

Minority Variants Associated with Transmitted and Acquired HIV-1 Nonnucleoside Reverse Transcriptase Inhibitor Resistance: Implications for the Use of Second-Generation Nonnucleoside Reverse Transcriptase Inhibitors

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Objectives: K103N, the most common nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutation in patients with transmitted resistance and in patients receiving a failing NNRTI-containing regimen, is fully susceptible to the new NNRTI, etravirine. Therefore, we sought to determine how often NNRTI-resistant mutations other than K103N occur as minority variants in plasma samples for which standard genotypic resistance testing detects K103N alone.

Methods: We performed ultradeep pyrosequencing (UDPS; 454 Life Sciences a Roche Company, Branford, CT) of plasma virus samples from 13 treatment-naive and 20 NNRTI-experienced patients in whom standard genotypic resistance testing revealed K103N but no other major NNRTI-resistance mutations.

Results: Samples from 0 of 13 treatment-naive patients vs. 7 of 20 patients failing an NNRTI-containing regimen had minority variants with major etravirine-associated NNRTI-resistant mutations ($P = 0.03$, Fisher exact test): Y181C (7.0%), Y181C (3.6%) + G190A (3.2%), L100I (14%), L100I (32%) + 190A (5.4%), K101E (3.8%) + G190A (4.9%), K101E (4.0%) + G190S (4.8%), and G190S (3.1%).

Conclusions: In treatment-naive patients, UDPS did not detect additional major NNRTI-resistant mutations suggesting that etravirine may be effective in patients with transmitted K103N. In NNRTI-experienced patients, UDPS often detected additional major NNRTI-resistant mutations suggesting that etravirine may not be fully active in patients with acquired K103N.

Key Words: drug resistance mutations, etravirine, nonnucleoside reverse transcriptase inhibitors, pyrosequencing, quasispecies, reverse transcriptase

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INTRODUCTION

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are essential components of antiretroviral (ARV) therapy.¹ However, NNRTIs have a low genetic barrier to resistance: a single mutation is often sufficient to cause resistance to the currently recommended first-generation NNRTIs, nevirapine and efavirenz. The reverse transcriptase (RT) mutation K103N is the most commonly occurring NNRTI-resistant mutation in patients with acquired^{2,3} and transmitted^{4,5} NNRTI resistance. K103N reduces susceptibility to efavirenz and nevirapine by ~20-fold and ~50-fold, respectively,³ but has no effect on susceptibility to the most recently approved NNRTI, etravirine.⁶

As a result of the low genetic barrier to NNRTI resistance, multiple NNRTI-resistant lineages often emerge in plasma samples from patients experiencing ongoing viral replication although receiving an NNRTI-containing regimen.^{7–9} Standard genotypic resistance testing (SGRT) performed by direct polymerase chain reaction (PCR) sequencing typically detects HIV-1 variants comprising $\geq 20\%$ of the viruses within a clinical sample but may miss less prevalent drug-resistant variants. To determine whether minority etravirine-resistant variants are present in patient samples,

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we performed ultradeep pyrosequencing (UDPS; 454 Life Sciences, a Roche Company, FLX technology, Branford, CT) of plasma samples from treatment-naive and NNRTI-experienced patients for whom direct PCR sequencing detected K103N but no other major NNRTI-resistant mutation.

METHODS

Patients and Samples

Patients included HIV-1-infected individuals followed within the Kaiser Permanente Medical Care Program of Northern California who had genotypic resistance tests performed at Stanford University Hospital between August 1998 and December 2007. Patient samples were characterized according to the following criteria: (i) presence of the RT mutation K103N, (ii) ARV treatment history, (iii) plasma HIV-1 RNA level, and (iv) availability of a cryopreserved sample.

Two types of samples meeting study criteria were identified: (1) samples from ARV-naive patients containing the K103N mutation (transmitted K103N) and (2) samples obtained from patients receiving a failing NNRTI-containing regimen (acquired K103N). To be eligible for study, plasma samples had to have plasma HIV-1 RNA levels ≥ 4.5 log copies per milliliter and an available cryopreserved sample. Samples with acquired and transmitted K103N were studied only if they lacked other major NNRTI-resistant mutations or known etravirine-resistant mutations.

Direct PCR Sequencing

Genotypic resistance testing for clinical purpose was carried out on all samples by direct PCR standard dideoxynucleotide sequencing as previously described.¹⁰ Briefly, reverse transcription-polymerase chain reaction (RT-PCR) products were obtained by plasma virus ultracentrifugation; followed by RNA extraction, reverse transcription using a high-fidelity RT enzyme, and PCR using *Taq* polymerase (The SuperScript One-Step RT-PCR with Platinum *Taq* System; Invitrogen, Carlsbad, CA). Direct PCR bidirectional sequencing encompassing HIV-1 protease and the first 250–350 codons of RT was performed using Big-Dye Terminators with products resolved electrophoretically on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). GenBank accession numbers of 14 previously submitted direct PCR sequences: AY030747, AY031476, AY030906, AF514165, AY030776, AY801580, AY032406, AY801679, AY801946, FJ983276, FJ983294, AY802041, AY802048, and FJ983334. GenBank accession numbers for the remaining 19 of the direct PCR sequences are pending.

Mutations

Etravirine-resistant mutations were defined as mutations associated in the DUET studies with a decreased virological response to etravirine: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S, and M230L.^{11,12} The mutations V90I, A98G, V106I, E138A, and V179D/T were considered to be less important indicators of etravirine resistance because they are polymorphic (particularly V90I and V106I) and have not been shown to have a phenotypic effect on etravirine susceptibility. Non-etravirine

NNRTI resistance mutations were defined as K101N/H, K103N/S/T, V106A/M, V108I, Y188C/F/H/L, G190E/Q, H221Y, P225H, F227L and P236L.¹³

Ultradeep Pyrosequencing

One milliliter of plasma was ultracentrifuged for 30 minutes, and RNA was extracted using the Roche Amplicor RNA extraction kit according to manufacturer's protocol. Reverse transcription was performed with random primers and Superscript III RT (Invitrogen) followed by RnaseH (Invitrogen) treatment to improve PCR efficiency. A first-round PCR was used to amplify a 1337 basepair product. Three second-round amplifications encompassed protease (positions 8 to 99) and the 5' part of RT genes (positions 1–238). Each primer consisted of a 5' 19-nucleotide UDPS adaptor, 1 of 7 patient-specific barcodes (ACTT, ATCA, TCTG, TACT, CTCT, CTCA, or CTAC), and the 3' HIV-1 target sequence (see Table, Supplemental Digital Content 1, <http://links.lww.com/QAI/A24>). PCR reactions were performed in a 50 μ L reaction mixture that contained 2.5 μ L of cDNA, 10 μ L Expand High FidelityPLUS Reaction Buffer, 2 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 0.5 mmol/L of each primer, and 2.5 U Expand High Fidelity PLUS DNA polymerase. cDNA titers were estimated by limiting dilution PCR of cDNA to confirm the presence of ≥ 100 amplifiable virus templates.

PCR products were purified by using AMPure beads (Agencourt Biosciences, Beverly, MA), quantified using Quant-iT Picogreen dsDNA reagent (Invitrogen), and pooled at equimolar concentrations. Clonal amplification on beads (emulsion PCR) was performed using reagents that enabled sequencing in both the forward and reverse directions (emPCR kits II and III; 454 Life Sciences). DNA containing beads were isolated and counted on a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). UDPS was performed on a Genome Sequencer FLX (454 Life Sciences), and each sample pool was loaded in 1 region of a 70 mm \times 75 mm PicoTiter plate (454 Life Sciences) fitted with a 4-lane gasket. Four PicoTiter plates were used to sequence 33 clinical samples and 4 plasmid control samples. The samples in the study shared plates with approximately an equal number of samples from other studies.

UDPS Coverage and Technical Error Rate

UDPS generated a median of 14,950 reads per sample with a median read length of 245 bases. This resulted in a median coverage of 3654 reads per base. An analysis based on 5 pNL43 clonal sequences performed on the same plates with the clinical samples yielded an overall mismatch error rate of 0.07%. The distribution of errors contained several outlier nucleotides for which the frequency of differences from pNL43 was slightly higher than would be expected from a Poisson distribution, in which rarely mismatch errors were present at a level between 1.0% and 2.0%. The approach we used to distinguish authentic minor variants from those that may have resulted from technical error was similar to that used in 2 previous publications^{14,15}: minority variants present in $\geq 2.0\%$ of sequence reads were considered highly unlikely to represent technical errors. Mutations present at levels between 0.5% and 1.9% had a higher risk of originating from a PCR

or sequencing artifact. Therefore, low prevalence variants occurring at a level of 0.5% or higher at positions for which there was an *a priori* suspicion of a possible mutation such as those at known drug resistance positions were also considered authentic variants.

RESULTS

Previously Untreated Patients With Virus Samples Containing Transmitted K103N

HIV-1 RT and protease sequences were obtained from 1334 ARV-naive patients between August 1998 and December 2007. Forty of the 42 patients (3.1% of the total) with the RT mutation K103N had no other detectable drug-resistant mutations. Twenty-four of these 40 patients had plasma HIV-1 RNA levels ≥ 4.5 log copies per milliliter. We identified cryopreserved aliquots of plasma for 17 of the samples with sufficiently high plasma HIV-1 RNA levels. From 13 of these cryopreserved aliquots, we successfully extracted ≥ 100 cDNA virus templates for UDPS.

Table 1 shows the plasma HIV-1 RNA levels, CD4 counts, RT mutations detected by SGRT, and RT mutations detected by solely by UDPS. The median CD4 count was 326 (range: 3–832). The median plasma HIV-1 RNA level was 5.2 log copies per milliliter. Five patients had plasma HIV-1 RNA levels between 4.5 and 4.9 log copies per milliliter, four had between 5.0 and 5.6, and another 4 had ≥ 5.7 log copies per milliliter, respectively. All virus samples belonged to subtype B. Five of the 13 patients had been infected within the year before presentation. The duration of infection for the remaining 8 patients was not known. SGRT detected a median of 6 amino acid differences (range: 4–14) from the consensus B sequence. In addition to K103N, the RT sequence from 1 patient had the accessory NNRTI-resistant mutation P225H.

UDPS confirmed each of the mutations detected by direct PCR sequencing in an unmixed form and 15 of the 19 mutations detected by SGRT as part of an electrophoretic mixture. UDPS detected a median of 6 reverse transcriptase amino acid mutations that were not detected by direct PCR sequencing (range: 0–10). The median number of additional silent reverse transcriptase mutations present in $\geq 2.0\%$ of sequence reads was 11 (range: 2–39), and the median number of residual consensus wild-type amino acids was 1 (range: 0–10). The 1 sample without any additional mutations detectable by UDPS was obtained in a patient undergoing primary HIV-1 infection (PID 22127). That sample, however, did have 7 silent minor variants detected only by UDPS.

The polymorphic etravirine-associated mutations V90I and V106I were each detected in 1 sample. No major etravirine-associated mutations were detected at a level above 0.5%. Five samples had 1 or more nucleoside reverse transcriptase inhibitor (NRTI)-resistant or NNRTI-resistant mutation including 30062, which had 4 NRTI-resistant mutations: M184V + L210W + T215Y + 219Q and 8048 had 1 NRTI-resistant mutation K65R and 1 NNRTI-resistant mutation P225H. No NNRTI-resistant mutations as high as 0.5% with the exception of K103S (a likely K103N revertant) which was detected in 0.6% and 0.9% of reads from samples

8048 and 28010, respectively. The sample from PID 30062—which contained 4 NRTI-resistant mutations—also had the following minority protease inhibitor (PI)-resistant mutations: M46I (0.8%), I84V (0.9%), and L90M (0.9%) (data not shown). No other minority PI-resistant variants were detected.

Six of the patients with transmitted K103N (16387, 22138, 25590, 27791, 27834, 30074) were treated with a regimen containing ritonavir-boosted atazanavir ($n = 4$) or lopinavir ($n = 2$) in combination with tenofovir (TDF) + lamivudine, emtricitabine (FTC), or didanosine. These patients experienced complete virological suppression for a median of 3 years (range: 2–4 years) through January 2009. Five patients were untreated for a median of 3.5 years (range: 2–5 years) through January 2009. One patient (8048) was treated with lopinavir/ritonavir in combination with stavudine + abacavir for 7 years but was poorly adherent and eventually developed the lopinavir resistance mutations (I54V and V82A). One patient (26412) was untreated for 3 months and then lost to follow-up.

NNRTI-Experiencing Patients With Virus Samples Containing Acquired K103N Mutations

Between August 1998 and December 2007, HIV-1 RT and protease sequences were obtained from 1057 NNRTI-experienced patients. By SGRT, 437 of these patients (41%) had a plasma sample containing K103N obtained although receiving a failing NNRTI-containing regimen including 160 patients (15%) whose virus samples exhibited no other major NNRTI-resistant mutations. Of these 160 plasma samples, 32 (20%) had plasma HIV-1 RNA levels ≥ 4.5 log copies per milliliter. Cryopreserved aliquots were available for 26 samples. For 20 of these aliquots, we successfully extracted ≥ 100 cDNA virus templates.

Table 2 shows the plasma HIV-1 RNA levels, CD4 counts, RT mutations identified by SGRT, and RT mutations detected solely by UDPS. The median CD4 count was 188 (range: 27–464). The median plasma HIV-1 RNA level was 4.9 log copies per milliliter (range 4.5–5.7). Eleven patients had plasma HIV-1 RNA levels between 4.5 and 4.9, 5 had 5.0–5.6, and another 4 had ≥ 5.7 log copies per milliliter, respectively. All virus samples belonged to subtype B. Direct PCR sequencing identified a median of 8.5 positions (range: 2–15) with differences from consensus B sequence. The accessory NNRTI-resistant mutations P225H ($n = 3$), V108I ($n = 1$), A98G ($n = 1$), and H221Y ($n = 1$) were detected in 6 of the samples. Twelve samples had established NRTI-resistant mutations including M184V/I in 9 samples and T215F/Y in 6 samples.

UDPS confirmed each of the mutations detected by SGRT in an unmixed form and 29 of the 37 mutations identified by SGRT as part of an electrophoretic mixture. UDPS also uncovered a median of 5.5 reverse transcriptase amino acid mutations that were not detected by direct PCR sequencing (range 0–11). A median of 8 additional silent reverse transcriptase mutations (range 1–19) were present in $\geq 2.0\%$ of sequence reads, and there was a median of 1 residual consensus wild-type amino acids (range: 0–6). The 1 sample in which UDPS did not detect any additional

TABLE 1. Clinical Characteristics and UDPS Results in 13 ARV-Naive Patients With HIV-1 Samples With the RT Mutation K103N

PID	Year	Months Infected	CD4 Count, cells/ μ L	HIV-1 RNA, Log Copies/mL	Mutations Detected by SGRT	Mutations Detected Only by UDPS	ETR Mutations†	Other NNRTI Mutations
8048	2000	NA	294	5.6	35I, 103N , 123E, 177E, 178V, 179I, 211K	48T(10), 65R(1.2) , 68G(2.1), <u>90I*(1.3)</u> , 103S(0.6), 135L(5.7), 192N(2.1), 195T(2.6), 225H(3.4)	(1)	2
30269	2006	<12	449	4.5	103N , 175H, 207N, 211K	<u>106I*(6.9)</u> , 123E(23), 162C(27), 179I(31), 196R(2.8)	(1)	0
22138	2004	NA	3	5.7	35I, 53DE, 83K, 103KN , 122EK, 200KAET	35T(8.5), 123N(10), 142V(33), 221Y(1.3) , 224K(5.5)	0	1
25590	2005	NA	17	5.7	20R, 35T, 39KAET, 40D, 82R, 98S, 103N , 122E, 123N, 196E, 200A, 207N, 211Q, 214L	35I(3.6), 39S(4.8), 47F(3.3), 48T(2.5), 50L(2.5), 73N(2.7), 102R(4.1), <u>103S(1.3)</u> , 123E(26), 219Q(1.4)	0	1
30062	2006	NA	392	4.5	103N , 122E, 174RQ, 177E, 178M, 179IV, 211K	184V(1.0) , 192N(4.5), 195M(2.2), 204Q(7.2), 210W(1.2) , 215Y(1.1) , 219Q(2.5)	0	0
27834	2006	<12	524	5.7	43E, 103N , 123E, 135T, 162A, 177E, 200A, 207E	48T(3.0), 60I(9.2), 67N(2.1) , 177K(8.4), 197R(5.6), 207K(2.2)	0	0
28010	2006	NA	525	4.5	60I, 83K, 103KN , 169D, 178L, 207E	32R(3.0), <u>103S(0.9)</u> , 122E(3.1), 200A(2.3)	0	1
16387	2003	<12	256	5.2	103N , 161PQ, 173EK, 207E, 225H	35I(9.8), 60I(14), 122E(3.8), 174R(2.3), 212S(6.6), 223R(3.5)	0	0
22127	2004	<3	832	5.2	39A, 103N , 122E, 142V, 162C, 200I		0	0
22219	2004	NA	607	4.5	103N , 122P, 162C, 173E, 200A, 211KR	192N(4.8), 203K(1.2)	0	0
26412	2006	<6	269	4.6	60I, 103N , 104R, 142VI, 178M, 200A, 211K	35I(27)	0	0
27791	2006	NA	326	5.6	103N , 123E, 180V, 192ND, 211K	102R(2.5), 166R(15), 177E(2.5), 177G(2.5), 178V(2.6)	0	0
30074	2006	NA	44	5.7	98S, 102RK, 103KN , 123E, 177E, 200A, 207K, 211K	20R(3.9), 203K(2.6), 207E(9.6)	0	0

ETR, etravirine.

Months infected, months since HIV seroconversion; ETR mutations: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S and M230L¹²; Other NNRTI mutations, major NNRTI-resistant mutations other than K103N and NNRTI-resistant mutations.

Mutations in bold are nonpolymorphic RT inhibitor resistance mutations. Underlined mutations are NNRTI-resistant mutations.

*Mutations with an asterisk are associated with etravirine resistance.

†Brackets indicate polymorphic mutations.

mutations had 4 silent minor variants identified only by UDPS in $\geq 8.5\%$ of sequence reads.

UDPS detected 1 or more NNRTI ($n = 12$) or NRTI ($n = 12$) resistance mutation in 14 of 20 samples: 10 samples had both NRTI-resistant and NNRTI-resistant mutations, 2 had NNRTI-resistant mutations alone, and 2 had NRTI-resistant mutations alone. Among the 12 samples with NNRTI-resistant mutations, 7 had a total of 11 major etravirine-resistant mutations including L100I ($n = 2$), K101E ($n = 2$), Y181C ($n = 2$), G190A/S ($n = 5$). Four samples had a total of 5 accessory etravirine-resistant mutations (V90I, V106I, V179D) including 2 of the 7 with major mutations. Ten of the 12 samples had a total of 14 nonetravirine-resistant mutations including V108I ($n = 6$), P225H ($n = 3$), K101N ($n = 1$), Y188C ($n = 1$), Y188F ($n = 1$), H221Y ($n = 1$), P236L ($n = 1$).

In conclusion, significantly more patients with acquired K103N than with transmitted K103N were infected with viruses containing nonpolymorphic etravirine-resistant mutations (7 of 20 vs. 0 of 13; $P = 0.03$; Fisher exact test). Furthermore, the overall number of nonpolymorphic etravirine-resistant mutations were significantly higher among those

with acquired vs. transmitted K103N (13 vs. 0 mutations; $P = 0.02$; Wilcoxon rank sum test). However, there was no significant difference in the number of polymorphic etravirine-resistant mutations (V90I, V106I, and V179D) between those with acquired and transmitted K103N (4 of 20 vs. 2 of 13; $P = NS$).

DISCUSSION

ARV-resistant viruses cause approximately 12%–15% of new HIV-1 infections in the United States.^{5,16,17} Although the prevalence of transmitted resistance does not seem to have changed much in the past 10 years, the proportion of transmitted viruses with NNRTI resistance has increased.^{18,19} Several investigators have shown that minority NNRTI-resistant variants may decrease the virological response to both initial and salvage NNRTI-containing regimens.^{20–24}

This study shows that ARV-naive HIV-1-infected patients in whom SGRT detects the NNRTI-resistant mutation, K103N, are generally not coinfecting with minority variants containing other major NNRTI-resistant mutations such as those that confer etravirine resistance. In contrast, in NNRTI-experienced

TABLE 2. Clinical Characteristics and UDPS Results in 20 NNRTI-Treated Patients With Plasma Samples Containing the RT Mutation K103N

PID	NNRTI	CD4 Count, Cells/μL	HIV-1 RNA, Log Copies/mL	Mutations Detected by Direct PCR Sequencing	Mutations Detected Only by UDPS	ETR Mutations†	Other NNRTI Mutations
6584	EFV	464	5.7	103NK , 118IV, 123E, 165IT, 174E, 214L	90I*(8.2), 101E*(3.8) , 101N(2.4) , 104N(3.1), 179D*(3.4), 188C(1.5) , 189I(2.3), 190A*(4.9)	2 (2)	2
6455	EFV	463	4.5	74LV , 101R, 102Q, 103N , 135V, 142V, 162A, 174H, 184V , 211K, 221HY , 234I/L	20R(13), 65R(9.7) , 67N(2.4) , 75I(14) , 100I*(32) , 108I(4.8) , 190A*(5.4) , 219N(2.7) , 224V(5.8), 225H(11.2) , 228R(5.0)	2	2
1872	NVP	239	4.5	20R, 41L , 42AE, 43KN, 64KR, 103N , 122E, 169D, 179I, 184MV , 215Y	36D(18), 181C*(3.5) , 190A*(3.2) , 196E(6.1), 210W(6.3) , 237N(5.9)	2	0
26423	EFV	27	5.7	35I, 75LV, 103N , 121Y, 122E, 135T, 176S, 200E, 211K, 225HP	62V(1.4), 69N(1.1), 74V(8.5) , 101E*(4.0) , 106I*(1.0), 190S*(4.8) , H221Y(1.1)	2 (1)	1
1838	DLV	91	4.9	20R, 35MI, 67N , 102Q, 103N , 122E, 162C, 184V , 200A, 203K, 207E, 210W , 211K, 215Y , 223E	6K(8.6), 53K(2.0), 74V(12) , 83K(58), 108I(28) , 111I(3.5), 118I(73), 181C*(7.0) , 219E(7.9) , 236L(1.2)	1	2
5248	EFV	120	5.3	103N , 122E, 184V , 207E, 225H	6K(2.7), 74V(13) , 100I*(14) , 108I(2.3) , 219E(1.1)	1	1
8350	EFV	261	4.5	41L, 101KQ, 103N , 108IV , 122E, 135T, 142V, 166R, 207E, 215Y	184V(1.2) , 190S*(3.1) , T200A(8.5), 225H(1.2)	1	1
4817	NVP	445	4.5	28A, 83K, 103N , 135T, 184V , 200A, 211K	4T(7.6), 67N(2.3) , 90I*(1.9), 142T(3.3), 171Y(20), 188F(5.0)	(1)	1
8077	NVP	64	5	68G, 103N , 122E, 162C, 165L, 200AITV, 204DE, 207E	47V(4.7), 90I*(10), 102E(4.4), 184V(0.8) , 207G(4.0)	(1)	0
16347	EFV	215	4.5	11KR, 60I, 101Q, 103N , 122E, 135T, 178M, 184V , 207E, 211K	20R(2.8), 21I(4.8), 43R(5.9), 108I(3.5) , 207D(13), 225H(4.5)	0	2
9651	NVP	335	4.5	35T, 39A, 67N , 70R, 74IL , 98G , 103N , 122E, 135L, 142IV, 184V , 215FIST , 218DE, 219Q	6K(5.3), 60I(29), 64R(3.8), 69N(4.6) , 108I(7.2) , 196E(3.3), 228H(4.0)	0	1
9666	EFV	87	4.6	41L , 75A, 103N , 123E, 135L/M, 162A, 177E, 211K, 215F	7I(35), 108I(31) , 178L(24), 184V(30)	0	1
3901	NVP	82	5.7	35T, 103N	41L(4.2) , 122E(19), 135T(15), 184V(4.8) , 210W(1.7) , 215Y(7.4)	0	0
5452	NVP	227	5	62VA, 103N , 122A, 123E, 135T, 177E, 184I , 197K, 202V, 211K	142V(3.4), 184V(7.7)	0	0
1613	EFV	41	4.8	102KR, 103N , 162C, 184V , 194AES, 200A, 215Y	10I(5.8), 11R(12)	0	0
3956	EFV	161	5.1	35T, 102Q, 103N , 122E, 162C, 173Q, 200A, 211K	6K (2.1), 27A (6.8), 39A (2.8), 60I (6.5), 169G (11), 177G (2.0)	0	0
7935	NVP	85	4.5	49R, 67N, 70R, 103N , 122E, 123N, 135T, 169D, 197P, 200I, 202V, 211K	9T(4.8), 11R (6.5), 39I (9.2)	0	0
8428	NVP	30	5.3	20R, 35LV, 103N , 122E, 207E, 225H	32T(7.1), 200S(7.9)	0	0
9650	EFV	247	5.7	41L , 98S, 103N , 135T, 162C, 197E, 211K, 215D	60I (10)	0	0
9905	EFV	322	4.5	20R, 102RK, 103N , 123E, 135T, 196E		0	0

ETR, efavirenz.

NNRTI, at sampling time point; ETR mutations: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S, and M230L¹²; Other NNRTI mutations are major NNRTI-resistant mutations other than K103N and NNRTI-resistant mutations.

Mutations in bold are nonpolymorphic RT inhibitor resistance mutations. Underlined mutations are NNRTI-resistant mutations.

*Mutations with an asterisk are associated with efavirenz resistance.

†Brackets indicate polymorphic mutations.

patients in whom SGRT detects only K103N, UDPS often detects additional major NNRTI-resistant mutations.

Consistent with other studies, we found that K103N—particularly K103N alone—was the most common pattern of transmitted genotypic NNRTI resistance, occurring in approximately 3.0% of all new infections. The relative absence of minority drug-resistant variants in ARV-naive

patients with K103N is consistent with the transmission bottleneck that occurs at the time of initial HIV-1 infection²⁵ and the absence of ongoing selective drug pressure after virus transmission. Nonetheless, minority variants were occasionally present in those with transmitted K103N. For example, PID 8048 had the NNRTI-resistant mutation K65R and the accessory NNRTI-resistant mutation P225H, and PID 30062

contained the NNRTI-resistant mutations M184V, L210W, T215Y, and K219Q.

The presence of multiple additional NNRTI-resistant mutations in patients with acquired K103N is consistent with the low genetic barrier to resistance associated with the first-generation NNRTIs. Indeed, patients developing virological failure although receiving an NNRTI often develop multiple independent virus lineages containing different NNRTI-resistant mutations that may not all be detectable by direct PCR sequencing.^{9,26,27} For example, in patients developing the nevirapine-resistant mutation Y181C, salvage therapy with efavirenz (which retains high levels of activity against viruses with Y181C) is often ineffective because the virus quasispecies in such patients often contains multiple other NNRTI-resistant mutations (such as K103N) at low levels that may not be detectable by SGRT.^{21,28,29}

We performed UDPS only on samples with plasma HIV-1 RNA levels ≥ 4.5 log copies per milliliter to ensure that ≥ 100 viral cDNA templates could be recovered from cryopreserved plasma despite the low efficiency of the recovery process.¹⁴ This restriction probably did not influence our findings in ARV-naïve patients because 60% had plasma HIV-1 RNA levels ≥ 4.5 log copies per milliliter. However, this restriction may have influenced our findings in NNRTI-experiencing patients because only 20% had plasma HIV-1 RNA ≥ 4.5 log copies per milliliter. The lower RNA levels among NNRTI-treated patients may be a consequence of the ongoing activity of other drugs in the patients' regimens and possibly the incomplete phenotypic resistance associated with K103N alone (at least to efavirenz). Whether NNRTI-treated patients with lower plasma HIV-1 RNA levels would be expected to have fewer or more NNRTI-resistant variants is not known.

The finding that etravirine-resistant minority variants were common in NNRTI-experienced patients receiving etravirine is consistent with the results of the recent clinical trial TMC125-C227 in which patients developing virological failure although receiving an NNRTI-containing regimen were less likely to respond to a salvage regimen containing etravirine than to a salvage regimen containing a ritonavir-boosted PI.³⁰ Patients in TMC125-227 whose viral sequences had K103N generally responded to therapy with etravirine plus 2 NRTIs. However, the number of patients in that study were insufficient to recommend the use of etravirine rather than a ritonavir-boosted PI in patients with K103N.

Based on previous studies, minority NNRTI-resistant variants present at levels of $\geq 2.0\%$ seem to be significantly associated with virological failure of an initial nevirapine-containing or efavirenz-containing ARV regimen.^{23,24} However, the impact of such minority variants on etravirine therapy has not been studied. Although current recommendations support using a boosted PI for treating patients with transmitted NNRTI resistance,^{1,31} our study suggests that further investigation of etravirine for the treatment of patients primarily infected with a K103N-containing virus is warranted.

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