Low-Abundance Drug Resistance Mutations: Extending the HIV Paradigm to Hepatitis B Virus

To the editor—The article by Margeridon-Thermet et al [1] in the May issue of the *Journal* shows how new technologies, such as ultra-deep pyrosequencing (UDPS), might become useful advanced diagnostic tools for the management of hepatitis B virus (HBV) infection, as they are in the management of human immunodeficiency virus (HIV) infection. Although HBV variability is limited by the presence of a double reading frame along most of the genome sequence and by the long life span of the infected cells, the virus occurs in quasispecies with a significant number of low-abundance variants [1–5], including those bearing drug resistance mutations. The role of these pre-existing variants is still controversial, because their effective selection as drug-resistant virus strains has not been demonstrated. In the case of HIV infection, the presence of low-abundance variants has been associated with a greater risk of viral treatment failure, at least for “low genetic barrier” compounds, such as nonnucleoside reverse-transcriptase inhibitors [6–8], whereas no such association has been reported for HBV. UDPS technology, with its ability to detect variants down to 1% of the quasispecies (and potentially less), could be useful in treatment-naïve patients at their first treatment, but, given the slow emergence of resistance to the latest powerful drugs—entecavir and tenofovir—the most interesting results might come from studies involving patients who experience treatment failure with lamivudine and/or adefovir and are in need of a treatment change. In these patients, the presence of key minority cross-resistance mutations might alter the outcome of subsequent treatments.

In a recently published study performed with UDPS technology [4], we describe the frequency of variants bearing drug resistance mutations in the reverse-transcriptase coding sequence of a subset of patients with chronic HBV infection, 8 of whom were treated (and experienced treatment failure) and 5 of whom were treatment naïve. Although the minor variants in the treated patients showed a number of novel mutations, 2 of the 5 treatment-naive patients had low-abundance variants, bearing known drug resistance mutations: A181S (1.1% frequency), M204I (1.5%), and V214A (7%) in one patient and V214A (1.9%) in the other patient. These patients had not received treatment, and therefore no conclusion can be drawn on the association with treatment outcome. However, monitoring the dynamic changes of minority variants during therapy failure by means of UDPS suggests that their presence is, indeed, predictive of subsequent therapy failure. In fact, in a patient successfully treated with adefovir for 3 years after the failure of lamivudine treatment, direct sequencing and InnoLipa DR (version 2; Innogenetics) detected only wild-type sequences at the very beginning of viral rebound (530 IU/mL HBV DNA in serum). However, adefovir resistance mutations (181V and 236T), co-existing with traces of the previous lamivudine resistance mutations (L80I, L180M, M204I, and V214A), were detectable by UDPS as minority variants (1%–12% of all sequences; Table 1). Three months later, after a 1-log increase in viral load, the lamivudine resistance mutations disappeared or their frequency was reduced to <2%, whereas adefovir mutations represented approximately one-half of the viral quasispecies and were detectable (as a mixed population) by both direct sequencing and InnoLipa DR.

In conclusion, HBV, similarly to HIV, replicates in quasispecies with several low-abundance variants, including mutations in drug resistance key residues. Whether traces of archived resistance or spontaneously emerging, some of these may play a role in the development of full-blown drug resistance or cross-resistance. UDPS analysis, although still expensive and labor intensive (but with good prospects for improvement), appears to be the method of choice for the detection of such variants. Important remaining issues are the understanding of the specific impact of single mutations—primary, accessory or compensatory—and their associations in the context of the genomic molecule that harbors them, as determinants of its evolutionary potential. Further studies are needed, including the development and application of powerful algorithms for haplotype reconstruction [9], to gain full insight into the evolutionary dynamics of minority variants of HIV, HBV and hepatitis C virus.

**Table 1. Evolving Frequency of Relevant Hepatitis B Virus (HBV) Resistance Mutations in a Patient who Experienced Adefovir Treatment Failure**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Time 1</th>
<th>Time 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>L80I</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>L180M</td>
<td>3.33</td>
<td>1.52</td>
</tr>
<tr>
<td>A181V</td>
<td>7.43</td>
<td>45.49</td>
</tr>
<tr>
<td>M204A</td>
<td>2.24</td>
<td>0.74</td>
</tr>
<tr>
<td>V214A</td>
<td>1.07</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>N236T</td>
<td>12.03</td>
<td>63.2</td>
</tr>
</tbody>
</table>

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References

Reply to Menzo et al
To the editor—Menzo et al [1] cite the recent publication by Solmone et al [2] of a study performed at the National Institute for Infectious Diseases Lazzaro Spallanzani in Rome, Italy, on the prevalence of minor hepatitis B virus (HBV) variants in HBV-infected individuals. The study by Solmone et al [2], like ours [3], used a new sequencing technology—ultra-deep pyrosequencing [4](UDPS; 454 Life Sciences)—to characterize the HBV reverse transcriptase (RT) quasispecies in plasma samples from nucleoside or nucleotide RT inhibitor (NRTI)-naive and experienced individuals. The results of the Solmone study generally paralleled the results of our study, although there were some differences in results and conclusions.

In NRTI-experienced individuals, Solmone et al [2] frequently detected minority NRTI resistance mutations that were not detected by standard Sanger sequencing. As in our study, minority RT mutations associated with resistance to the L-nucleoside NRTIs (L80I, V173L, L180M, A181T/V, and M204I/V) and the acyclic phosphonate NRTIs (A181T/V and N236T) were often detected only by UDPS. We agree with the conclusion of Menzo et al that, in NRTI-treated individuals, the detection of minority drug resistance mutations may help quantify the risk of cross-resistance to the most commonly used salvage therapy NRTIs: adefovir, tenofovir, and entecavir [5].

In NRTI-naive individuals, Solmone et al [2] detected minority NRTI resistance variants in 2 of 5 patients. In one patient, the nonpolymorphic NRTI resistance mutations A181S and M204I were detected in 1.1% and 1.5% of UDPS sequence reads, respectively, and the polymorphic mutation Q214A was detected in 7.0%. In a second patient, Q214A was detected at a prevalence of 1.7%. Although we also detected NRTI resistance mutations at a low prevalence (in 2 of 17 individuals), we believe it is premature to consider these mutations to be clinically significant. First, mutations present at a low prevalence may be technical artifacts, which in our experience is most commonly caused by errors in performing polymerase chain reaction [3]. Second, APOBEC-induced G to A hypermutation is common in HBV quasispecies [6] and was responsible for low levels of the mutations A181T, A194T, and M204I in several of the samples that we studied. Hypermutated viruses are unlikely to be replication competent.

Solmone et al [2] emphasized that UDPS detected many minority variants that were not previously reported. We have also been intrigued by the possibility of a relationship between mutations detected as minority variants by UDPS and those reported as the dominant variant in different virus samples. Therefore, we have performed an analysis that used the data from our study and have arrived at a conclusion different from that of Solmone et al [2]. Our different conclusions, however, may have resulted from how each of our groups defined previously reported mutations. Solmone et al [2] appear to have restricted their search to mutations of the same genotype as the samples undergoing UDPS, whereas we searched reported mutations belonging to all HBV genotypes.

There were 2 parts to our analysis. First, we created a catalog of reported HBV RT mutations. We downloaded the April 2009 build of the GenBank Viral Sequence Database and performed a BLAST search using a consensus HBV sequence. The search returned 6935 HBV RT sequences encompassing codons 80–250 from 240 independent GenBank submissions: 188 PubMed references and 52 direct submissions. Approximately 2500 sequences were