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The K101P and K103R/V179D Mutations in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confer Resistance to Nonnucleoside Reverse Transcriptase Inhibitors

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Genotypic patterns associated with nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance in the absence of well-characterized resistance mutations were identified using a database ($n > 47{,}000$) of phenotype-genotype data. Among samples with no known NNRTI mutations, the most resistant samples contained K101P ($n = 35$) or a combination of K103R and V179D ($n = 41$). Site-directed mutagenesis confirmed the importance of these mutations.

Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) of human immunodeficiency virus type 1 (HIV-1) are an important component of highly active antiretroviral therapy regimens (14). Resistance to NNRTIs is common in virus from treated patients and is typically accompanied by the development of one or more recognized mutations in the target enzyme. These mutations, derived from a combination of in vitro and in vivo data, form the basis for predicting NNRTI resistance in genotype-based resistance assays (13).

The availability of large databases of matched protease-RT genotype and drug susceptibility data, as well as the increasing clinical use of combined phenotype-genotype resistance assays, enables more thorough and comprehensive analyses of concordance between the interpretation of both types of assays (6, 8, 12). While concordance rates are high for NNRTIs, infrequent cases of reduction in phenotypic susceptibility not explained by known mutations can be dramatic (10-fold to more than 100-fold change in 50% inhibitory concentration [IC_{50}]), in a range that is clinically significant. We investigated the underlying causes for such discordance in a large database of genotype and phenotype resistance assay results from clinical samples submitted to the Monogram Biosciences (formerly ViroLogic) Clinical Reference Laboratory for routine testing.

As of April 2005, the Monogram Biosciences phenotype-genotype database contained data for more than 47,000 individual clinical samples, 24,151 of which lacked any commonly recognized NNRTI resistance mutations (A98G, K101E, K103N/S, V106A/M, P225H, M230L, P236L, or any substitution at position L100, Y181, Y188, G190, or F227). There were 577 samples that displayed a fold change in IC_{50} (FC) for at least one NNRTI of fivefold or greater, and 196 had an FC of 10-fold or greater. Several samples had IC_{50}s above the highest concentration tested in the assay: 28 samples for nevirapine (NVP), 10 samples for delavirdine (DLV), and 1 sample for efavirenz (EFV). For statistical purposes, these samples were assigned FC values of 400-, 250-, and 700-fold for NVP, DLV, and EFV, respectively. These maximum FC values are derived from the ratio of the highest drug concentration tested to the distribution of IC_{50}s for the drug-sensitive reference virus over a 1-year time period.

To explore the genotypic correlates of this unexplained NNRTI resistance, we performed univariate analysis, testing for the association of all mutations in RT which occur in more than 1% of the samples with an elevated NNRTI FC. We used an arbitrary threshold of fivefold, which is higher than the biological cutoff for EFV and NVP (9), since clinical cutoffs for NNRTIs have not been determined. For each specific mutation, the number of samples containing or lacking the mutation with an FC of >5 for each NNRTI was compared to the number of samples with an FC of <5 using the Fisher exact test. The Benjamini adjustment for multiple comparisons (1) was used (variables with adjusted P values of <0.05 were considered significant). The mutations with the strongest association with NNRTI resistance (odds ratio of >5 for at least one NNRTI) are listed in Table 1. For EFV, the I31L, K101H/P, K103R, V179D/E, and K238T mutations were most strongly associated with an FC of >5 (odds ratio, >10) (see Table 1). K101P had the highest odds ratio for all three NNRTIs.

To attempt to distinguish between mutations primarily responsible for the resistance phenotype and those that are linked to them, regression tree analysis using CART 5.0 software (Salford Systems, San Diego, CA) was performed. This approach, also known as binary recursive partitioning, identifies "predictor" variables (the presence or absence of mutations in RT) that best separate the population into two groups with respect to the target variable (NNRTI FC). Each group thus defined (a "node") is successively divided further until additional separation does not improve the overall predictive value of the model. This analysis identified K101P and the combination of K103R and V179D as strong predictors of high EFV FC (Fig. 1). The presence of L74V or the absence of V35I, in addition to K101P, was associated with further reductions in susceptibility, although the number of samples in these nodes of the tree was small. Similarly, the K103R V179D R277K combination was associated with additional reductions

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TABLE 1. Mutations associated with reduced susceptibility to NNRTIs[a]

Mutation (n^b)	Drug											
	NVP				DLV				EFV			
	% FC < 5[c]	% FC > 5[d]	OR[e]	*P* value[f]	% FC < 5	% FC > 5	OR	*P* value	% FC < 5	% FC > 5	OR	*P* value
I31L (50)	0.2	2.9	16.9	**<0.0001**	0.2	1.8	10.8	**<0.0001**	0.2	3.8	20.4	**<0.0001**
K64N (97)	0.4	1.9	5.1	**0.0082**	0.4	0.6	1.5	0.6854	0.4	1.3	3.2	0.3369
L74I (285)	1.1	5.2	4.6	**<0.0001**	1.2	2.3	1.9	0.1299	1.1	6.3	5.5	**0.0003**
V75I (171)	0.7	1.9	2.8	0.0657	0.7	1.0	1.4	0.6325	0.7	3.8	5.5	**0.0084**
K101P (40)	0.02	11.7	694	**<0.0001**	0.03	6.8	227	**<0.0001**	0.02	22.5	1349	**<0.0001**
K101Q (344)	1.3	10.0	7.6	**<0.0001**	1.4	4.9	3.6	**<0.0001**	1.4	5.6	4.0	**0.0054**
K101H (43)	0.1	3.6	26.5	**<0.0001**	0.1	3.5	31.4	**<0.0001**	0.2	1.9	11.2	**0.0194**
K103R (562)	2.1	17.2	8.0	**<0.0001**	2.1	11.9	5.6	**<0.0001**	2.1	32.5	15.3	**<0.0001**
V108I (324)	1.2	10.7	8.7	**<0.0001**	1.3	4.3	3.3	**<0.0001**	1.3	7.5	5.8	**<0.0001**
D123K (146)	0.6	1.9	3.3	**0.0388**	0.6	1.8	3.2	**0.0128**	0.6	3.1	5.3	**0.0189**
E138A (602)	2.5	5.2	2.1	**0.0221**	2.2	16.4	7.5	**<0.0001**	2.5	2.5	1.0	1.1412
E138K (57)	0.2	0.0	0.0	1.0601	0.2	1.4	6.9	**0.0009**	0.2	0.0	0.0	1.1345
E138G (79)	0.3	1.6	5.2	**0.0152**	0.3	1.6	5.4	**0.0013**	0.3	1.9	5.9	0.0645
T139R (55)	0.2	1.3	6.1	**0.0217**	0.2	1.6	8.2	**0.0001**	0.2	0.6	2.8	0.5773
T165L (61)	0.2	2.3	10.0	**0.0001**	0.2	1.2	5.3	**0.0068**	0.2	1.9	7.8	**0.0396**
V179D (350)	1.2	19.7	16.3	**<0.0001**	1.0	24.6	25.5	**<0.0001**	1.2	45.6	39.5	**<0.0001**
V179E (117)	0.4	3.2	7.2	**<0.0001**	0.3	9.8	33.9	**<0.0001**	0.4	15.6	40.7	**<0.0001**
D218E (552)	2.2	8.4	3.8	**<0.0001**	2.2	4.7	2.1	**0.0059**	2.2	11.3	5.1	**<0.0001**
K219H (32)	0.1	0.6	5.1	0.1546	0.1	1.2	11.1	**0.0003**	0.1	0.6	4.8	0.4376
K219N (209)	0.8	3.2	3.9	**0.0025**	0.8	1.6	1.9	0.1742	0.8	4.4	5.2	**0.0051**
H221Y (172)	0.7	4.5	6.8	**<0.0001**	0.7	2.7	4.0	**0.0004**	0.7	5.6	8.3	**<0.0001**
K238T (59)	0.2	4.9	26.3	**<0.0001**	0.2	2.0	9.8	**<0.0001**	0.2	2.5	10.9	**0.0062**
E297P (80)	0.3	2.3	7.4	**0.0006**	0.3	1.2	3.9	**0.0223**	0.3	1.3	3.8	0.2659
E300A (37)	0.1	1.9	14.9	**<0.0001**	0.2	0.0	0.0	1.0052	0.2	0.0	0.0	1.0104

[a] Mutations with a *P* value of <0.05 and odds ratio of >5 for at least one NNRTI are listed.
[b] *n*, no. of samples.
[c] Percentage of NNRTI-sensitive samples (FC < 5.0) with mutation.
[d] Percentage of NNRTI-resistant samples (FC > 5.0) with mutation.
[e] Odds ratio (ratio of percent resistant with mutation to percent sensitive with mutation).
[f] Fisher's exact test with Benjamini correction; mixtures counted as mutant; bold if *P* value is <0.05.

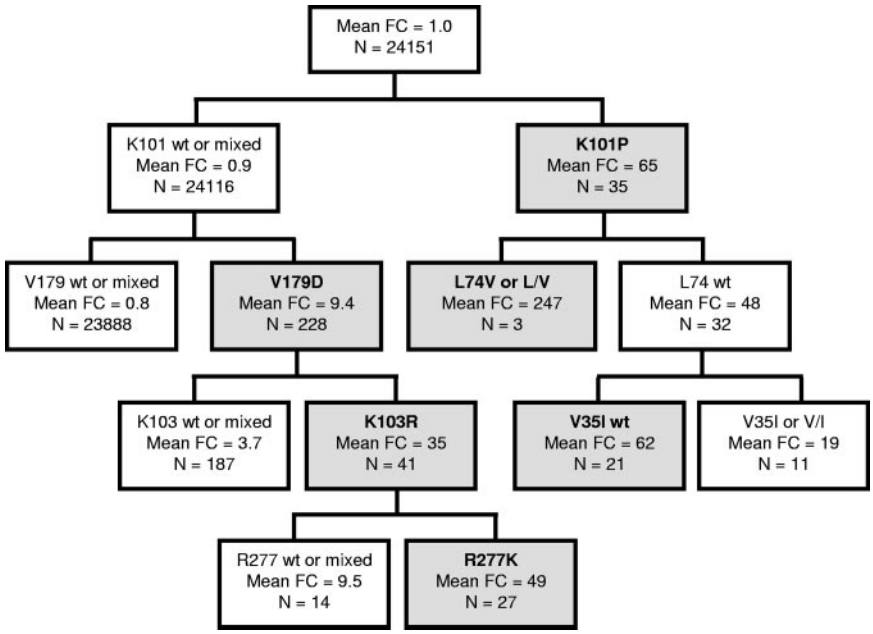

FIG. 1. Regression tree analysis for efavirenz (EFV). Fold change in IC_{50} (FC) for EFV was the target variable, and all individual mutations present unmixed in greater than 1% of samples with NNRTI resistance and Fisher's exact test *P* value of >0.1 (after adjustment for multiple comparisons) were considered potential predictor variables. Mutations were scored as 0 if absent and 1 if present; mixtures were scored as 0.5. Mutations in bold type and gray shading represent those associated with an increased EFV FC.

TABLE 2. NNRTI susceptibility in clinical samples containing K101P, K103R, V179D, or K103R plus V179D[a]

Mutation(s) (n^b)	NVP FC				DLV FC				EFV FC			
	Mean	Median	Range	% > 10[c]	Mean	Median	Range	% > 10	Mean	Median	Range	% > 10
K101P (35)	278	400	21.5–400	100	51	28	2.5–250	83	65	29	5.7–700	94
K103R only (298)	1.0	0.8	0.2–10	0.3	1.2	0.8	0.1–23	0.7	0.8	0.7	0.1–12	0.3
V179D only (171)	2.2	1.4	0.3–27	2.9	5.4	3.8	0.2–42	10	3.3	2.2	0.3–68	3.5
K103R + V179D (41)	72	16	1.2–400	71	67	27	2.1–250	80	35	16	0.9–196	73

[a] Clinical samples containing the indicated mutations (excluding mixtures) but lacking any known NNRTI resistance mutation (A98G, K101E, K103N/S, V106A/M, P225H, M230L, P236L, or any mutation at position L100, Y181, Y188, G190, or F227) were identified. Samples with IC_{50}s above the highest drug concentration tested in the assay were assigned a maximum FC value based on the ratio of the highest concentration tested to the lowest reference virus IC_{50} measured over a 1-year period. These values are 400-, 250-, and 700-fold for NVP, DLV, and EFV, respectively. Nucleotide sequences for the RT region of samples with K101P or K103R plus V179D have been submitted to GenBank (accession ID DQ224082-DQ224157).

[b] *n*, no. of samples.

[c] % > 10, percent samples for which the FC of the NNRTI was > 10.

in susceptibility. Results were similar for the other NNRTIs in that K101P and K103R plus V179D were found to strongly predict high-level resistance (data not shown).

The patterns of NNRTI resistance in the clinical samples containing K101P, K103R, V179D, or the combination of K103R and V179D are summarized in Table 2. Among 35 samples with K101P (excluding those with a mixture of K and P at position 101), mean FC values ranged from 51- to 278-fold, and 83 to 100% of samples had a FC of >10 for the various NNRTIs. The presence of K103R alone was not associated with significant changes in NNRTI susceptibility. Samples with the V179D mutation alone had a mean FC between 2.2- and 5.4-fold, with between 3 and 10% having an NNRTI with a FC of >10-fold. However, the combination of K103R and V179D (again, excluding mixtures at either of these positions) had a synergistic effect on NNRTI resistance, with a mean FC of 35- to 72-fold and 71 to 80% over 10-fold.

To confirm that these mutations were responsible for the NNRTI resistance observed in clinical samples, they were introduced into the NL4-3 reference virus using site-directed mutagenesis (11). Mutant vectors were sequenced to confirm the absence of unwanted mutations. The K101P mutation conferred high-level resistance to all three NNRTIs (mean FC, 26- to 171-fold [Table 3]). Results from the K103R and V179D single mutants closely mimicked those observed in clinical samples, and the combination of the two mutations conferred 16- to 27-fold resistance.

The K101P and K103R mutations identified above occur at positions where other well-known NNRTI resistance mutations have been reported (7, 10, 15). Other variant amino acids have also been observed at these positions, such as K103S, -T, -H, or -Q (3) and K101H or -Q (Table 1).

It is notable that some mutations usually thought to be responsible for resistance to nucleoside reverse transcriptase inhibitors (NRTIs) were found to be associated with unexplained NNRTI resistance. For example, L74I, V75I, and K219H/N were found to be significantly associated with an elevated FC for at least one NNRTI (Table 1). While this may reflect the likely linkage between NRTI and NNRTI resistance mutations in viruses exposed to combination treatments including both classes of reverse transcriptase inhibitors, it is also possible that they play a direct role in NNRTI resistance or in compensation of replication capacity defects related to the NNRTI resistance mutations. Such effects have been reported previously for mutations at positions 74 and 75 (2, 4, 5).

The treatment histories of the patients from which the samples used in this study were obtained are unknown. However, since all of the samples with K101P also had recognized nucleoside RT inhibitor resistance-associated mutations, and since it was not found in otherwise drug-sensitive (by genotype) samples, it is likely that this mutation is not a naturally occurring polymorphism. For K103R plus V179D, 5 of 41 samples lacked any NRTI or protease inhibitor resistance mutations; individually either mutation is commonly found in samples without any other resistance-associated mutation. Thus, it is formally possible that the combination occurs naturally in the absence of drug selection, as a consequence of drug pressure, or both. Additional clinical cohort data will be required to clarify this issue.

The results presented here demonstrate that two mutational patterns, an isolated K101P mutation ($n = 35$) or the combination of K103R and V179D ($n = 41$), confer large reductions in susceptibility to all three approved NNRTIs. Current genotype interpretation algorithms do not account for these two patterns and should be updated to reflect these data. Additional mutational patterns not including these mutations are also likely to be uncovered in future analyses, since only approximately 19% of the samples with at least one NNRTI FC of >5 have one of the two patterns. These rare samples continue to be reliably assessed only by the use of phenotypic resistance assays.

TABLE 3. Site-directed mutagenesis results

Mutation (s)	Mean IC_{50}(nM) ± SD (FC)[a]		
	NVP	DLV	EFV
None (NL4−3 WT[b])	93 ± 8.3 (1.0)	33 ± 3.9 (1.0)	1.8 ± 0.13 (1.0)
K101P	16,597 ± 3413 (171)	1,344 ± 348 (47)	50 ± 5.6 (26)
K103R	101 ± 6.8 (1.1)	33 ± 9.6 (1.0)	2.3 ± 0.029 (1.3)
V179D	198 ± 23 (2.1)	129 ± 33 (3.9)	6.2 ± 0.013 (3.4)
K103R + V179D	1,488 ± 442 (16)	921 ± 266 (27)	32 ± 6.1 (19)

[a] Values are averages for two (K101P, K103R, V179D) to four (NL4−3, K103R + V179D) experiments.

[b] WT, wild type.

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