Improved detection of resistance at failure to a tenofovir, emtricitabine and efavirenz regimen by ultradepth sequencing

Eve Todesco1–3*, Christophe Rodriguez4,5, Laurence Morand-Joubert1,2,6, Mélanie Mercier-Darty4, Nathalie Desire1–3, Marc Wirden1–3, Pierre-Marie Girard1,2,7, Christine Katlama1,2,8, Vincent Calvez1–3 and Anne-Geneviève Marcelin1–3

1Sorbonne Universités, UPMC Univ. Paris 06, UMR S_1136, Institut Pierre Louis d’Épidémiologie et de Santé Publique, F-75013 Paris, France; 2INSERM, UMR S_1136, Institut Pierre Louis d’Épidémiologie et de Santé Publique, F-75013 Paris, France; 3Department of Virology, Hôpital Pitié-Salpêtrière, AP-HP Paris, France; 4Department of Virology, Hôpital Henri Mondor, Université Paris-Est, Créteil, France; 5INSERM U955, Créteil, France; 6Department of Virology, Hôpital Saint-Antoine, AP-HP Paris, France; 7Department of Infectious Diseases, Hôpital Saint-Antoine, AP-HP Paris, France; 8Department of Infectious Diseases, Hôpital Pitié-Salpêtrière, AP-HP Paris, France

*Corresponding author. Department of Virology, Bât. CERVI, Hôpital Pitié-Salpêtrière, 83 Bd de l’Hôpital, 75013 Paris, France. Tel: +33-1-42177401; Fax: +33-1-42177411; E-mail: eve.todesco@psl.aphp.fr

Received 25 September 2014; returned 5 November 2014; revised 3 December 2014; accepted 12 December 2014

Objectives: Resistant minority variants present before ART can be a source of virological failure. This has been shown for NRTIs, NNRTIs and CCR5 inhibitors. However, very few data are available for the detection of such minority resistant variants that could be selected at virological failure and not detected using classical Sanger sequencing.

Methods: We studied 26 patients treated with tenofovir, emtricitabine and efavirenz with their first virological failure (defined as two consecutive viral loads >50 copies/mL). We performed standard Sanger sequencing and ultradeep sequencing (UDS; Roche 454 Life Sciences) in plasma at failure. For UDS, mutations >1% were considered. We compared the presence of reverse transcriptase mutations between the two techniques, using the latest ANRS algorithm.

Results: UDS detected more resistance mutations in 38.5% of cases (10/26 patients) and the genotypic sensitivity score (GSS) was reduced for 6 of them (23.1%). The GSS was impacted more often for NRTIs than for NNRTIs, for which most mutations were already detected by Sanger sequencing. Resistant minority variants were detected even in patients with low viral load at failure.

Conclusions: These results strongly argue for the use of next-generation sequencing in patients failing on an NRTI+NNRTI regimen, as UDS has the potential to modify the choice of the subsequent regimen.

Keywords: HIV-1 drug resistance, minority variants, virological failure, resistance genotyping

Introduction

The sequencing techniques currently used in routine work only detect viral populations if they account for >15%–20% of the total viral population. However, minority resistant variants (MRVs), whose rates by definition are under the detection threshold of Sanger sequencing, seem to be important. Indeed, it has been shown that the presence of HIV-1 drug-resistant minority variants may increase the risk of virological failure in first-line treatment. This was particularly demonstrated for NNRTI-based regimens. It was also shown that the existence of a minority subpopulation using the CXCR4 co-receptor at baseline was a risk factor for virological failure during CCR5 inhibitor treatment. The next-generation sequencing (NGS) technologies detect and quantify these MRVs at a far lower frequency. However, very few data are available for the detection of such MRVs that could be selected at virological failure and most of the data were obtained in patients experiencing virological failure many times or during intermittent ART. The objective of the study was to evaluate the selection of MRVs and the underestimation of resistance mutations detected by current sequencing in patients with their first virological failure to a tenofovir, emtricitabine and efavirenz regimen.

Methods

Patients

Blood plasma from 26 patients treated with tenofovir, emtricitabine and efavirenz who had experienced their first virological failure (defined as two consecutive viral loads >50 copies/mL) were studied. All of them achieved a viral load <50 copies/mL before failure. These patients could...
have been treated previously (but without having any virological failure before). Patients were followed by the Department of Infectious Disease of Pitie-Salpetriere and Saint Antoine Hospitals (Paris, France). Antiretroviral histories were obtained from the existing electronic database and/or medical record. We performed standard Sanger sequencing and ultradepth sequencing (UDS; Roche 454 Life Sciences) in the sample corresponding to the second detectable viral load >50 copies/mL. All patients were still under therapy at the time of genotypic resistance testing, meaning that the virus was still under drug selection pressure and that resistance mutations could be detected if they existed.

Sanger sequencing
The sample corresponding to the second detectable plasma HIV-1 RNA > 50 copies/mL was used for genotypic testing. Reverse transcriptase (RT) resistance genotypic analysis was conducted according to the Agence Nationale de Recherches sur le SIDA (ANRS) consensus method. Any sequences found to have a mixture of wild-type and mutant amino acid residues at single positions were considered to have the mutant at that position. Resistance was interpreted according to the latest version of the ANRS algorithm (www.hivfrenchresistance.org). Subtype determination was performed using the HIV module of SmartGene (SmartGene, Zug, Switzerland), the methodology of which is based on BLAST. The database of the HIV module is composed of sequences of different subtypes and circulating recombinant forms (CRFs) published by Los Alamos on http://www.hiv.lanl.gov. In the case of inconclusive results, the HIV SmartGene module provides the ability to perform a phylogenetic analysis.

UDS
After RNA extraction (NucliSENS® Easy MAG™, bioMérieux Clinical Diagnostics), reverse transcription into DNA was done using RT enzyme and DNA was then amplified by PCR (TianOne Tube RT-PCR Kit®, Roche Applied Science) using specific primers for the RT gene. A nested PCR was performed with a high-fidelity Taq polymerase (QS® High-Fidelity DNA Polymerase, New England Biolabs), each primer consisting of an adapter, a ‘key’ of four nucleotides, a specific patient sequence (identifier of 10 nucleotides) and the target sequence. PCR products were purified with AMPure® Beads (Agencourt, Biosciences), quantified using a Qubit® 2.0 Fluorometer (Life Technologies) and pooled equimolarly. Pyrosequencing on GS Junior (Roche 454 Life Sciences) was performed according to the manufacturer’s recommendations.

RT sequencing by UDS was performed on two fragments: RT1 (RT amino acids 17–140) and fragment RT2 (RT amino acids 133–247). Amplicon Variant Analyser (AVA) was used to analyse the UDS results. The presence of RT mutations was compared between the two sequencing methods. For UDS, mutations with a frequency of >1% were considered. Resistance mutations were interpreted using the latest ANRS algorithm (http://www.hivfrenchresistance.org). The genotypic susceptibility score (GSS) was calculated (drug with full susceptibility, 1; intermediate resistance, 0.5; full resistance, 0) with and without taking account of MRVs.

Statistical analysis
Patients were separated into two groups: with and without additional mutations detected by UDS. Fisher’s exact test and the Mann–Whitney test were used to compare baseline characteristics of patients for categorical (subset B, previous treatment) and continuous (viral load, CD4 cells count, time before failure) variables, respectively, between the groups. In univariate analysis, subtype (B/non-B), previous treatment (yes/no), viral load, CD4 cell count and time under the tenofovir, emtricitabine and efavirenz regimen before failure were investigated as potential factors relevant to the occurrence of MRVs. All factors with P<0.2 were retained for multivariate analysis and a logistic regression model was used to investigate whether previous variables were independent predictors of the occurrence of MRVs.

Results

UDS coverage
A median of 4790 and 6455 reads per nucleotide position was amplified for RT1 and RT2, respectively, which allowed accurate detection of variants down to a frequency of 1%. The average error rate in controls (cellular clone 8E5) was 0.0038.

Patients
A large proportion of patients were infected with HIV-1 subtype B (12/26). One patient was infected with a subtype G virus, one with a subtype A virus and 12 with CRFs (6 CRF02, 2 CRF01, 1 CRF09, 1 CRF14, 1 CRF06 and 1 CRF11). Viral load at failure ranged from 156 to 118 553 copies/mL [2.2 to 5.1 log; median=1228 copies/mL (3.1 log), with 13 patients having a viral load <1000 copies/mL. The median CD4 cell count was 369 cells/mm³ (range 60–1134 cells/mm³). The median time under tenofovir, emtricitabine and efavirenz treatment before failure was 11 months (range 1–61 months). None of the patients had previous virological failure, even though some of them had received another treatment before the tenofovir, emtricitabine and efavirenz regimen.

Sequencing
Six patients did not harbour any resistance mutation detected by either bulk sequencing or UDS. Among these six patients, only one had received treatment previously. Ten patients had exactly the same mutations detected by both methods; four of them were previously treated.

Finally, 10 patients (38.5%) had more mutations detected by UDS than by bulk sequencing. Eight of these 10 patients had received one or more lines of treatment before the tenofovir, emtricitabine and efavirenz regimen. Characteristics of these patients are described in the following paragraphs and Table 1. UDS detected MRVs affecting NRTI resistance in viruses from seven patients (eight MRVs). The M41L mutation was identified in viruses from three patients who had received NRTIs before. The M184V mutation was detected in virus from one patient who had previously been treated with emtricitabine. However, the K65R mutation was present in two patients never treated with tenofovir before the start of the tenofovir, emtricitabine and efavirenz regimen. In addition, one L74I and one K219Q mutation were also detected. The NRTI GSS was decreased for five of the seven patients.

Additional NRTI mutations were found in the plasma of five patients (five MRVs). The V179I mutation was identified in viruses from two patients who had received efavirenz before. The Y188H, H221Y and M230V mutations were each detected in single patients who had never been treated with NNRTIs. The NNRTI GSS was reduced in two of the five patients (in these two patients ripivirine susceptibility was affected by the additional mutations H221Y or M230V).

Finally, in 6 of the 10 patients who had more mutations detected by UDS, the GSS calculated by UDS was reduced (by up to 3.5) compared with the GSS calculated using Sanger sequencing results.

Of note, MRV were detected even in patients with low viral load at failure (six cases of patients with viral load <1000 copies/mL). To complete this work, we searched for genotypes before the start of tenofovir, emtricitabine and efavirenz treatment. We
**Table 1.** Characteristics of patients who had more mutations detected by UDS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Viral load (copies/mL)</th>
<th>Previous drugs*</th>
<th>Time under TDF/FTC/EFV (months)</th>
<th>Mutations detected by both Sanger sequencing and UDS*</th>
<th>Drug resistance detected both by Sanger sequencing and UDS*</th>
<th>Mutations detected by UDS only (coverage of the mutation: number of reads)</th>
<th>Drug resistance detected by UDS only*</th>
<th>Reduction of GSS*c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>248</td>
<td>TDF FTC EFV</td>
<td>42</td>
<td>K103N V118I G190A</td>
<td>V179I (3241)</td>
<td>5.56 NVP EFV</td>
<td>no</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>612</td>
<td>3TC ddl d4T NVP EFV</td>
<td>10</td>
<td>K103N V179I</td>
<td>M41L (6840)</td>
<td>5.03 NVP EFV</td>
<td>no</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>156</td>
<td>no</td>
<td>10</td>
<td>K103N K70R M184V</td>
<td>K65R (6924)</td>
<td>8.86 NVP EFV</td>
<td>—</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>11891</td>
<td>no</td>
<td>10</td>
<td>K103N K101E K103N K65R M184V A98S L100 K103N E138A</td>
<td>V179M (6296)</td>
<td>3.95 M41L (2796)</td>
<td>no</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>14267</td>
<td>ZDV ABC 3TC EFV</td>
<td>10</td>
<td>M184V K103N P225H</td>
<td>V179M (3386)</td>
<td>2.28 NVP EFV RVP ETR</td>
<td>no</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2108</td>
<td>3TC</td>
<td>1.5</td>
<td>M184V K103N</td>
<td>L74I (4096)</td>
<td>16.96 H221Y (3945)</td>
<td>ABC ddi</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>909</td>
<td>d4T TDF 3TC/FTC</td>
<td>11.5</td>
<td>M41L M184V T215Y K103N</td>
<td>M230V (3296)</td>
<td>4.83 NVP EFV RVP</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>9860</td>
<td>ZDV ABC 3TC/FTC EFV</td>
<td>12</td>
<td>M184I K103N Y188H</td>
<td>K65R (4602)</td>
<td>4.13 NVP EFV RVP</td>
<td>—</td>
<td>3.5</td>
</tr>
<tr>
<td>9</td>
<td>336</td>
<td>ZDV d4T 3TC NVP</td>
<td>12</td>
<td>M184V T215Y A98G K101Q K103N G190A</td>
<td>M41L (6500)</td>
<td>5.95 NVP EFV RVP</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>296</td>
<td>TDF FTC</td>
<td>10</td>
<td>no</td>
<td>M184V (15372)</td>
<td>12.28 NVP EFV RVP</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*3TC, lamivudine; ABC, abacavir; d4T, stavudine; ddl, didanosine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; NVP, nevirapine; RPV, rilpivirine; TDF, tenofovir; ZDV, zidovudine.

*Drug with intermediate resistance in bold.

Mutations detected by UDS only (coverage of the mutation: number of reads).

*bNRTIs are listed on the first line and NNRTIs on the second.

Reduction of Sanger GSS by the presence of MRVs (1, drug with full susceptibility; 0.5, intermediate resistance; 0, full resistance).
found only 4/10 available Sanger genotype results in the treatment-naive status (Table 1; patients 3, 4, 6 and 8). Only a K103T mutation was found (patient 8). However, unfortunately, no samples were available so we could not perform UDS.

**Statistical analysis**

In univariate analysis, the presence of MRVs detected only by UDS was associated with a longer time under tenofovir, emtricitabine and efavirenz before failure (P=0.0585) and, non-significantly, with previous treatment (P=0.0992). In multivariate analysis, there was only a non-significant relationship between the presence of MRVs detected only by UDS and prior treatment (P=0.097).

**Discussion**

In patients failing a tenofovir, emtricitabine and efavirenz regimen, the use of UDS instead of classical Sanger sequencing on plasma samples at failure detected more resistance mutations to ART in 38.5% (10/26 patients) of cases and, more relevantly, the GSS was reduced in 23.1% (6/26 patients) of cases, with a potential impact on salvage treatment.

Overall, total concordance was found between Sanger sequencing and UDS for all mutations detected by bulk sequencing. Additional mutations were found in approximately half of the samples in which resistance mutations were detected by Sanger sequencing (10/19 patients). It seems that patients who were more likely to have MRVs were previously treated patients and those who were treated for a longer time with tenofovir, emtricitabine and efavirenz.

When the presence of MRVs impacted the GSS, it was more often for NRTIs than for NNRTIs. This was presumably because NNRTIs, which are drugs with a low genetic barrier, were already widely affected by the mutations found by Sanger sequencing (K103N was already detected by Sanger sequencing in 9 of 10 viruses with additional mutations in UDS). Thus, our study emphasizes the interest of UDS for NRTI resistance at first failure, whereas the interest of UDS was previously highlighted for NNRTI resistance in first-line treatments. 3,4 We detected MRVs in 6 of 13 patients with a viral load <1000 copies/mL, but it is generally accepted that the value of UDS is questionable for such low viral loads because of the stochastic distribution of low-copy-number samples, and therefore viral loads <1000 copies/mL had not been studied by UDS previously. 3,4 However, despite a certain variability in detecting MRVs, it seems interesting to study the low viral loads, especially since it has been shown that, even with a small number of mutated copies/mL, some variants of resistance could be correlated with virological failure. 7 Obviously, further studies are needed to follow prospectively the impact of these MRVs on the virological response to subsequent regimens. However, these results reinforce recently published data and could argue in favour of the use of UDS in patients failing on an NRTI+NNRTI regimen, 15 even in patients with a viral load at failure <1000 copies/mL.

**Acknowledgements**

We thank Géraldine Lemallier and Philippe Grange for their technical assistance.


**Funding**

This work received financial support from the Agence Nationale de Recherches sur le SIDA (ANRS), the European Community’s Seventh Framework Programme (FP7/2007-2013) under the project ‘Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN)’ (grant agreement no. 223131) and Roche Diagnostics France.

**Transparency declarations**

None to declare.

**References**