Plasma samples were collected from all patients from baseline (week 0) to week 104; the samples selected for UDPs are listed in Table 1. Representative samples collected 4 weeks before baseline (week $-4$) were also selected for pyrosequencing. To optimize PCR and UDPs, HBV DNA levels were taken into consideration and the viral load of all selected samples was $>2 \times 10^5$ copies ml$^{-1}$. A total of 76 plasma samples from 26 patients were included in this study (Table 1).

PCR amplification and UDPs. HBV DNA was extracted from 200 μl plasma samples using the QIAamp DNA blood minikit (Qiagen). For samples with HBV DNA level $<1 \times 10^4$ copies ml$^{-1}$, 500 μl plasma aliquots were used for HBV DNA extraction. Primer pairs, listed in Table S4, were designed to amplify four partially overlapping segments covering the complete region of the RT. PCR amplifications were performed using FastStart High-Fidelity Enzyme (Roche) and the PCR products were purified using an Omega gel extraction kit (Omega Bio-tek) and quantified by a Nanodrop 1000 (Thermo Scientific).

Each gel-purified PCR product was separated into two parts; one part was pooled in equimolar quantities and subjected to UDPs, and the other part was subjected to direct Sanger sequencing to facilitate the UDPs data-cleaning processes and to confirm the authenticity of the sample. UDPs in this study was performed on the 454 Life Sciences platform (GS-FLX; Roche Applied Science) in both directions of the sequence according to the manufacturer’s instructions.

UDPs error rate. To estimate the error rate during the process of PCR amplification and pyrosequencing, a plasmid DNA was included as an internal control. A patient sample was used as template to amplify a fragment spanning nucleotide positions 58 to 1885 of the HBV genome, and the PCR product was inserted into the pGem-T Easy vector (Promega) to generate PT-HBV1.8K. The plasmid was PCR amplified with the same primers used for patients’ plasma samples and subjected to both UDPs and Sanger sequencing in parallel, and included in each UDPs run. A total of three UDPs runs were performed in this study and the mean mismatch rate of the overall plasmid sequence was 0.05–0.13 %. Over the three runs, a mean of 3.8 nt per sequence had a mismatch error rate $>1 \%$ and a mean of 1 nt per sequence had a mismatch error rate $>2 \%$ but did not exceed 5 %. Based on this estimate and results from a previous publication (Eriksson et al., 2008), we used an exclusion cut-off value of $>1 \%$ to estimate mutation frequency.

UDPs data analysis. The initial UDPs-generated sequence reads were first identified using the time- and point-specific bar-codes contained in the middle part of the primer sequence (Table S4). The reads were then filtered using the following criteria: (1) $>325$ bases and $<500$ bases in length; (2) no undetermined bases; and (3) read spanned the region of interest (aa 1–344 of the RT gene). The remaining reads were further aligned with corresponding Sanger sequences to correct insertions and deletions. A set of 980 HBV sequences was obtained from NCBI and used to create genotype-specific consensus sequences for RT. The filtered sequence reads were aligned to their respective consensus sequences and Sanger sequences by using the Smith–Waterman algorithm, and mutations in corresponding sites were calculated. For the prevalence of variants and mutations, we set ‘low prevalence’ at $<20 \%$ and ‘dominance’ at $>90 \%$ of HBV quasispecies (Le et al., 2009).

Calculation of quasispecies complexity. The heterogeneity of viral quasispecies was evaluated by a complexity metric based on the number of variants and their prevalence detected in the population. Quasispecies complexity at the aa and nt level was estimated for each site using $S_i$ according to the following formula: $S_i = \frac{-\Sigma f_i \ln f_i}{\ln p}$, where $f_i$ represents the frequency of a particular variant in the quasispecies (Domingo et al., 2006; Nishijima et al., 2012). The mean viral complexity in each sample was determined by calculating the total $S_i$ at each position and dividing by the total length nt or aa number. The Wilcoxon signed-rank test was performed to evaluate the changes of complexity between week $-4$ and baseline, between baseline and week 12 of the responder group, and between baseline and week 12 of the non-responder group. Changes between the responder group and the non-responder group at baseline or week 12 were compared using the Mann–Whitney U-test. In all tests, $P<0.05$ was considered statistically significant.

In vitro assay of the effect of mutations rtM204I and rtL80I/V on the susceptibility of HBV to LDT. To evaluate the effects of the mutations rtM204I and rtL80I/V on the susceptibility of HBV to LDT in vitro, a series of HBV replication-competent plasmids including single mutation of rtL80I, rtL80V or rtM204I, and double mutations of rtL80I/rtM204I and rtL80V/rtM204I, were generated. The single mutations were constructed based on a pTrEx-E-1-HBV plasmid containing 1.1-fold overlength HBV genome (jiang et al., 2010) by overlap extension PCR (Ho et al., 1989) with the primers listed in
Table S4. Double mutations were acquired using the same method with plasmids containing single mutations as templates. All the mutations were confirmed by Sanger sequencing analysis.

The human hepatoma cell line Huh7 (American Type Culture Collection) was maintained and transfected as described previously (Cao et al., 2014). In brief, Huh7 cells were seeded in 6 cm dishes (2 x 10^5 per dish) and transfected with 4.5 μg of the constructs using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were digested with trypsin (Invitrogen), washed three times with PBS and resuspended in DMEM containing 2% FBS and 1% DMSO. Then the cells were seeded into 24-well plates (2 x 10^5 per well) and cultured in the presence of LDT at the indicated concentration for another 72 h. To control the transfection efficiency, a GFP expression vector was co-transfected to monitor the transfection efficiency. The final concentrations of LDT used in this study were 0.01, 0.1, 1, 10 and 100 μM.

For Q-PCR analysis, 200 μl supernatants were harvested and treated with DNase I (Invitrogen) for 24 h to eliminate contaminant HBV vectors. Then the secreted HBV DNA was extracted using the QIAamp DNA blood mini-kit (Qiagen). Q-PCR was carried out using SYBR Premix Ex Taq TM II (TaKaRa) by 40 cycles as follows: denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec. Primers used for Q-PCR are qRT-FW and qRT-RW (Table S4). All extracted DNA was tested for DNA vector contamination by Q-PCR using primers qRT-Amp-FW and qRT-Amp-RW, designed to amplify the pTriEx-1 backbone of the HBV vectors (Table S4). The proportion of DNA vector in all of the samples was below 1% of the total HBV DNA. Each of the transfections and Q-PCR analysis was performed in triplicate. The mean amount of HBV DNA in the LDT-treated cultures was standardized relative to that of the untreated WT plasmid (defined as 1.0). EC_50 was estimated by SigmaStat software (version 3.0; SPSS). Statistical significance was analysed by one-tailed t-test. P<0.05 was considered statistically significant.

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