'Sentinel' mutations in standard population sequencing can predict the presence of HIV-1 reverse transcriptase major mutations detectable only by ultra-deep pyrosequencing

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Objectives: This proof-of-concept study aimed to identify whether mutations considered not yet relevant for drug resistance (but located at key drug-resistance positions) can act as 'sentinels' of minority resistant variants in HIV-1 drug-naive patients.

Methods: We focused our attention on three reverse transcriptase (RT) mutations (T69S, L210M and K103R) easily detected by standard population sequencing [i.e. the genotypic resistance test (GRT)]. Ultra-deep pyrosequencing (UDPS) of HIV-1 RT was performed using GS-FLX Roche, on plasma RNA from 40 drug-naive patients infected with HIV-1 subtype B without primary resistance detected by GRT. Only RT drug resistance mutations detected at >0.1% in both forward and reverse directions were considered. Associations between GRT sentinel mutations and UDPS drug resistance were assessed using Fisher's exact test.

Results: UDPS detected drug resistance mutations in 18/40 drug-naive patients. Patients carrying HIV-1 strains with T69S and L210M by GRT showed a trend to greater infection by minority drug-resistant variants than control patients infected by HIV-1 without these mutations (5/10 and 7/10 versus 3/10; P = not significant). No association was found for K103R by GRT. Notably, T69S and L210M (but not K103R or control viruses) were associated with GRT minority drug-resistant variants with a prevalence >1% (3/10 and 4/10 versus 0/20 in K103R and controls; $P=0.03$ and $P=0.008$, respectively). Moreover, the presence of L210M or T69S viruses by GRT significantly correlated with that of minority thymidine analogue mutations by UDPS (6/20 patients carrying HIV-1 strains with T69S/L210M versus 0/20 patients carrying HIV-1 having K103R or none of these mutations; $P=0.03$).

Conclusions: This proof-of-concept study suggests the existence of genetic markers, detectable by routine testing, potentially acting as sentinel mutations of minority drug resistance. Their identification may help in the selection of patients at high risk of resistance in reservoirs without the necessity of using UDPS.

Keywords: HIV-1 RT mutations, minority variants, drug resistance, UDPS

Introduction

At present, 25 antiretroviral drugs are available for the treatment of HIV-1. The combined use of several (3 or 4) of these drugs, known as highly active antiretroviral therapy (HAART), has successfully suppressed HIV-1 replication and has dramatically improved the prognosis of HIV-1-infected patients. However, when viral load rebound occurs during failing therapy against HIV-1 infection, viruses with mutations conferring drug resistance can be selected and become dominant in the viral population.[1] In HAART-treated patients with detectable plasma HIV-1 RNA, the prevalence of resistance to at least one drug can reach 80%.[2,3]

The frequent selection of drug-resistant strains during treatment failure can in turn increase the risk of their transmission (both predominant and minority species) to new individuals who are naive to the antiretroviral drugs (drug-naive individuals). The transmission of drug-resistant HIV-1 strains is well documented and frequently associated with suboptimal virological response to initial antiretroviral therapy.[4–8]

Based on the above considerations, the identification of resistance in drug-naive individuals (also including the minority

species) is important if a suboptimal response or, worse, virological failure to first-line antiretroviral therapy, is to be avoided.[9–12]

The genotypic resistance test (GRT) is considered the standard of care for detecting drug-resistant strains and is therefore recommended in both drug-naive and HAART-treated patients.[5] However, the sequencing procedure currently used is based on the Sanger method, which cannot detect viral variants below a detection threshold of about 15%–20% of the viral quasispecies. Below this level, drug-resistant variants may therefore be missed by GRT, suggesting that this test might underestimate the presence of drug-resistant viruses.

Recently, more sensitive assays, such as ultra-deep pyrosequencing (UDPS), have allowed the assessment of antiretroviral drug resistance mutations even when present as minority species (with a prevalence <20% of the entire viral population).[13–15] However, the implementation of these sensitive tests in clinical practice remains limited due to their current high cost and complexity.

The aim of this paper was to identify new genetic markers acting as 'sentinel' mutations in HIV-1 reverse transcriptase (RT) that are detected by population sequencing and are able to predict the presence of drug-resistant minority species in patients with no evidence of resistance by GRT. The potential sentinel mutations considered in this study were three RT mutations at positions already associated with drug resistance:[16] T69S, L210M, and K103R. These mutations are thought not to be involved in phenotypic resistance to RT inhibitors in either clinical isolates or site-directed mutagenesis experiments.[17,18] Analysis of 3370 individuals infected with HIV-1 subtype B in our database, all naive to antiretroviral therapy, showed that these atypical mutations were generally present at low frequencies (T69S, 1.9%; L210M, 0.7%; and K103R, 1.4%). Interestingly, their prevalence increased from 0.8%, 0.1% and 0.4% in samples collected before 2004 to 2.2%, 0.9% and 1.7% in samples collected after 2004, respectively.

Patients and methods

Study population

Among 3370 patients infected with HIV-1 subtype B who were naive to antiretroviral therapy and under care at 'L. Spallanzani' Hospital in Rome, Italy, between January 2004 and December 2009, 40 patients were selected: 30 infected by HIV-1 virus with T69S ($n=10$), L210M ($n=10$) or K103R ($n=10$) by standard GRT; and 10 infected by HIV-1 virus having none of these mutations, used as controls.

All 40 patients matched the following criteria: no previous exposure to antiretroviral drugs; a viral load >10000 copies/mL at the time of testing; and complete absence of primary transmitted resistance by standard GRT.[19]

All patients were coded for anonymity; demographic characteristics, self-reported route of transmission and date of sampling were analysed, together with virological, clinical and immunological parameters.

HIV-1 sequencing

Standard population sequencing

HIV genotype analysis was performed on all 40 plasma samples using a commercial sequencing kit (ViroSeq HIV-1 genotyping system; Abbott Laboratories).[20] Briefly, after 2 h of ultracentrifugation, HIV-1 RNA was extracted, retrotranscribed with murine leukaemia virus RT and amplified with Amplitaq-Gold polymerase by using two different sequence-specific primers for 40 cycles. The amplified products (containing the entire protease and the first 335 amino acids of the RT open reading frame; 1302 nucleotides) were full-length-sequenced in sense and antisense orientations using an automated sequencer (ABI 3100) with seven different overlapping sequence-specific primers.[20] Sequences having a mixture of wild-type and mutant residues at single positions were considered to have the mutant(s) at that position.

UDPS

After RNA extraction (performed as for standard sequencing), UDPS of RT was performed on all 40 of the samples.

UDPS was carried out using the 454 Life Sciences platform (GS-FLX; Roche Applied Science), using the HIV prototype primer set and sequencing protocol of the 2009 HIV Alphasite Study, supported by Roche.[21] Briefly, three overlapping cDNA sequences provided the source material for eight partially overlapping amplicons spanning the entire protease and RT codons, which were generated using barcoded primers. PCR products were then clonally amplified on capture beads in water-in-oil emulsion micro-reactors, and pyrosequencing was performed, following the standard approach for PCR amplicon sequencing. For each sample a Standard Flowgram Format (SFF) file was obtained, from which nucleotide sequence data were extracted.

GS Amplicon Variant Analyzer (AVA) software was used to analyse the UDPS results, as described by Simen *et al.*[14] Sequences from both orientations and from overlapping amplicons were combined into a single alignment, and primer regions were automatically trimmed to avoid artefacts from the nucleotide content of the synthesized primers.

UDPS generated a median of 7404 sequences per sample with a median sequence length of 245 bases. At least 300 sequences per base was considered the minimum accepted sequence coverage for each position of interest. Median coverage was 1200 sequences per base (range 300–6145).

Determination of drug-resistant minority variants

To estimate the prevalence of RT drug-resistant minority variants in the selected group of drug-naive patients, we used the list of drug resistance mutations reported in bold by Johnson *et al.*[16] and by the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/, last update June 2008). For each patient and for each drug resistance mutation, we determined the percentage of RT mutated sequences as well as their viral concentration (mutational load), calculated using the formula: percentage (%) of sequences containing each mutation×contextual viral load.[22]

Drug resistance mutations detected at a level of >2.0% of viral species in both forward and reverse directions were considered authentic minor variants. Drug resistance mutations detected at a level of >0.1% and <2.0% of viral species in both forward and reverse directions were considered authentic minority variants only if the relationship presented by Wang *et al.*[23] between the number of required sequences per position (required coverage) and the frequency of a given minority sequence variant (detection threshold) was guaranteed.

We also analysed all mutations found in the same amplicon to investigate any drug resistance pattern. Further analysis (i.e. haplotype reconstruction) was not attempted, due to the RT length and the high number of overlapping amplicons used to cover it.

Statistical analysis

Potential differences among the four groups of patients were assessed with the χ^2 test for trend for categorical variables and the Kruskal–Wallis test for continuous variables.

The association between sentinel mutations revealed by standard GRT and drug-resistant minor variants revealed by UPDS was assessed using Fisher's exact test.

The role of minority drug-resistant variants found in drug-naive patients in the virological outcome was also evaluated in patients starting their first-line treatment ($n=32$). The time needed to reach a plasma HIV-1 RNA level of <50 copies/mL after the start of treatment was determined, and compared between patients with and without drug-resistant minority variants using the Wilcoxon rank-sum test.

Results

Study population

Table 1 shows the epidemiological and viro-immunological characteristics of the 40 patients analysed.

At the time of the GRT, performed after a median time of 0.8 years [interquartile range (IQR) 0.1–3.7] since HIV diagnosis, patients had a median CD4 cell count of 239 cells/mm^3 (IQR 66–345) and a median viral load of 5.0 log copies/mL (IQR 4.5–5.5). No differences were reported among the four groups of patients, with the exceptions of HIV exposure and CD4 cell count at baseline (Table 1).

After GRT, 32 patients started an antiretroviral treatment. Among patients for whom the therapeutic regimen was known, 14 started a regimen containing two nucleoside RT inhibitors (NRTIs) plus one protease inhibitor (PI), 10 started with two NRTIs plus one non-NRTI (NNRTI), 2 started with three NRTIs plus one PI and 1 started with two NRTIs. Two other patients started a regimen containing raltegravir in combination with two NRTIs and two NRTIs plus one PI, respectively.

UDPS results for each patient analysed are reported in Table 2.

Coverage profile and UDPS results

The mean number of sequences obtained for each RT position is shown in Figure 1.

Focusing our analysis on the RT positions known to be associated with RT inhibitor (RTI) resistance, we found that UDPS detected minority variants with at least one RT resistance mutation in 18/40 patients (45.0%); of these, 7 patients carried drug resistance minority variants with prevalence >1%. Ten individuals carried only NRTI minority variants, three individuals carried only NNRTI minority variants and five individuals carried both NRTI and NNRTI minority variants.

The thymidine analogue mutations (TAMs) were the most frequently observed mutations (6/40 patients), with a prevalence in the viral quasispecies ranging from 0.40% for T215Y and K70R to 1.81% for D67N in the viral population. Other mutations found as minor species were the NRTI mutation F77L, involved in the Q151M complex, and the NNRTI mutations G190A/E, each observed in 4/40 patients. K103N and M184V mutations were also found by UDPS in two patients with a prevalence of 2.6% and 4.2%, respectively, and in four patients with a prevalence <2% (ranging from 0.49% to 1.14%).

Additional mutations, including the T215 revertants A/N/I/D and the NNRTI V106I and V179D mutations, were found in 4, 3 and 2 patients, respectively, with a prevalence ranging from 0.32% to 1.35% for the T215 revertants and from 0.85% to 4.6% for the NNRTI mutations.

Role of sentinel RT mutations in predicting minority resistant variants

Prevalence of drug-resistant minority variants

By UDPS, patients infected with HIV-1 virus who had T69S or L210M by standard GRT showed a trend to greater infection by

Table 1. Demographic, clinical and virological characteristics of the 40 patients

		Samples with mutations detected by GRT				
	Overall	control virus	K103R	T69S	L210M	P value[a]
Number of patients	40	10	10	10	10	
Year of HIV-1 diagnosis, median (IQR)	2005 (2003–06)	2002 (2000–06)	2005 (2003–05)	2005 (2002–06)	2006 (2005–08)	NS
Time since HIV-1 diagnosis (years), median (IQR)	0.8 (0.1–3.7)	2.9 (0.1–6.5)	0.3 (0.2–4.1)	0.9 (0.2–3.2)	0.4 (0.1–2.0)	NS
Male, n (%)	34 (85.0)	9 (90.0)	10 (100.0)	6 (60.0)	9 (90.0)	NS
HIV exposure, n (%)						
MSM	9 (22.5)	3 (30.0)	0 (0.0)	1 (10.0)	5 (50.0)	0.03
heterosexual	8 (19.5)	1 (10.0)	2 (20.0)	5 (50.0)	0 (0.0)	0.03
injection drug users	7 (17.1)	3 (30.0)	3 (30.0)	1 (10.0)	0 (0.0)	NS
other or unknown	16 (39.0)	3 (30.0)	5 (50.0)	3 (30.0)	5 (50.0)	NS
Age (years), median (IQR)	39 (33–44)	42 (40–44)	44 (41–49)	38 (36–39)	33 (27–41)	NS
Patients starting a first-line regimen, n (%)	32 (80.0)	9 (90.0)	7 (70.0)	10 (100.0)	6 (60.0)	
Viraemia (log copies/mL), median (IQR)	5.0 (4.5–5.5)	5.0 (4.8–5.6)	5.0 (4.6–5.3)	5.3 (4.5–5.7)	4.8 (4.3–5.2)	NS
CD4 cell count (cells/mm^3), median (IQR)	239 (66–345)	142 (46–272)	70 (25–189)	170 (64–327)	559 (270–897)	0.02
No. of DNA samples available	9	2	4	1	2	

MSM, men who have sex with men; NS, not significant.
[a]Statistically significant differences among the four groups of patients were assessed by the χ^2 test for trend for categorical data and the Kruskal–Wallis test for continuous variables.

Table 2. Clinical characteristics, UDPS results, HAART therapy and virological response for 40 antiretroviral-naive persons analysed by standard direct PCR Sanger sequencing

| Patient number	Transmission route	Date of diagnosis	CD4 count (cells/mm³)[a]	HIV-1 RNA (log copies/mL)[b]	Atypical mutation[c]	Minority drug resistance (% of reads with mutation)[d]
2580	unknown	15/08/2003	235	4.03	L210M	**M184V (1.14)**; **K219Q (0.8)**; V179D (1.06)
4027	homosexual	01/10/2005	1134	5.70	L210M	**M41L (0.76)**; **L74V(2.38)**; **V108I (1.51)**
5581	homosexual	15/12/2004	1128	4.36	L210M	**F77L (0.27)**
7755	homosexual	27/06/2008	270	5.24	L210M	**M41L (1.75)**; **M184V(0.49)**; **T215FY (0.40)**; **K103N (2.6)**; **G190A (0.35)**
8889	homosexual	27/07/2009	820	4.08	L210M	**F77L (0.55)**; **G190E (0.95)**
8893	homosexual	NA	NA	4.21	L210M	**A62V (0.82)**; **F77L (0.57) G190E (1.1)**
7567	unknown	09/01/2006	271	4.55	L210M	none
8932	homosexual	01/01/2009	NA	5.47	L210M	**V75I (0.89)**; **G190E (0.47)**
4780	homosexual	01/07/2004	783	5.23	L210M	none
4095	homosexual	23/12/2005	336	4.79	L210M	none
1749	heterosexual	01/03/1999	727	4.30	T69S	none
3268	heterosexual	01/11/2003	63	4.63	T69S	**D67N (1.81)**
3742	unknown	01/01/2006	65	5.70	T69S	**V75I (0.70)**; V106I (0.85)
4004	unknown	01/01/2002	264	5.34	T69S	**L210W (0.53)**; V106I (4.6)
5603	heterosexual	01/05/2006	457	4.48	T69S	**K103N (4.2)**
6534	drug user	01/05/2007	30	5.70	T69S	T215N (0.38)
6565	homosexual	12/06/2006	345	4.28	T69S	**A62V (1.70)**; **K70R (0.40)**
7587	unknown	01/01/2002	271	5.23	T69S	none
2667	heterosexual	15/10/2004	20	5.70	T69S	none
8502	heterosexual	01/03/2009	76	5.93	T69S	none
3012	drug user	16/02/2005	90	5.70	none	none
4845	homosexual	17/02/2006	281	5.62	none	**V106A (0.97)**
7070	heterosexual	01/09/2007	19	4.95	none	**F77L (0.92)**; T215I (1.1)
2773	drug user	NA	174	4.80	none	none
7253	homosexual	20/11/2006	487	5.02	none	none
7644	drug user	NA	1269	4.06	none	none
5171	unknown	01/01/2000	21	4.47	none	**M184V (0.54)**
5202	homosexual	01/08/2000	31	5.70	none	none
5732	unknown	NA	243	5.06	none	none
7169	unknown	NA	111	5.44	none	none
2990	homosexual	01/01/2005	25	5.47	K103R	none
3726	drug user	01/01/2000	337	4.87	K103R	**F77L (0.76)**
202	unknown	01/07/2005	69	5.70	K103R	T215D (1.35)
4402	unknown	NA	8	4.48	K103R	T215A (0.32); **M184V (0.67)**
6335	drug user	NA	189	5.20	K103R	none
2729	unknown	01/09/2004	25	4.12	K103R	none
2799	heterosexual	01/01/2003	147	5.32	K103R	none
3267	unknown	15/04/2005	70	4.91	K103R	none
7817	drug user	01/07/2008	NA	5.09	K103R	**V108I (0.56)**
8648	heterosexual	17/04/2005	344	4.15	K103R	none

[a]CD4 cell count at GRT.
[b]Plasma HIV-1 RNA level at GRT.
[c]Atypical mutation at drug resistance position detected by bulk sequencing.
[d]Percentages for minority variants are shown in parentheses. Drug resistance mutations are shown in bold, while revertants and minor resistance mutations are shown in non-bold font.

minority RT drug-resistant variants than control patients (5/10 and 7/10 versus 3/10; not significant, $P = 0.20$) (Figure 2). Conversely, for patients carrying HIV-1 virus with K103R by standard GRT, UDPS revealed the same level of minority resistance (3/10) as control viruses.

Of note, minority drug-resistant species with a prevalence of >1% (predictors of poor treatment outcomes[24–26]) were observed only in patients infected with T69S or L210M viruses. In particular, three patients with T69S and four patients with L210M carried minority RT resistance variants with a mutation
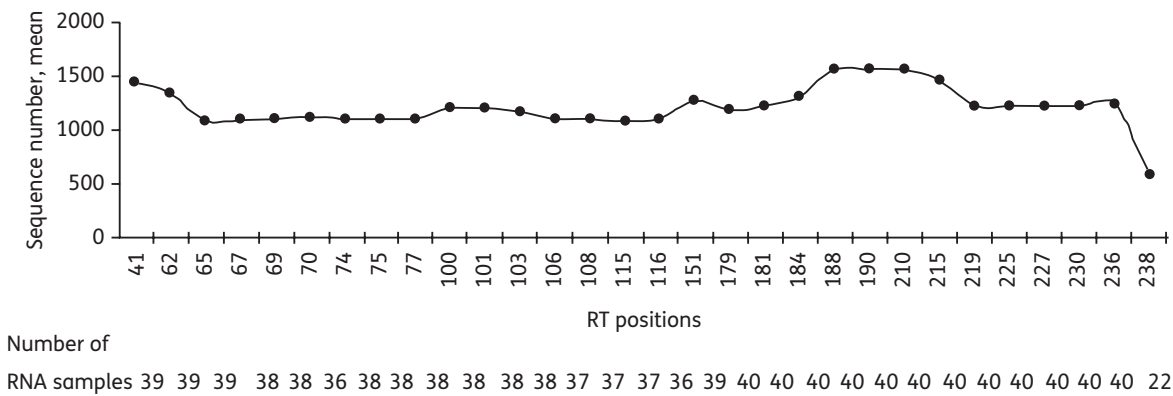
Figure 1. Coverage profile for each drug resistance position in RT. The mean numbers of sequences for RT drug resistance-related positions analysed in the study are shown in the figure. We analysed only positions per sample where at least 300 sequences were generated for each position (range of number of sequences/position analysed, 303–3371).
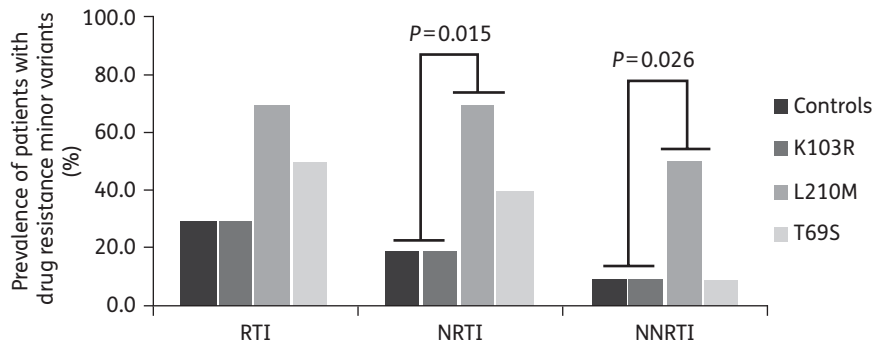
Figure 2. Prevalence of patients with RTI-, NRTI- and NNRTI-resistant minority variants, stratified by control, K103R, L210M and T69S viruses.

prevalence >1%, versus none in controls and in K103R samples (3/10 and 4/10 versus 0/20; $P=0.03$ and $P=0.008$, respectively) (Figure 3a).

The higher level of minority drug resistance in patients with L210M viruses was also confirmed by analysing the mutational load. As shown in Figure 3(b), six drug resistance mutations in L210M viruses reached a load of 2650–11900 copies/mL. These mutations were the TAM M41L, the abacavir/didanosine resistance mutation L74V, V75I, involved in the 151 complex, and the NNRTI resistance mutations V108I and K103N. This suggests a greater chance of these mutations being selected if there is incomplete control of virus replication.

Association of L210M and T69S with specific drug resistance mutations

When specific drug resistance mutations were analysed, TAMs were detected as minority species exclusively in patients with L210M and T69S sentinel mutations. In particular, six patients, 3/10 with T69S and 3/10 with L210M, carried TAMs versus 0/10 control and 0/10 K103R viruses ($P=0.03$). TAM1 and TAM2 pathways were also equally distributed in these patients; the mutations M41L (0.76% and 1.14%), T215FY (0.40%) and K219Q (0.80%) were found in sequences from patients with L210M by GRT, while D67N (2.82%), K70R (0.40%) and L210W (0.53%) were found in sequences from patients with T69S by GRT.

Combination of drug resistance mutations

Sequences obtained by UDPS were also analysed in order to determine patterns of drug resistance mutations in our samples.

Among the 40 drug-naive patients analysed, only patients with L210M or T69S viruses by GRT showed more than one major RT resistance mutation by UDPS (6/10 patients with L210M and 1/10 with T69S, versus 0/20 in controls and patients with K103R viruses; $P=0.01$) (Figure 2). In three patients, all carrying L210M by GRT, drug resistance mutations were localized in the same viral strain. In particular, in sample no. 7755, 0.33% of viral strains expressing NRTI T215F also carried NRTI M184V and NNRTI G190A. In sample 4027 all minority variants expressing the TAM M41L (0.76% of the viral population) also carried the mutation L74V, while in sample 2580 all the minority species with K219Q (0.8%) also carried the NRTI resistance mutation M184V.

These data suggest that the mutation L210M detected by standard GRT could be a sentinel of drug resistance minority species, including multidrug-resistant minority variants.

Response to HAART

Thirty-two out of 40 patients (17 infected with no drug-resistant minority variants and 15 infected with drug-resistant minority variants) started an antiretroviral regimen after genotypic testing (all after 2009). Thirty (93.7%) out of 32 patients
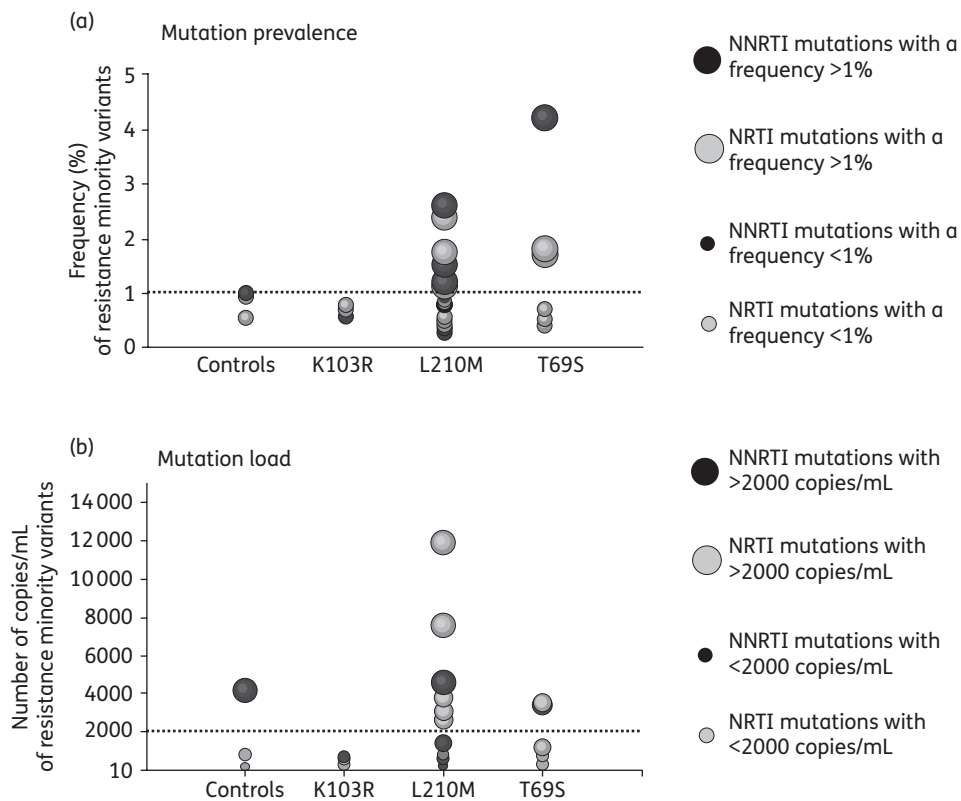
Figure 3. Prevalence of RTI resistance mutations (a) and RTI mutational load (b) in the 40 drug-naive patients. Each dot represents one drug resistance mutation; grey dots represent NRTI resistance mutations and black dots represent NNRTI resistance mutations. Drug resistance loads were estimated using the formula: percentage (%) of sequences containing each mutation×contextual viral load.

reached virological success in a median time of 15.8 weeks (IQR 12.0–19.6) after HAART initiation. Interestingly, patients with minority drug-resistant variants with a prevalence of >1% reached virological success in a median time of 19.9 weeks (IQR 14.8–21.0), later than patients with either no or low-prevalence minority variants (14.1 weeks; IQR 10.7–16.0) ($P=0.08$).

Analysing the plasma HIV-RNA levels from baseline to week 24, we also found that, whereas at weeks 4 and 8 the proportion of patients who reached virological success (HIV-RNA level <50 copies/mL) was not different between patients who carried minor resistance mutations with a prevalence >1% and those who did not (0/7 versus 0/18 and 0/7 versus 2/18), at weeks 12 and 24 the proportion of patients who reached virological success was slightly lower in patients who carried minor resistance mutations with a prevalence >1% compared with those who did not [2/7 versus 13/21 ($P=0.12$) and 3/6 versus 18/21 ($P=0.06$)].

Discussion

In our group of 40 HIV-1-infected individuals naive to antiretroviral drugs and with no evidence of resistance by bulk sequencing, minority drug-resistant variants were detected by UDPS in 18/40 patients with a prevalence ranging from 0.40% to 4.2% of the entire viral population. The prevalence of drug-naive patients with minority drug resistance was relatively higher

than previously reported.[26–28] However, it is important to note that, so far, most of the results on minority variants have been obtained by investigating only a few mutations using allele-specific PCR technology (such as searching for K103N, M184V and Y181C); thus, the frequency of minority resistant variants in these studies would certainly be higher if more mutations had been searched for.

Another reason that could explain the high prevalence of minority drug resistance mutations in our study may arise from the selection criterion used; in fact, the presence of specific sentinel mutations in HIV-1 RT, studied for their ability to predict minority drug resistance, may be responsible for the increased drug resistance prevalence in the study population.

Indeed, in our study we showed that the prevalence and patterns of minority drug-resistant strains in drug-naive patients appear to be dependent on specific mutations that are detectable by standard GRT and located at known RT drug resistance positions, potentially acting as sentinel markers for minority drug resistance mutations. In particular, patients infected with HIV-1 viruses carrying L210M and T69S mutations harbour minority NRTI- and NNRTI-resistant variants more frequently than those infected by control and/or K103R viruses. Even if these sentinel markers have low prevalence in the drug-naive population, their prevalence is similar to that observed for the classical transmitted drug resistance mutations,[29,30] confirming their potential role as sentinel markers of transmitted drug resistance.

The drug resistance mutations more often detected by UDPS in this study were the NRTI mutations, TAMs (together with T215 revertants) and NNRTI mutations G190A/E and K103N. It is interesting to note that the most common drug resistance mutations observed also by standard GRT in drug-naive individuals were the NNRTI resistance mutation K103N, the NRTI T215 revertants and TAMs.[29–31]

The null or modest viral fitness cost conferred by these mutations on the virus supports the persistence of these mutants in the untreated population and highlights their potential for frequent transmission.

Of note, the atypical mutation L210M could be a sentinel marker not only of undetected drug resistance but especially of multidrug-resistant minority variants. Indeed, all three patients harbouring minority HIV-1 strains with more than one major drug resistance mutation carried L210M by standard GRT.

The sentinel role of L210M may also be relevant from an epidemiological point of view; patients with this mutation acquired HIV predominantly through the homosexual route, which is known to be more frequently associated with an increased risk of drug resistance transmission.[29,32] The co-presence of this mutation with the homosexual transmission route could thus represent an additional marker of acquired drug resistance.

In addition, the L210M mutation is also associated with a high detection threshold of drug-resistant minority variants, frequently up to 1% and with a mutational load up to 2000 copies/mL (3/10 patients). In this regard, Goodman et al.[9] recently showed that the presence of the NNRTI mutation K103N at a level >2000 copies/mL strongly correlated with virological failure to a first-line efavirenz-containing regimen. In our cohort of patients, the NNRTI mutation K103N was found in two patients at >2000 copies/mL: in one L210M-expressing patient its frequency was 2.6%, corresponding to 4564 copies/mL, and in one T69S-expressing patient its frequency was 4.2%, corresponding to 3383 copies/mL. Both patients responded to their first-line therapy (not containing NNRTIs).

Indeed, in this study we have also investigated the virological response to the first-line regimen in a small group of patients according to the presence of resistant variants as a minority population. Interestingly, patients with minority drug resistance variants with a prevalence of >1% reached virological success later than patients with either no or low-prevalence minority variants. This finding is consistent with previous studies, suggesting that transmitted resistance, even if in minority species, could lead to suboptimal response to first-line therapy.[10,12,14,26]

However, a limitation of this study is the absence of a long-term follow-up for our patients, all of whom started the HAART regimen after 2009, and the fully active treatment received. Moreover, we cannot exclude the possibility that the small number of patients analysed may have reduced the statistical power of the study, in particular preventing any other effect of these minority drug resistance variants on the virological response from being seen.

In conclusion, this proof-of-concept study suggests that specific mutations revealed by standard GRT, such as the RT mutations L210M and T69S, could be markers of the presence of undetected drug resistance mutations, but could also act as sentinels of minority viral strains carrying multiple drug resistance mutations. Due to their low replication capacity in the absence of drug pressure, drug-resistant variants generally fade away after transmission, and may became present only as minority variants in the viral population replicating in the new host. A detailed map of the association between sentinel mutations, detected by GRT, and minor drug-resistant variants, detectable only with high-resolution methods, may therefore help when selecting patients at high risk of resistance in reservoirs. These mutations can thus provide a surrogate marker, easily detectable by routine testing, of minority drug resistance mutations.

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