

## Prevalence of Drug-Resistant Minority Variants in Untreated HIV-1–Infected Individuals With and Those Without Transmitted Drug Resistance Detected by Sanger Sequencing

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Minority variant human immunodeficiency virus type 1 (HIV-1) nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations are associated with an increased risk of virological failure during treatment with NNRTI-containing regimens. To determine whether individuals to whom variants with isolated NNRTI-associated drug resistance were transmitted are at increased risk of virological failure during treatment with a non-NNRTI-containing regimen, we identified minority variant resistance mutations in 33 individuals with isolated NNRTI-associated transmitted drug resistance and 49 matched controls. We found similar proportions of overall and nucleoside reverse transcriptase inhibitor–associated minority variant resistance mutations in both groups, suggesting that isolated NNRTI-associated transmitted drug resistance may not be a risk factor for virological failure during treatment with a non-NNRTI-containing regimen.

**Keywords.** HIV-1; drug resistance; minority variant; next-generation sequencing; antiretroviral therapy.

Standard genotypic resistance testing using Sanger sequencing is recommended upon diagnosis of human immunodeficiency virus type 1 (HIV-1) infection to identify individuals to whom drug-resistant HIV-1 was transmitted. Sanger sequencing typically detects drug resistance mutations (DRMs) present in at least 20%–30% of circulating plasma viruses [1]. However,

DRMs present below this threshold, commonly referred to as “minority variant DRMs,” have been shown to increase the risk of virological failure during an initial nonnucleoside reverse transcriptase inhibitor (NNRTI)–containing regimen [2].

The prevalence of transmitted drug resistance (TDR) in the United States ranges from 10% to 20%, with NNRTIs being the most commonly affected drug class [3, 4]. Nucleoside reverse transcriptase inhibitor (NRTI)–associated and NNRTI-associated DRMs emerge commonly in patients with virological failure during an NNRTI-containing regimen. However, in the absence of selective antiretroviral pressure, transmitted NRTI-associated DRMs decay more rapidly than transmitted NNRTI-associated DRMs [5]. Therefore, we investigated whether individuals with NNRTI-associated TDR detected by Sanger sequencing are more likely than those without TDR detected by Sanger sequencing to harbor minority variant DRMs that would increase their risk of virological failure even during a non-NNRTI-containing regimen.

To determine whether TDR is associated with an increased prevalence of minority variant DRMs, we performed Illumina next-generation sequencing of a portion of the reverse transcriptase coding region from plasma samples collected before antiretroviral therapy from individuals with isolated NNRTI-associated TDR and individuals without TDR. For accurate identification and quantification of minority variants, we used the Primer ID method, which corrects for polymerase chain reaction (PCR) bias and for PCR and sequencing errors and reveals the sampling depth of the viral population [6].

### METHODS

#### Study Population

The study population included HIV-1–infected, antiretroviral-naïve individuals in the Kaiser Permanente Medical Care Program–Northern California (KPNC) undergoing Sanger sequencing at Stanford Health Care Diagnostic Virology Laboratory between April 2004 and September 2012. The TDR prevalence in this population during the study period was 12.5%. The TDR group comprised 33 individuals with isolated NNRTI-associated TDR, defined as having variants with  $\geq 1$  NNRTI-associated surveillance DRM but no NRTI-associated or protease inhibitor–associated surveillance DRMs detected by Sanger sequencing [7]. The control group comprised 49 antiretroviral-naïve individuals without any TDR by Sanger sequencing who were matched to the TDR group for HIV-1 RNA level, CD4<sup>+</sup> T-cell count, sampling year, and antiretroviral regimen where possible. Additional inclusion criteria were a viral load of  $>10\,000$  copies/mL and an available cryopreserved sample for analysis by next-generation sequencing. The Stanford University and KPNC institutional review boards approved this study.

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## Sanger Sequencing

RNA extracted from plasma was reverse transcribed to complementary DNA, using SuperScript One-Step reverse transcription PCR with Platinum *Taq* (ThermoFisher Scientific, Waltham MA). AmpliTaq DNA polymerase was used for nested PCR analysis. Bidirectional sequencing encompassing protease and the first 250 codons of reverse transcriptase was performed using Big Dye Terminator Cycle Sequencing with electrophoretic resolution of products on an ABI 3730 sequencer (ThermoFisher Scientific). The GenBank accession numbers for the Sanger sequences are in the Supplementary Materials.

## Primer ID Illumina Next-Generation Sequencing

Primer ID Illumina/MiSeq sequencing was performed using a previously published protocol [6]. RNA was extracted from plasma, using the QIAamp viral RNA mini kit (Qiagen, Germantown, MD). Barcoded primers (Primer IDs) and Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) were used to generate complementary DNA (primer sequences are in the Supplementary Materials). The complementary DNA was purified to remove the unincorporated Primer ID oligos and then used as a template in 2 rounds of PCR, with MiSeq-indexed primers incorporated in the second round of PCR. Sequencing libraries were purified, quantified, and pooled for sequencing by Illumina MiSeq 300-base paired-end platform. The Illumina bcl2fastq pipeline (v.1.8.4) was used to for initial processing of sequence data. The Primer ID consensus pipeline was then used to create template consensus sequences for each starting viral RNA genome by pooling reads with identical Primer IDs, with the number of unique Primer IDs in a run defining the sampling depth of the viral RNA population [6] (available at: <https://github.com/SwanstromLab/PID>). The HXB2 numbering for the sequenced regions was 2648–3257 (reverse transcriptase codons 35–235). To distinguish mutations present in the viral RNA from the low rate of residual error, a Poisson threshold set at 10 times the frequency of the residual error was determined for each sample. Additionally, all template consensus sequences containing stop codons were excluded [6].

## Statistical Analysis

We defined minority variant mutations as amino acid differences from the consensus B sequence that were detected by next-generation sequencing but not Sanger sequencing. We compared the prevalence and frequencies of minority variant DRMs between the TDR and control groups, using the Fisher exact test and Wilcoxon rank sum test, respectively. We defined potential APOBEC-associated DRMs as DRMs that could result from G→A substitutions within the canonical APOBEC3F (GA→AA) or APOBEC3G (GG→AG) dinucleotide contexts: D67N, E138K, M184I, G190S/E, and M230I [8]. Human APOBEC3F/G enzymes are retroviral restriction factors that generate de novo viral mutations in a characteristic sequence context to impair viral fitness.

We performed 2 analyses of clinical outcomes: (1) we compared the proportions of individuals with virological failure between the TDR and control groups, and (2) we compared the proportions of individuals with virological failure between individuals with and those without minority variant DRMs conferring reduced susceptibility to any antiretroviral in the regimen they received. Reduced susceptibility to an antiretroviral was defined as intermediate or high-level resistance according to the Stanford HIV Drug Resistance Database interpretation system (version 8.2) [9]. Virological failure was defined as a confirmed HIV RNA level of >75 copies/mL at week 48. The Fisher exact test was used for both comparisons.

## RESULTS

### Characteristics of the TDR and Control Groups

Overall, 90% of study individuals were male, 55% were white, and the median age was 42 years. The median CD4<sup>+</sup> T-cell count was 339 cells/mm<sup>3</sup>, and the median viral load was 4.8 log copies/mL. Fifty percent and 35% of patients initiated a regimen containing raltegravir or boosted atazanavir, respectively. Eighty-eight percent of regimens included the NRTIs emtricitabine and tenofovir. The median year of pretherapy Sanger sequencing was 2010. Demographic and clinical characteristics were similar between the TDR and control groups, with the exception that more control individuals initiated a regimen not containing raltegravir or boosted atazanavir (Supplementary Table 1).

### Primer ID Illumina Sampling Depth

The median number of template consensus sequences was 619 overall (interquartile range [IQR], 257–1462). It was higher in the control group (1045; IQR, 394–1663) than in the TDR group (445; IQR, 169–960;  $P = .03$ , by the Wilcoxon rank sum test). The median lower limit of detection of a minority variant with 95% confidence for a sample was 0.5% overall (IQR, .2%–1.2%), 0.7% in the TDR group (IQR, .3%–1.8%), and 0.3% in the control group (IQR, .2%–.8%;  $P = .03$ , by the Wilcoxon rank sum test). The lowest limit of detection was 0.04% in the TDR group and 0.03% in the control group.

### Distribution of Drug Resistance Mutations

Overall, 110 DRMs were detected in 66 of 82 study individuals (80%). Of the 110 DRMs, 35 were NNRTI-associated DRMs detected by Sanger sequencing among the 33 individuals in the TDR group. Each of these mutations was also detected by next-generation sequencing. The additional 75 DRMs were minority variants, as they were detected only by next-generation sequencing. Overall, minority variant DRMs were detected in 49 study individuals (60%). The most-common minority variant DRMs were G190E (in 16 individuals), K219R (in 11), and K101E, M184I, and K219E (in 5 each; Table 1). Figure 1 shows that there were 2 main distributions of DRMs: those occurring at low frequencies detectable only by next-generation sequencing and those occurring at high frequencies that were detected

**Table 1. Minority Variant Drug Resistance Mutations (DRMs) in the Control Group and the Group With Transmitted Drug Resistance (TDR)**

Variable	Control Group (n = 49)	TDR Group (n = 33)	P <sup>a</sup>
Minority variant DRM pattern, patients, no. (%)			
Any	30 (61)	19 (58)	.82
NRTI only	22 (45)	10 (30)	.25
NNRTI only	19 (39)	11 (33)	.65
NNRTI and NRTI	11 (22)	2 (6)	.06
Minority variant DRMs, median frequency, % (number of occurrences)			
Any	0.2 (48)	0.2 (27)	.82
NRTI			
Overall	0.2 (25)	0.2 (16)	.29
M41L	0	0.3 (1)	
K65R	0	0.2 (1)	
D67N <sup>b</sup>	0.2 (3)	0	
K70E	0	0.1 (1)	
K70R	0	0.1 (1)	
V75M	0.1 (1)	0	
M184I <sup>b</sup>	0.4 (5)	0	
M184V	0.1 (1)	0.6 (1)	
L210W	11.4 (1)	0	
T215I	0.2 (2)	0.5 (2)	
T215S	0.2 (3)	0	
K219E	1.1 (1)	0.1 (4)	
K219N	0.7 (1)	0	
K219Q	0.8 (1)	0	
K219R	0.2 (6)	0.4 (5)	
NNRTI			
Overall	0.2 (23)	0.5 (11)	.10
K101E	0.2 (3)	5.7 (2)	
K103N	4.4 (1)	0.3 (1)	
K103S	0	0.9 (4)	
Y181C	0.2 (3)	0.1 (1)	
Y181V	0.2 (1)	0	
Y188H	0.1 (1)	0	
G190E <sup>b</sup>	0.2 (13)	1.2 (3)	
P225H	1.5 (1)	0	

Frequency is calculated as the percentage of an individual's virus population that bears a specific DRM.

Abbreviations: NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor.

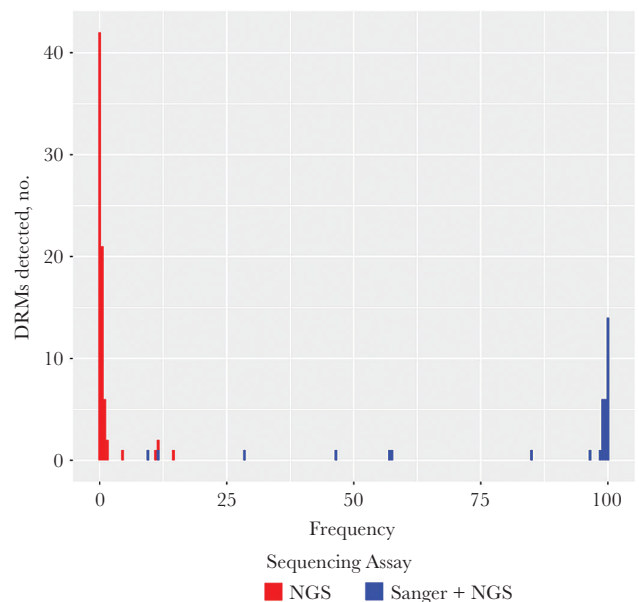
<sup>a</sup>Comparisons of the proportions of patients with a particular minority variant DRM pattern between the control and TDR groups were made using the Fisher exact test. Comparisons of the DRM frequencies among those individuals with a particular DRM in the control and TDR groups were made using the Wilcoxon rank sum test.

<sup>b</sup>Mutations that could potentially arise from APOBEC-mediated G→A editing [8].

by both sequencing methods. Among the 75 minority variant DRMs, 48 occurred at frequencies of 0.1%–0.3%, whereas 27 occurred at frequencies of 0.4%–14%.

#### Comparison of Minority Variants in the TDR and Control Groups

There was no difference in the proportion of individuals in the TDR (19 of 33 [58%]) and control (30 of 49 [61%]) groups with minority variant DRMs ( $P = .82$ ; Table 1). NRTI-associated minority variant DRMs were present in 39% of individuals overall, including 10 (30%) in the TDR group and 22 (45%) in



**Figure 1.** Histogram of drug resistance mutation (DRM) frequency, by method of sequencing. The histogram plots the number of DRMs detected at each frequency (percentage of the total virus population). DRMs in red were detected only by next-generation sequencing (NGS), and those in blue were detected by both Sanger sequencing and NGS.

the control group ( $P = .25$ ). NNRTI-associated minority variant DRMs were present in 37% of individuals overall, including 11 (33%) in the TDR group and 19 (39%) in the control group ( $P = .65$ ). Combined NRTI-associated plus NNRTI-associated minority variant DRMs were present in 16% of individuals overall, including 2 (6%) in the TDR group and 11 (22%) in the control group ( $P = .06$ ). There was no difference in the median frequency of minority variant DRMs observed in the TDR and control groups (0.2% in both groups;  $P = .82$ ).

Of the 75 minority variant DRMs, 24 (32%) were potential APOBEC-associated DRMs, including G190E in 16 individuals, M184I in 5 individuals, and D67N in 3 individuals. There was no difference in the proportions of potential APOBEC-associated minority variant DRMs between the TDR and control groups.

#### Virological Outcome

There was no difference in the proportions of individuals with virological failure between the TDR and control groups (2 of 33 vs 5 of 49;  $P = .70$ ). Poor medication adherence was documented in 4 of 7 individuals with virological failure. Two individuals in the control group and none in the TDR group underwent repeat Sanger sequencing. The DRM M184V was detected during virological failure in one of the control individuals who lacked baseline minority variant DRMs.

Two of 33 individuals in the TDR group and 8 of 49 in the control group had minority variant DRMs associated with intermediate or high-level resistance to  $\geq 1$  antiretroviral in their treatment regimen ( $P = .30$ ). One of the 8 patients in

the control group with a pretherapy minority variant DRM (M184I at a frequency of 0.1%) developed virological failure during treatment with a boosted atazanavir-based regimen. Repeat resistance testing was not performed, and the individual achieved resuppression following substitution of lopinavir for atazanavir.

## DISCUSSION

We used Primer ID Illumina next-generation sequencing to quantify the number of minority variant DRMs in 33 antiretroviral-naive individuals with isolated NNRTI-associated TDR detected by Sanger sequencing and 49 matched controls without TDR. The overall proportion of individuals with minority variant DRMs was 60%. This proportion is higher than that reported in previous next-generation sequencing studies, which have reported detecting minority variant DRMs in 20%–35% of antiretroviral-naive individuals [10]. Previous next-generation sequencing studies, however, generally ignored minority variant DRMs detected at levels of <1.0% because of the high probability that, in the absence of a method such as Primer ID, variants detected at a lower frequency include errors introduced during PCR amplification and sequencing [11].

The minority variant DRMs we observed likely represent a combination of transmitted DRMs that faded to levels no longer detectable by Sanger sequencing and de novo mutations resulting from HIV-1 reverse transcriptase misincorporation or APOBEC-mediated editing. Indeed, most of the DRMs in this study had a frequency either close to 100% or close to 0%. This bimodal distribution is consistent with 2 sources of DRMs. The finding that nearly one third of the minority variant DRMs were potential APOBEC-associated DRMs suggests that many of these were de novo mutations. In particular, G190E, which was present in 16 individuals, is rarely detected by Sanger sequencing and is extremely unfit [12].

Previous noncomparative studies have shown that individuals with TDR detected by Sanger sequencing may have additional minority variant DRMs [13, 14]. In this study, however, we found that individuals with TDR were not more likely than those without TDR to have additional minority variant DRMs. A potential explanation for the similar prevalence of minority variant DRMs among those with and those without NNRTI-associated TDR is that minority variant DRMs resulting from de novo mutation are likely to be evenly distributed among individuals with and those without TDR, potentially obscuring differences arising from the fading of transmitted DRMs.

The absence of an excess of minority variant DRMs among individuals with isolated NNRTI-associated TDR may help explain previous reports that antiretroviral-naive individuals with isolated NNRTI-associated TDR who receive a

non-NNRTI-containing regimen achieve similar rates of virological suppression as compared to individuals without TDR [15]. However, our study was underpowered to assess the clinical significance of minority variant DRMs because only 10 individuals had minority variant DRMs associated with reduced susceptibility to any of the antiretrovirals in their regimen.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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