Effect of Hepatitis B Virus Reverse Transcriptase Variations on Entecavir Treatment Response

Danny Ka-Ho Wong,1,2 Malgorzata Kopaniszen,1 Katsumi Omagari,4 Yasuhito Tanaka,4 Daniel Yee-Tak Fong,3 Wai-Kay Seto,1 James Fung,1,2 Fung-Yu Huang,1 An-ye Zhang,1 Ivan Fan-Ngai Hung,1 Ching-Lung Lai,1,2 and Man-Fung Yuen1,2

1Department of Medicine, 2State Key Laboratory for Liver Research, and 3School of Nursing, University of Hong Kong, Hong Kong SAR, China; and 4Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Japan

Background. Entecavir therapy often reduces hepatitis B virus (HBV) DNA to an undetectable level, but HBV DNA remain detectable in some patients. We investigated whether baseline HBV reverse transcriptase (rt) polymorphism and quasispecies complexity and diversity were associated with treatment response.

Methods. Pretreatment HBV DNA levels, HBV rt sequence, serology, and quasispecies complexity and diversity from 305 entecavir-treated patients were determined. These data were tested for their association with year 1 virological outcome, defined by optimal response (undetectable HBV DNA; lower limit of detection, ≤12 IU/mL) or partial response (detectable HBV DNA).

Results. Four rt variants were more frequently detected in the 64 partial responders than in the 241 optimal responders (all \(P < .05\)). Multivariate analysis revealed that high baseline HBV DNA level (\(P < .0001\); odds ratio [OR], 2.32), HBV e antigen (HBeAg) positivity (\(P < .001\); OR, 3.70), and rt124N (\(P = .002\); OR, 3.06) were associated with a partial entecavir response. Compared with the optimal responders, the partial responders had a lower quasispecies complexity and diversity.

Conclusions. Apart from the known factors (high baseline HBV DNA level and HBeAg positivity), a novel single nucleotide polymorphism (rt124N) and lower quasispecies complexity and diversity were associated with partial entecavir response at year 1.

Keywords. antiviral therapy; hepatitis B; chronic viral hepatitis; drug response.
on the predictive HBV rt sequences for entecavir treatment are scarce.

In this study, our primary aim was to investigate whether there are some pretreatment HBV rt sequence variations that can predict response to entecavir. Identifiable sequence variations would be subjected to a molecular docking stimulation analysis to determine the possible underlying mechanisms [17]. It is not known whether viral quasispecies heterogeneity will affect entecavir response. The secondary aim was to examine whether there was an association between baseline quasispecies complexity and diversity with response to entecavir.

METHODS

Study Subjects
We recruited 370 patients with chronic hepatitis B who started entecavir therapy between January 2002 and September 2009 in the Liver Clinic at the Queen Mary Hospital, Hong Kong. None of these 370 patients had received prior NA therapy or interferon/pegylated-interferon therapy or had other chronic liver diseases, including chronic hepatitis C virus and hepatitis D virus coinfection, autoimmune diseases, and alcoholic liver diseases. All 370 patients had taken 0.5 mg of entecavir continuously, year 1, and year 3 (if applicable) HBV DNA levels measured. Liver cirrhosis was defined by the presence of cirrhosis-related complications, such as ascites, and esophageal/gastric varices, with or without ultrasonographic evidence of small-sized or nodular-surfaced liver. All patients consented to have baseline and subsequent serum samples stored for analysis. This study was approved by the Institutional Review Board of The University of Hong Kong and Hospital Authority Hong Kong West Cluster, Hong Kong.

HBV DNA Levels and Definition of Treatment Response
HBV DNA levels were measured by the Cobas TaqMan HBV Test (Roche Diagnostics, Branchburg, NJ), which has a lower limit of detection of 12 IU/mL (60 copies/mL) and a linear range of up to $1.1 \times 10^8$ IU/mL (6.4 $\times 10^8$ copies/mL). For statistical and numerical analysis, samples with a viral load of $>1.1 \times 10^8$ IU/mL were regarded to have a viral load of $1.1 \times 10^8$ IU/mL. Optimal and partial virological responses were defined as undetectable HBV DNA by the Cobas TaqMan assay ($\leq 12$ IU/mL) and detectable HBV DNA (>12 IU/mL), respectively, at the end of 1 year of treatment [18].

Direct Polymerase Chain Reaction (PCR) Sequencing of the Gene Encoding HBV rt
HBV DNA was isolated from 200 µL of serum samples, using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The HBV polymerase rt domain was PCR amplified using the Platinum Taq High Fidelity DNA Polymerase, the forward primer HBV56s (5′-CCTGCTGGTGCTCCAGTTC-3′), and the reverse primer HBV1234a (5′-GACACAAAGGTCCCACG CAT-3′). The 1.1-kb amplicon was sequenced bidirectionally, using the BigDye Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems Prism 3730xl Genetic Analyzer (Life Technologies, Carlsbad, CA).

Clonal Sequencing
PCR amplicons were cloned into the TA cloning sequencing vector system (Life Technologies) and transformed into competent Escherichia coli JM109 cells. For each isolate, 10–20 white colonies (median, 18 colonies) were picked and sequenced, as described above.

DNA Sequence Analysis
HBV DNA sequences were assembled and aligned using the CLC Main Workbench 6.6.2 (CLC Bio, Katrinebjerg, Denmark). HBV quasispecies heterogeneity was evaluated by 2 parameters: quasispecies complexity and quasispecies diversity. Quasispecies complexity is a measure of heterogeneity within a sample, while quasispecies diversity refers to the relatedness (genetic distance) of individual genomes within the population. Complexity is measured by normalized Shannon entropy (Sn) at both nucleotide and amino acid levels, using the following formula: $Sn = -\sum_i [p_i \times \ln p_i]/\ln N$, where $p_i$ is the observed proportion of each different sequence of the mutant spectrum, and $N$ the total number of clones compared [19]. Normalized Sn ranges from 0 (when all clones are conserved) to 1 (when all clones are different). Quasispecies diversity was assessed by the mean genetic distance (d) at both nucleotide and amino acid levels, the number of synonymous substitutions per synonymous site (dS), and the number of nonsynonymous substitutions per nonsynonymous site (dN), using the MEGA software [20, 21].

Molecular Docking Simulation
As there are no data on the crystal structures of the HBV rt, the crystal structures of the HIV type 1 (HIV-1) rt heterodimer (Protein Data Bank accession number 3KLI) were used as a template for molecular modeling [22]. Protein-compound docking simulation was performed by the Sievegene module in the myPresto Program and an in-house program OPPIH (Option Program for myPresto In HTML tools) [23, 24].

Statistical Analysis
Statistical analyses were performed using SPSS 18.0 (SPSS, Chicago, IL). Continuous variables with normal and skewed distributions were compared using the Student t test and the Mann–Whitney test, respectively. Categorical variables were tested using the $\chi^2$ test or the Fisher exact test as appropriate. Bonferroni correction was used to control for type I error in multiple comparisons. Stepwise logistic regression was performed to test the factors associated with complete or
RESULTS

Baseline Characteristics

Full-length HBV rt DNA was successfully amplified by PCR in 305 patients. Of these 305 patients, 114 (37%) were HBV e antigen (HBeAg) positive and 191 (63%) were HBeAg negative at baseline. At baseline, 87 patients (29%) had clinical evidence of liver cirrhosis. At year 1, 64 patients (21%) had partial virological response to entecavir (HBV DNA level, >2 IU/mL). The median HBV DNA level at year 1 for the partial responders was 110 IU/mL (range, 12.4 × 106–5.12 × 106 IU/mL). The baseline characteristics of the 305 patients are listed in Table 1.

The sex ratios, baseline albumin levels, and bilirubin levels were comparable between the partial and optimal responders. Compared with the optimal responders, the partial responders were younger, had greater proportions of HBeAg-positive patients, and had higher baseline HBV DNA and alanine aminotransferase (ALT) levels. Compared with the optimal responders, the partial responders also had a greater proportion of patients with HBV genotype B and a smaller proportion of patients with cirrhosis.

HBV rt Sequence Analysis

The amino acid sequence for the whole HBV rt region (344 amino acids) was studied. Major known drug resistance mutations (rtL80I/V, I169T, V173L, L180M, T184G, A181V/T, T184I, S202I, M204I/V, N236T, and M250V) were not detected in these patients, except that, in one patient, concomitant rtL80I and rtL180M mutations were detected. Of the 344 rt amino acid positions, 217 amino acid residues were conserved among these 305 patients. Twenty rt variations were found only in partial responders (distributed among 17 partial responders) but not in any of the optimal responders (Supplementary Table 1). Because of the rarity of these cases, association between these polymorphisms and partial/slow response was not statistically different.

Association analysis revealed that 17 amino acid variants were detected more frequently in the 64 partial responders than in the 241 optimal responders (Table 2). Since multiple comparisons of 127 nonconserved amino acid positions were performed, Bonferroni correction was used to control for type I error. After Bonferroni adjustment, 4 rt variants, namely rt53N, rt118N, rt124N, and rt332S, occurred significantly more frequently in the partial responders than in the optimal responders (Table 2). Of these 305 patients, 52 harbored HBV with all 4 variants (rt53N, rt118N, rt124N, and rt332S). The proportion of patients harboring all of these 4 variants was higher in the partial responders (25/64 [39%]) than in the optimal responders (27/241 [11%]; P < .0001).

We investigated the HBV rt sequence at year 1 in the partial responders. We successfully amplified the HBV rt region in 18 partial responders, all with an HBV DNA level of >500 IU/mL at year 1. HBV entecavir resistance mutations were not detected.

Table 1. Baseline Characteristics of Patients With an Optimal or Partial Response to Entecavir

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Optimal Responders (n = 241)</th>
<th>Partial Responders (n = 64)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean ± SD</td>
<td>48.3 ± 13.1</td>
<td>41.5 ± 10.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Sex, male:female</td>
<td>167:74</td>
<td>42:22</td>
<td>.574</td>
</tr>
<tr>
<td>HBeAg positivity, patients, no. (%)</td>
<td>67 (28)</td>
<td>47 (73)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HBV DNA level, log10 IU/mL</td>
<td>6.2 (2.3–8.0)</td>
<td>8.0 (4.4–8.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HBV genotype, B:C</td>
<td>65:176</td>
<td>30:33a</td>
<td>.002</td>
</tr>
<tr>
<td>Cirrhosis, patients, no. (%)</td>
<td>78 (32)</td>
<td>9 (14)</td>
<td>.004</td>
</tr>
<tr>
<td>ALT level, U/L</td>
<td>77 (11–3000)</td>
<td>110 (20–2144)</td>
<td>.004</td>
</tr>
<tr>
<td>Albumin level, g/L</td>
<td>42 (22–51)</td>
<td>42 (25–48)</td>
<td>.459</td>
</tr>
<tr>
<td>Total bilirubin level, µmol/L</td>
<td>12 (4–261)</td>
<td>13 (2–216)</td>
<td>.256</td>
</tr>
</tbody>
</table>

Continuous parameters are expressed as median value (range), unless otherwise indicated.

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus.

Table 2. Reverse Transcriptase (rt) Variants With Significantly Different Distribution Among Patients With an Optimal or Partial Response to Entecavir

<table>
<thead>
<tr>
<th>rt Variant</th>
<th>Percentage of Variants Found in Optimal Responders</th>
<th>Percentage of Variants Found in Partial Responders</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt9H</td>
<td>68.8</td>
<td>53.0</td>
<td>.025</td>
</tr>
<tr>
<td>rt16T</td>
<td>50.0</td>
<td>29.6</td>
<td>.002</td>
</tr>
<tr>
<td>rt53N</td>
<td>51.6</td>
<td>27.4</td>
<td>.00024</td>
</tr>
<tr>
<td>rt109S</td>
<td>54.7</td>
<td>35.3</td>
<td>.005</td>
</tr>
<tr>
<td>rt118N</td>
<td>48.4</td>
<td>25.3</td>
<td>.00034</td>
</tr>
<tr>
<td>rt121N</td>
<td>51.6</td>
<td>29.5</td>
<td>.001</td>
</tr>
<tr>
<td>rt124N</td>
<td>50.0</td>
<td>23.7</td>
<td>.000038</td>
</tr>
<tr>
<td>rt127R</td>
<td>46.9</td>
<td>29.0</td>
<td>.007</td>
</tr>
<tr>
<td>rt131N</td>
<td>50.0</td>
<td>28.2</td>
<td>.001</td>
</tr>
<tr>
<td>rt134N</td>
<td>35.9</td>
<td>16.2</td>
<td>.00048</td>
</tr>
<tr>
<td>rt151Y</td>
<td>51.6</td>
<td>30.3</td>
<td>.001</td>
</tr>
<tr>
<td>rt221Y</td>
<td>53.1</td>
<td>37.8</td>
<td>.026</td>
</tr>
<tr>
<td>rt222A</td>
<td>40.6</td>
<td>21.6</td>
<td>.002</td>
</tr>
<tr>
<td>rt238H</td>
<td>50.0</td>
<td>31.1</td>
<td>.005</td>
</tr>
<tr>
<td>rt271M</td>
<td>48.4</td>
<td>26.1</td>
<td>.001</td>
</tr>
<tr>
<td>rt278V</td>
<td>70.3</td>
<td>54.8</td>
<td>.025</td>
</tr>
<tr>
<td>rt332S</td>
<td>43.8</td>
<td>20.1</td>
<td>.00010</td>
</tr>
</tbody>
</table>

rt variants with significant different distribution after Bonferroni correction are shown in bold.
Compared with baseline, no change in the variants at year 1 at positions rt53, rt118, rt124, and rt332 was observed, indicating that these variants persisted over time in these patients.

The relationship between these 4 rt variants and other baseline parameters were studied. The 4 rt variants were distributed equally in patients of different sexes and HBeAg statuses (all $P > .05$). However, these 4 variants were found more frequently among patients without cirrhosis, those with HBV genotype B, and those with a high HBV DNA level (all $P < .05$).

Multivariable logistic regression analysis was performed to determine the independent effects of the baseline factors of age, HBeAg status, liver cirrhosis, ALT level, HBV DNA level, HBV genotype, and rt variants on partial entecavir response at year 1. We found that high baseline HBV DNA levels ($P < .0001$; odds ratio [OR], 2.32; 95% confidence interval [CI], 1.61–3.36), HBeAg positivity ($P < .001$; OR, 3.70; 95% CI, 1.79–7.65), and rt variant rt124N ($P = .002$; OR, 3.06; 95% CI, 1.53–6.11) were associated with partial entecavir response at year 1.

We also studied whether these HBV variants were associated with suboptimal entecavir response if other cutoff HBV DNA levels were used. Of these 305 patients, 13 (4.3%) had an HBV DNA level of >2000 IU/mL at year 1. A higher proportion of patients with an HBV DNA level of >2000 IU/mL had the rt124N variant, compared with patients with an HBV DNA level of ≤2000 IU/mL (7/13 [53.8%] vs 82/292 [28.1%]; $P = .046$). However, no significant difference in the variant frequencies of rt53N, rt118N, and rt332S was found ($P = .281$, .057, and .076, respectively). When other cutoff HBV DNA levels, such as 1000 IU/mL and 200 IU/mL, were used, no significant differences in the HBV variant frequencies were found (data not shown).

**Molecular Docking Simulation**

We used a molecular docking simulation model to assess whether rt124N causes a steric hindrance to the binding of entecavir to HBV rt [17]. On the basis of the crystal structure of the HIV-1 rt, we constructed a molecular model of the catalytic binding pocket of the HBV rt heterodimer (Figure 1). In this model, residue rt124 was positioned behind the helix, which consists of rt180L and rt184T. As shown in Figure 1A, rt124Y, the prevailing rt sequence for HBV genotype C, did not interfere with binding of entecavir to the rt catalytic pocket. In contrast, rt124N, the prevailing sequence of HBV genotype B, was in close proximity to entecavir and might cause a slight interference to the binding of entecavir to rt (Figure 1B).

**HBV Quasispecies Complexity and Diversity**

Quasispecies complexity and diversity were compared between the optimal and partial responders. As shown in Table 3, the optimal responders had a significantly higher quasispecies complexity than the partial responders at the nucleotide level ($P = .036$), and the quasispecies complexity at the amino acid level also tended to be higher in the optimal responders ($P = .087$). Similarly, the optimal responders had a greater quasispecies diversity than the partial responders in terms of all 4 parameters (mean genetic distance at both the nucleotide and amino acid levels, number of synonymous substitutions per synonymous site, and number of nonsynonymous substitutions per nonsynonymous site; all $P < .05$; Table 3). In other words, lower baseline quasispecies complexity and diversity were associated with partial response to entecavir at year 1.

**Longer-Term Entecavir Treatment Response**

We also determined whether pretreatment rt variations were associated with differences in longer term treatment response. Among the 64 suboptimal responders, 55 patients had continuous entecavir treatment with year 3 HBV DNA data available. At year 3, 14 of 55 patients (25%) still had detectable HBV.
DNA, while HBV DNA became undetectable in the majority of patients (41/55 [75%]). rt124N variant was found in 9 of 14 patients (64%) with a detectable year 3 HBV DNA level and in 18 of 41 patients (44%) with an undetectable year 3 HBV DNA level (P = .314).

We further investigated the role of rt124N in patients who tested positive for HBeAg and had a high HBV DNA level at baseline, the 2 risk factors for suboptimal responses at year 1 that were identified in the present study, and also for a slower 3-year entecavir response, which was identified by a previous study [25]. Among the 55 suboptimal responders with year 3 HBV DNA data available, 32 (58%) were HBeAg positive and had an HBV DNA level of > 8 logs at baseline. Of these 32 HBeAg-positive patients with a high baseline HBV DNA level, 9 (28%) and 23 (72%) had detectable and undetectable HBV DNA at year 3, respectively. The proportion of patients with rt124N was higher in those with detectable year 3 HBV DNA (7/9 [78%]) than in those with undetectable year 3 HBV DNA (8/23 [35%]; P = .049).

**DISCUSSION**

The present study showed that 21% of patients still had detectable HBV DNA after 1 year of therapy, which is comparable to the detectability rate of HBV DNA at year 1 in other studies [3, 5, 6, 25–27]. Although HBV DNA may become undetectable in some partial responders upon long-term treatment, it has been shown that patients with detectable HBV DNA after 1 year of entecavir treatment have a lower probability of having undetectable HBV DNA at year 3 [3]. This partial treatment response also has a long-term clinical implication, as it has been demonstrated that patients with intermediate levels of HBV DNA still carry an increased risk of development of hepatocellular carcinoma and cirrhosis-related complications [28]. A recent study has also demonstrated that a posttreatment cutoff HBV DNA level of <2000 IU/mL is not sufficient to indicate a reduced probability of disease progression, pinpointing the importance of complete HBV DNA suppression (to a level of <12 IU/mL) as the so-called optimal response [29]. Thus, investigating the molecular reasons for the slow/incomplete virological response to entecavir is of clinical importance. To examine this, studies with 2 important properties are required: (1) a large number of subjects for testing, because only 20%–30% of patients are expected to have suboptimal responses, and (2) a sensitive and robust viral sequence revelation of the whole genomic constitution of the HBV rt region. Our present study is specifically designed to solve these issues.

In the present study, HBV rt sequence analysis of 64 partial responders and 241 optimal responders showed that 4 variations (rt53N, rt118N, rt124N, and rt332S) were found to be present in a higher proportion in the partial responders. Detailed analyses showed that these 4 rt variants were also associated with other baseline parameters, such as HBV genotype and cirrhosis. In silico analysis showed that they were mostly associated with HBV genotype B, which was found to be more prevalent in the partial responders in the present study. However, logistic regression analysis showed that HBV genotype B was not an independent factor for partial response to entecavir. This is in accordance with the findings observed in other studies that the difference in the entecavir response has not been observed with different HBV genotypes in different studies [5, 6]. The prevalence of the 4 rt variants was also lower in patients with cirrhosis, which was also found to be associated with a better entecavir response in this present study. This is in accordance with a recent study showing that cirrhosis predicts early HBV DNA clearance upon entecavir therapy [30]. However, it should be noted that the patients with cirrhosis were generally HBeAg negative, had a lower HBV DNA level, and were older (P = .013, .002, and < .0001, respectively; data not shown), all of which were associated with a better entecavir response. Thus, a multivariate analysis was performed to identify the independent factors associated with a partial entecavir response.

Multivariate analysis showed that high baseline HBV DNA level, HBeAg positivity, and the rt variant rt124N were
associated with a partial entecavir response. This agrees with a previous study from our center, which found that patients who tested positive for HBeAg and had an HBV DNA level of ≥8 logs copies/mL (7.3 logs IU/mL) had a lower rate of HBV DNA undetectability at years 1–3 [25]. One novel finding of the present study is that the rt124N variant was significantly associated with a partial entecavir response at year 1. Furthermore, rt124N may also be associated with a higher chance of persistently detectable HBV DNA at year 3. Although the percentage of subjects with the rt124N variant was comparable between patients with detectable and those with undetectable HBV DNA at year 3 (64% vs 44%, respectively), it may be due to the limited number of subjects being tested. In particular, among the HBeAg-positive patients with a high baseline HBV DNA level, rt124N was significantly more frequently found in the patients who still had detectable HBV DNA at year 3. These data suggested that rt124N, although not the sole factor, is associated with a slower long-term response to entecavir, particularly when other coexisting adverse factors are present.

At the molecular level, the effect of rt124N on entecavir response was further studied by adopting our molecular docking simulation model. HBV rt124 is located in the rt fingers domain, but it is not inside the catalytic pocket within the palm domain of rt [17, 31]. Thus, it is less likely that rt124 variants will cause a remarkable conformational change to the entecavir binding site. Nevertheless, our molecular docking simulation model indicated that rt124N caused a slight interference with entecavir binding, suggesting that the rt124N may be slightly less susceptible to entecavir, without completely abolishing the binding. This may partly explain the slower response to entecavir in some patients who still achieve undetectable HBV DNA upon continuation of treatment beyond the first year. However, this modeling is based on the assumption that HBV rt forms a dimerized structure like that of HIV-1 rt. Should the crystal structure of HBV rt be available, the steric effect of HBV rt124N to entecavir binding will have to be confirmed with an HBV-based model. It should also be noted that the direct effect of rt124N should be best studied by an in vitro phenotypic assay. Nevertheless, this present study served as an initial identification of the HBV variants. An in vitro phenotypic study is required as a subsequent confirmation.

Another interesting finding from the clonal sequencing data is that the optimal responders had a higher quasispecies complexity and diversity than the partial responders. Studies of quasispecies complexity and diversity in NA-treated patients with chronic hepatitis B are rare. Our present finding differs from that in a recent study by Liu et al., which involved 31 entecavir recipients and showed that the baseline quasispecies complexity and diversity are comparable between the optimal and partial responders [21]. However, our study had a greater power because of the greater number of patients (n = 97) being studied.

The reason for the higher baseline quasispecies complexity and diversity in optimal responders in this present study is unclear. From the classical view of viral genetics, high quasispecies diversity would imply that the population has an increased possibility to harbor drug-resistant variants. However, according to the quasispecies theory, all variants within a viral population form an interacting network and react as a whole unit in response to stimuli such as antiviral therapy [19]. Under this quasispecies theory, a high quasispecies diversity may imply that the viral population reaches a critical status, termed self-organized criticality, in which the quasispecies network is maintained at an optimal capability [32]. At such a self-organized criticality status, the viral quasispecies population would be extremely sensitive to external perturbation [19, 33]. Therefore, a viral population with a high quasispecies diversity will be more prone to the external pressure exerted by entecavir. In accordance with the same line of thought, it has been demonstrated that higher baseline HCV quasispecies diversity and complexity are associated with an early virological response to interferon and ribavirin therapy [33]. It is also possible that, in the optimal responders with higher baseline complexity and diversity, the viral quasispecies were “less-fit” subpopulations coexisting in the total viral population with no single “good-fit” and replicatively competent dominant strain. Consequently, they are more susceptible to entecavir.

In conclusion, apart from the known factors of high baseline HBV DNA level and HBeAg positivity, a novel single nucleotide polymorphism, rt124N, was found to be a significant factor associated with partial entecavir response. In addition, lower baseline quasispecies complexity and diversity were also found to be associated with partial response to entecavir. These 4 factors may exert additive or synergistic influences on the suboptimal responsiveness to entecavir treatment.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
References