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Minority resistant HIV-1 variants and the response to first-line NNRTI therapy

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ABSTRACT

Background: The presence of low-frequency HIV-1 variants with mutations making them resistant to non-nucleoside reverse-transcriptase inhibitors (NNRTI) could influence the virological response to first-line NNRTI therapy.
Objectives: This study was designed to describe the proportions and quantities of NRTI and NNRTI-resistant variants in patients with successful first-line NNRTI therapy.
Study design: We evaluated the presence of drug-resistance mutations (DRMs) prior to treatment initiation in 131 naive chronically HIV-1-infected patients initiating NNRTI-based first-line therapy. DRMs were detected by ultradeep pyrosequencing (UDPS) on a GS Junior instrument (Roche).
Results: The mean HIV RNA concentration was 4.78 ± 0.74 log copies/mL and the mean CD4 cell count was 368 ± 184 CD4 cells/mm^3. Patients were mainly infected with subtype B (68%) and 96% were treated with efavirenz. The sensitivity threshold for each mutation was 0.13–1.05% for 2000 reads. Major NRTI-resistant or NNRTI-resistant mutations were detected in 40 patients (33.6%). The median frequency of major NRTI-resistant mutations was 1.37% [IQR: 0.39–84.1], i.e.: a median of 556 copies/mL [IQR: 123–37,553]. The median frequency of major NNRTI-resistant DRMs was 0.78% [IQR: 0.67–7.06], i.e.: a median of 715 copies/mL [IQR: 391–3452]. The genotypic susceptibility score (GSS) of 9 (7.3%) patients with mutations to given treatment detected by UDPS was 1.5 or 2.
Conclusions: First-line NNRTI-based treatment can produce virological success in naïve HIV-1-infected patients harboring low-frequency DRMs representing <1% of the viral quasispecies. Further studies are needed to determine the clinical cut-off of low-frequency resistant variants associated to virological failure.

1. Background

HIV-1 drug resistance genotyping is recommended to optimize first-line antiretroviral therapy and thus prevent virological failure [1]. Current tests for drug resistance fail to detect low-frequency drug-resistance mutations (DRMs) present in less than 15–25% of the total quasispecies because they are based on bulk population sequencing [2,3]. Several studies have used recently developed ultra-sensitive methods, including allele-specific PCR [4,5] and ultra-deep sequencing [6–8], to show that low percentages of viruses with DRMs could contribute to subsequent treatment failure. There is no consensus as to whether more sensitive techniques should be used routinely to detect and quantify minor populations of drug resistant HIV-1 variants that are not detected by bulk population sequencing.

Low-frequency variants of HIV-1 with altered drug sensitivity could be clinically relevant in two main settings. First, when detecting minority variants that are resistant to non-nucleoside reverse transcriptase inhibitors (NNRTI) before starting first-line NNRTI-based treatment [9,10] and, second, when detecting low frequencies of CXCR4-using variants before using CCR5 antagonists [11].

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There is still confusion about which HIV-1 minority variants are clinically significant and how their presence affects clinical practice. Most studies published to date used techniques focusing on limited predefined DRMs [9]. Few have used ultra-deep sequencing methods which give access to all known DRMs and allow haplotyping [12–15]. The enhanced sensitivity of these assays facilitates the detection of minority variants and any resistance artifacts generated by the method [8,16]. However, appropriate clinical cut-offs and the proportion of low-frequency DRMs associated with virological success or failure are missing.

2. Objectives

NNRTI-based treatment is the recommended first-line highly active antiretroviral therapy (HAART); it is the most commonly prescribed treatment for naïve patients [17,18]. The presence of low-frequency NNRTI DRMs could influence the virological response. This study was designed to describe the proportion and quantity of minority resistant variants observed in patients successfully treated with first-line NNRTI therapy.

3. Study design

3.1. Population

A total of 131 naïve patients chronically infected with HIV-1, given first-line NNRTI therapy between 2008 and 2011 and who experienced virological success (HIV-1 RNA <50 copies/mL) 6 and 12 months after treatment initiation, were studied. All were followed at Toulouse University Hospital (France). The concentrations of HIV-1 RNA in plasma samples were measured by real-time RT-PCR (COBAS^{TM} Ampliprep/COBAS^{TM} Taqman HIV test; Roche Diagnostics; sensitivity: 20 copies/mL). All patients gave their informed consent for these virological studies.

3.2. Bulk population sequencing

Genotypic resistance tests were performed at treatment initiation. The HIV-1 reverse transcriptase gene was amplified by nested PCR and sequenced from bulk PCR products in both directions by the dideoxy chain 10 termination method (BigDye Terminator; Applied Biosystems) on an ABI 3130 DNA sequencer. The prevalence of resistant HIV-1 populations was analyzed using the latest International AIDS Society list of mutation (2013) [19] and the French resistance algorithm (2013, v23) (www.hivfrenchresistance.org).

3.3. Ultra-deep pyrosequencing (UDPS)

Plasma samples, collected for virological analyses at treatment initiation and stored frozen at −80 °C, were used to perform UDPS. HIV-1 RNA was extracted from 1 mL samples of plasma with virus loads of 708–3,090,295 copies/mL using QIAamp RNA extraction kits (Qiagen, Courtaboeuf, France). The next-generation ultradeep pyrosequencing (UDPS) HIV-1 assay currently being developed by Roche for the GS Junior system was used to amplify HIV-1 before UDPS. Briefly, first-strand cDNA was generated with two gene-specific oligonucleotides. This was used to produce four partly overlapping amplicons covering the HIV-1 pol gene (protease and reverse transcriptase gene codons 1–251). These were purified and quantified. Equimolar amounts of all four amplicons from each sample were pooled and subjected to clonal amplification on beads using reagents that enabled sequencing in both the forward and reverse directions. The beads were isolated and those bearing enriched DNA were counted. Ultra-deep pyrosequencing was carried out on 500,000 beads loaded onto the PicoTiter plate and sequenced on a Genome Sequencer Junior (Roche-454 Life Sciences). The amplicon nucleotide sequence reads were aligned with a consensus sequence HXB2. The capacities of two analytical programs, GS Amplicon Variant Analyzer (AVA) software and DeepCheck®-HIV, to analyze the ultra-deep pyrosequencing results were compared. GS Amplicon Variant Analyzer (AVA) software describe all the mutations whereas DeepCheck®-HIV describe only mutation with frequencies >1%. Data presented were based on AVA analysis. We used the latest International AIDS Society list of mutation (2013) [19] to identify resistance mutations and the French resistance algorithm (2013, v23) to interpret the involvement of each codon in resistance to each drug. Mutations conferring drug resistance on HIV alone are considered to be major mutations, while mutations that confer resistance only when associated with other mutations are defined as minor mutations. The genotypic sensitivity score (GSS) was interpreted using the ANRS algorithm: each antiretroviral was assigned a score 1 if susceptible, 0.5 for intermediate resistance and of 0 for high resistance.

Table 1
Description of mutations observed on each plasmid clones by sanger sequencing.

Clone	Subtype	Mutations (sanger sequencing)
1	B	V179I
2	B	V179I G190A T215D
3	B	WT[a]
4	B	WT
5	CRF06	WT
6	B	WT
7	CRF02	K103 N
8	B	WT
9	B	L100I, K103 N, E138 K, Y188L
10	B	M41L, A98G, G190A, V179I, L210 W, T215S

[a] WT: wild type.

3.4. UDPS technical error rate

We assessed the frequency of errors resulting from PCR amplification and pyrosequencing of the codon of interest by comparing the pyrosequencing data from a panel of 10 plasmid clones with their respective clonal sequence obtained by the Sanger method. Five plasmid clones had a wild type sequence and the others had one to six mutations on the polymerase gene (Table 1). The mean error rate and confidence interval were determined, and the upper confidence limit of the error rate was used to calculate the sensitivity of pyrosequencing for a given number of reads. Poisson distribution was used to distinguish authentic variants from artifactual sequences resulting from errors arising during PCR amplification and ultra-deep pyrosequencing. Only those variants yielding a P value of <0.001 according to the Poisson model were considered authentic. The detection threshold as a function of the read number was determined for each position. For 2000 reads, the thresholds of UDPS were as follow: M41 (0.15%), K65 (0.20%), D67 (1.05%), K70 (0.15%), L74 (0.25%), M184 (0.20%), L210 (0.18%), K219 (0.15%) for NRTI DRMs and L100 (0.50%), K101 (0.55%), V106 (0.15%), E138 (0.39%), Y181 (0.15%), Y188 (0.20%), G190 (0.15%), H221 (0.24%) for NNRTI DRM.

4. Results

4.1. Study population and drug resistance in bulk population sequencing

The demographics and other baseline characteristics of the patients are shown in Table 2. HIV subtype B was found in 89 (68%) patients and CRF02 in 22 (16.8%). Most patients were treated with a

Table 2
Baseline characteristics of patients.

Characteristic	
Male, *n* (%)	95 (73%)
Age, mean ± SD[a]	39 ± 10
Plasma HIV-1 RNA load (log copies/mL), mean ± SD	4.78 ± 0.74
CD4 cells count (cells/mm^3), mean ± SD	368 ± 184
ART[b] regimen	
TDF + FTC + EFV	118 (90%)
ABC + 3TC + EFV	4 (3%)
AZT + 3TC + EFV	2 (1.5%)
3TC + DDI + EFV	1 (1%)
AZT + 3TC + NVP	2 (1.5%)
TDF + FTC + NVP	4 (3%)
Mean time between HIV diagnosis and treatment initiation (years), mean ± SD	2 ± 4

[a] SD: standard deviation.
[b] ART: antiretroviral treatment.

regimen containing efavirenz (96%). Prior to treatment, DRMs were detected in 10 (9.3%) patients by bulk sequencing. Major NRTI DRMs were detected in 4 (3.7%) patients and major NNRTI DRMs were detected in 6 (5.7%) (Table 3).

4.2. UDPS detection of resistance-associated mutations

The sensitivity threshold determined for each mutation ranged from 0.13 to 1.05% for 2000 reads. The UDPS method did not amplify 8 samples (6%). Major DRMs were detected in 40 (33.6%) patients prior to treatment initiation (Table 3). About half (50–63%) of the major NRTI or NNRTI mutations identified accounted for less than 1% of the quasispecies, 15–19% accounted for 1–20%, and 19–38% accounted for over 20%. Similarly, 24–46% of minor mutations identified accounted for less than 1% of the viral quasispecies, 15–26% for 1–20%, and 38–50% for over 20%. The mutations identified were of very low abundance (<1% of the quasispecies) in 23 patients (18.6%), of low abundance (1–20% of the quasispecies) in 8 patients

Table 3
Patients with major drug resistance mutations (following list of IAS 2013).

ID	HIV-1 subtype	HIV-1 RNA (log copies/mL)	DRM[a] (sanger method)	NNRTI[b] mutations (UDPS method)	NRTI mutations (UDPS method)	GSS (based on DRM identified by UDPS method)
1	B	5.73	None	V106A (0.27%)	None	3
2	B	6.38	E138A	E138A (99.36%)	None	3
3	B	5.72	None	None	**M41L (10.86%)**[c]	3
4	B	3.04	None	**E138A (42.6%)**	None	3
5	B	5.11	None	K101E (0.67%), H221Y (0.86%)	None	3
6	B	4.89	None	G190E (0.62%)	None	3
7	CRF02	5.23	ND[d]	None	M41L (55.99%)	3
8	B	4.49	L210W	None	K65R (0.38%), L210W (100%)	1.5
9	B	5.18	None	L100I (0.74%), E138K (0.34%), G190E (0.77%)	None	2
10	B	3.52	None	G190E (0.4%)	None	3
11	Non-B	4.95	None	E138K (0.68%)	None	3
12	B	5.18	None	None	K65R (0.54%)	1.5
13	B	4.05	V108V/I	V108I (46.86%), **Y188H (1.37%)**	None	3
14	B	4.92	None	None	K70R (0.34%)	3
15	B	5.26	M41L	E138K (0.49%)	M41L (81.3%)	3
16	B	4.89	E138A	E138A (99.23%)	None	3
17	CRF14	4.76	M41L	None	M41L (100%)	3
18	CRF01	4.97	ND	Y188C (0.3%)	K65R (0.27%)	1.5
19	NON B	4.84	None	G190E (0.82%)	None	3
20	B	3.98	None	None	**D67N (1.76%)**	3
21	B	5.26	E138A	E138A (99.26%)	None	3
22	B	5.13	E138A	E138A (3.07%)	None	3
23	B	3.52	None	V106A (0.65%)	None	3
24	B	5.02	M41L, K219E	None	M41L (100%), K219E (92.46%)	3
25	B	3.95	None	None	**K70R (14.3%)**	3
26	B	4.47	E138A	E138A (13.96%)	None	3
27	B	5.24	None	None	L210W (0.77%)	3
28	B	3.65	None	**K101E (8.39%)**	None	3
29	B	5.24	None	None	M184V (0.17%)	2
30	B	4.61	E138A	E138A (99.72%)	None	3
31	B	5.07	None	G190E (0.32%)	None	3
32	B	4.8	None	E138K (0.69%)	None	3
33	B	3.64	None	E138K (0.95%)	None	3
34	B	3.74	ND	None	D67N (98.95%), **K70R (2.28%)**, L210W (0.40%)	3
35	D	4.46	None	None	L74V (0.98%)	3
36	B	4.25	None	None	M184V (0.20%)	2
37	B	6.35	None	Y181C (0.79%)	None	2
38	B	3.75	ND	None	K70R (0.39%)	3
39	CRF02	3.81	None	None	M184I (0.39%)	2
40	CRF02	3.25	None	None	K65R (0.40%)	1.5

[a] DRM: resistance-associated mutation.
[b] NNRTI: non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor.
[c] Boldface: minority DRMs >1%, not detected by Sanger.
[d] ND: no data.

(6.5%) and of high abundance (>20% of the quasispecies) in 12 (9.7%) patients. All DRMs with a frequency of >1% were identified by both analysis programs, and the frequencies described by them were identical.

Major NRTI mutations were observed in 21 patients (17%) (1 of whom also had minor DRMs) and minor mutations alone in 6 patients (4.8%). The median frequency of major NRTI DRMs was 1.37% [IQR: 0.39–84.1] corresponding to a median copy numbers of resistant variants of 556 copies/mL [IQR: 123–37,553]. The median frequency of minor NRTI DRMs was 0.31% [IQR: 0.1–0.72] corresponding to a median copy numbers of resistant variants of 149 copies/mL [IQR: 40–481].

Major NNRTI mutations were observed in 22 patients (17.9%) (11 of whom had both major and minor DRMs) and minor mutations in 30 patients (24%). The median frequency of major NNRTI DRMs was 0.78% [IQR: 0.61–7.06], corresponding to a median copy numbers of resistant variants of 715 copies/mL [IQR: 391–3452]. The median frequency of minor NNRTI DRMs was 20.24% [IQR: 1.06–99.2], corresponding to a median copy numbers of resistant variants of 8720 copies/mL [IQR: 721–110,306].

4.3. Low-frequency resistant variants to given treatment and virological response

DRMs conferring resistance to given treatment were observed in 9 (7.3%) patients with a GSS of 1.5 or 2 (Table 3). All these DRMs represented less than 1% of the viral quasispecies. The median frequency of NRTI DRMs conferring resistance to the NRTI used in 7 patients was 0.38 [IQR: 0.25–0.43], representing a median copy number of resistant variants of 184 [IQR: 33–425] copies/mL. The global genotypic susceptibility score (GSS) was 2 for three of them and 1.5 for the other four. EFV DRMs (L100I, Y181C) were detected in 2 patients given EFV, the median frequency was 0.77% [IQR: 0.74–0.79], representing a median copy number of resistant variants of 1165 copies/mL [IQR: 1120–3420]. The GSS for these patients was 2.

With a 3-year follow-up, all the patients with major DRMs were in virological success (whatever the mutation frequency in the quasispecies) whereas virological failure was observed in 4 patients without DRMs.

5. Discussion

Low frequency drug-resistant HIV-1 variants in naïve patients arise due to the highly error-prone replication of HIV or to direct transmission from hosts harboring resistant HIV-1 variants. Our data show that low-frequency drug-resistant variants are common in antiretroviral naïve patients chronically infected with HIV-1. Using an UDPS method, we observed that 33.6% of HIV-1 infected patients who were successfully treated with NNRTI-based first-line regimen harbored a virus with major DRMs and that 7.3% of patients harbored DRMs corresponding to the given treatment.

The sensitivity of UDPS for detecting low-frequency variants depends on the coverage of reads obtained by base and on the single-read substitution error rate. We measured the sequence error rates resulting from PCR amplification and UDPS of plasmid clones at different codons. The sensitivity thresholds were lower (0.13–1.05%) than those previously described (0.25–3%) [12]. This could be due to a new amplification protocol. The amplicons encompassing the reverse transcriptase and the protease used in this study were longer and less numerous than those used previously. This may explain the differences between error rates, as errors vary with the position of the base on the read, the transition from the previous base and the sequence length [16,20].

Prevalences of 4.7–6.2% of NRTI-resistant viruses and of 2.3–4.9% of NNRTI-resistant viruses have been reported by using bulk genotyping tests [21–24]. Similar rate were observed in the present cohort. Using the UDPS method, the prevalences of DRMs to NRTI previously detected in antiretroviral naïve HIV-1 infected patients were 14–25% and 11–15% for DRMs to NNRTI [6,14]. The prevalence of DRMs for NRTI found in this study was similar, while that of DRMs for NNRTI was somewhat greater. The greater prevalence of NNRTI is probably due to the recent description of new DRMs at position E138. The most common major mutations found in our cohort were M41L, D67N, K70R and L210W for NRTI, and E138A/K, G190E for NNRTI. These mutations have been commonly described in antiretroviral-naïve patients presenting resistant variants [23–26]. Any one of these mutations can influence the response to one of the three NNRTI molecules (efavirenz, nevirapine, rilpivirine) in first-line regimens. We did not detect K103N, although it is the most common pattern of transmitted genotypic NNRTI resistance – prevalence about 3% [22,24,27]. The prevalence of major DRMs was not different between B and non-B subtypes. A good concordance was observed between bulk genotyping and UDPS, except for one sample harboring E138A at 42.6% but not detected by bulk sequencing. This is either due to a lack of bulk genotyping to detect this mutation in this specific sample, or to an overestimation of the proportion by UDPS. Unfortunately, there was no sample left for this sample to document this discordance by cloning and sequencing.

Several studies have investigated the effect of drug-resistant minority variants in antiretroviral naïve patients starting NNRTI-based first-line treatment. Some have reported that low-frequency NNRTI resistance mutations double the risk of virological failure in patients given a NNRTI-based regimen [9,10]. Others, however, found that low frequencies of viruses resistant to NNRTI had no impact on treatment outcome [13,15]. There is very little evidence for an association between the presence of low-frequency NRTI-resistance mutations and virological failure. Our study found low frequencies drug-resistant variants in one third of the patients who experienced virological success. The rates of DRMs detected by UDPS in populations of antiretroviral naïve patients in previous studies were similar (28–30.5%), independent of the treatment outcome [6,14]. The overall prevalence of DRMs in patients successfully treated with a first-line regimen containing a protease inhibitor was 33.3% at baseline [14]. Another study on antiretroviral-naïve subjects starting first-line NNRTI treatment found that the prevalence of all DRMs detected was 68%, while 12% harbored DRMs whose frequency was >1% [15]. The rate of patients harboring DRMs with a frequency of >1% was similar in our study.

A dose-dependent association between drug-resistant minority variants and the risk of virological failure has been described [9]. Previous studies suggested that minority NNRTI-resistant variants accounting for at least 1% or >3 log copies/mL of the total population could be associated with virological failure [2,28,29]. Clinical cut-offs have been mainly defined based on data for low-frequency K103N mutations [9,30]. As there is little or no data available for other mutations, the cut-offs associated with the virological response are not known. While the number of patients is low, our finding suggest that frequency <1% or absolute quantity <3 log copies/mL of resistant variant in the viral quasispecies did not influence antiretroviral efficacy in this population.

In summary, our UDPS has identified a greater proportion of patients chronically infected with HIV-1 who harbored drug-resistant variants than did standard genotyping. These low-frequency drug-resistant variants were relatively abundant in this cohort of successfully treated patients, but they had no impact on treatment outcome. Further investigations are needed to determine cut-off values for each variant related to virological failure.

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Competing interests

None to declare.

Ethical approval

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