JOURNAL OF VIROLOGY, July 2006, p. 7186–7198 Vol. 80, No. 14
0022-538X/06/$08.00+0 doi:10.1128/JVI.02084-05
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Involvement of Novel Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mutations in the Regulation of Resistance to Nucleoside Inhibitors

Valentina Svicher,[1]† Tobias Sing,[2]† Maria Mercedes Santoro,[1] Federica Forbici,[3] Fátima Rodríguez-Barrios,[4] Ada Bertoli,[1] Niko Beerenwinkel,[2]‡ Maria Concetta Bellocchi,[3] Federigo Gago,[4] Antonella d'Arminio Monforte,[5]§ Andrea Antinori,[3] Thomas Lengauer,[2] Francesca Ceccherini-Silberstein,[1]* and Carlo Federico Perno[1,3]

Department of Experimental Medicine, University of Rome Tor Vergata, Rome,[1] National Institute for Infectious Diseases L. Spallanzani, Rome,[3] and Institute of Infectious and Tropical Diseases, University of Milan, Milan,[5] Italy; Max Planck Institute for Informatics, Saarbrücken, Germany[2]; and Department of Pharmacology, University of Alcalá, Alcalá de Henares, Spain[4]

Received 4 October 2005/Accepted 14 April 2006

We characterized 16 additional mutations in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) whose role in drug resistance is still unknown by analyzing 1,906 plasma-derived HIV-1 subtype B *pol* sequences from 551 drug-naïve patients and 1,355 nucleoside RT inhibitor (NRTI)-treated patients. Twelve mutations positively associated with NRTI treatment strongly correlated both in pairs and in clusters with known NRTI resistance mutations on divergent evolutionary pathways. In particular, T39A, K43E/Q, K122E, E203K, and H208Y clustered with the nucleoside analogue mutation 1 cluster (NAM1; M41L+L210W+T215Y). Their copresence in this cluster was associated with an increase in thymidine analogue resistance. Moreover, treatment failure in the presence of K43E, K122E, or H208Y was significantly associated with higher viremia and lower CD4 cell count. Differently, D218E clustered with the NAM2 pathway (D67N+K70R+K219Q+T215F), and its presence in this cluster determined an increase in zidovudine resistance. In contrast, three mutations (V35I, I50V, and R83K) negatively associated with NRTI treatment showed negative correlations with NRTI resistance mutations and were associated with increased susceptibility to specific NRTIs. In particular, I50V negatively correlated with the lamivudine-selected mutation M184V and was associated with a decrease in M184V/lamivudine resistance, whereas R83K negatively correlated with both NAM1 and NAM2 clusters and was associated with a decrease in thymidine analogue resistance. Finally, the association pattern of the F214L polymorphism revealed its propensity for the NAM2 pathway and its strong negative association with the NAM1 pathway. Our study provides evidence of novel RT mutational patterns that regulate positively and/or negatively NRTI resistance and strongly suggests that other mutations beyond those currently known to confer resistance should be considered for improved prediction of clinical response to antiretroviral drugs.

During its spread among humans, human immunodeficiency virus type 1 (HIV-1) has developed an extraordinary degree of genetic diversity, mainly due to the intrinsic inability of HIV-1 reverse transcriptase (RT) to carry out proofreading of DNA during replication (21) and exacerbated by the high rate of viral replication in vivo ($\approx 10^9$ viral particles produced daily). Among the different areas of the viral genome, the *pol* gene, encoding enzymes such as reverse transcriptase and protease, is subjected not only to natural evolutionary forces but also to selective pressure imposed by pharmacological treatment (9, 10, 16, 36).

The HIV-1 reverse transcriptase enzyme is responsible for the conversion of the single-stranded RNA genome into a double-stranded DNA that is later integrated into host genomic DNA (14, 43). Owing to its pivotal role in the HIV-1 life cycle, the reverse transcriptase represents an attractive target for antiviral therapy. To date, 11 out of 21 compounds approved for the treatment of HIV-1 infection are reverse transcriptase inhibitors. In particular, they consist of the group of seven nucleoside analogue reverse transcriptase inhibitors (NRTIs; zidovudine, stavudine, lamivudine, didanosine, abacavir, zalcitabine, and emtricitabine), one acyclic nucleoside monophosphate (tenofovir, generally considered in the class of NRTIs), and three nonnucleoside analogue reverse transcriptase inhibitors (NNRTIs; nevirapine, efavirenz, and delavirdine) (2, 9, 11, 28).

When antiviral therapy fails to be fully suppressive, new viral variants can emerge, allowing HIV-1 to escape from reverse transcriptase inhibitors by accumulating mutations, either alone or in multiple clusters, that affect the long-term therapy targeting reverse transcriptase (3, 13, 20, 35). To date, two mechanisms are known to contribute to decreased NRTI susceptibility (9, 26). Several mutations or groups of mutations in HIV-1 reverse transcriptase can promote resistance by selectively impairing the ability of the enzyme to incorporate the nucleoside analogue into DNA. These mutations include M184V, K65R, and L74V and the Q151M complex (A62V, V75I, F77L, F116Y, and Q151M) (33, 36, 42). On the other hand, a specific set of mutations collectively termed "nucleo-

* Corresponding author. Mailing address: Department of Experimental Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy. Phone: 39-06-72596553. Fax: 39-06-72596039. E-mail: ceccherini@med.uniroma2.it.
† V.S. and T.S. contributed equally to this work.
‡ Present address: University of California, Berkeley, Calif.
§ For the ICONA study group.

Downloaded from jvi.asm.org by on December 4, 2009

side analogue mutations" (NAMs; M41L, D67N, K70R, L210W, T215Y, and K219E/Q) can confer resistance by promoting a phosphorolysis reaction that selectively removes the nucleoside analogue from the terminated DNA chain (1, 22, 24). The NAMs occur gradually under the selection pressure imposed by the thymidine analogues (zidovudine and stavudine) and can promote resistance to almost all nucleoside and nucleotide analogues. Recent studies have suggested the existence of two distinct pathways of NAM resistance, defined by different mutation patterns (NAMs 1 [M41L, L210W, and T215Y] and NAMs 2 [D67N, K70R, and K219Q/E]), whose evolution seems to be strictly influenced by viral replication (15, 44). On the other hand, resistance to NNRTIs is mediated by the appearance of mutations at the hydrophobic NNRTI binding pocket that reduce the affinity of the inhibitor for the enzyme (9, 36).

To date, mutations at 61 residues in HIV-1 reverse transcriptase have been related to treatment with the experimentally tested reverse transcriptase inhibitors. Of these, 18 sites are involved in resistance to the eight currently approved NRTIs and 16 sites are involved in resistance to the three currently approved NNRTIs (20; Stanford HIV Drug Resistance Database, http://hivdb.stanford.edu).

Since resistance to reverse transcriptase inhibitors is a very complex phenomenon, it is conceivable that more mutations (and associations of mutations) than currently known are involved in the development of drug resistance and therefore lead to therapeutic failure. For instance, recent studies have identified novel positions or mutations positively associated with NRTI treatment (7, 8, 13); however, their exact role in the development of NRTI resistance remains unclear. Thus, we focused our attention on 16 uncharacterized mutations in HIV-1 reverse transcriptase and used computational and statistical methods to define their association with specific NRTIs and NRTI resistance mutations at therapeutic failure. A better definition of the mutational pathways that regulate the evolution of drug resistance in vivo is a key element for the design of effective anti-HIV chemotherapy. Moreover, our study underlines the importance of computational methods as important tools for the interpretation of HIV genetic variability and suggests that additional mutations beyond those currently known to be associated with NRTI resistance should be considered to define precise algorithms able to predict resistance to antiretroviral drugs.

MATERIALS AND METHODS

Patients. The study included 1,906 patients enrolled either in the Italian Cohort of Antiretroviral-Naïve (ICONA) patient study or in different clinical centers in central Italy. Five hundred fifty-one patients were naïve for treatment with antiretroviral drugs, and 1,355 were failing their last antiretroviral regimen containing at least one NRTI (865 were failing NRTIs but not NNRTIs, and 490 were failing NRTIs plus NNRTIs). Drug-naïve patients underwent genotypic tests between January 1997 and July 2004, while NRTI-treated patients were tested between January 1998 and July 2004. Overall, drug-treated patients were exposed to an average of four NRTIs and 1,271 (93.8%) were receiving highly active antiretroviral therapy. In detail, 84.2% of patients had been treated with lamivudine, 73.6% with zidovudine, 70.7% with stavudine, 60.0% with didanosine, 21.7% with abacavir, 15.3% with zalcitabine, and 10.5% with tenofovir. At the time of genotypic analysis, 64.9% of patients were receiving treatment with lamivudine (with a median time of 450 days), 48.1% with stavudine (median time, 492 days), 34.5% with zidovudine (median time, 436 days), 33.2% with didanosine (median time, 393 days), 12.5% with abacavir (median time, 363 days), 7.2% with tenofovir (median time, 221 days), and 2.7% with zalcitabine (median time, 359 days). Patients carrying non-B subtypes of HIV *pol* were excluded from this analysis. Data for all patients were stored in a specifically designed anonymous database that included mutational, demographic, immunologic, virologic, and therapeutic parameters.

HIV sequencing. HIV genotype analysis was performed on plasma samples by means of a commercially available kit (ViroSeq HIV-1 genotyping system; Abbott Laboratories) (6, 29). Briefly, RNA was extracted, retrotranscribed by murine leukemia virus RT, and amplified with Amplitaq-Gold polymerase enzyme by using two different sequence-specific primers for 40 cycles. Pol-amplified products (containing the entire protease and the first 335 amino acids of the reverse transcriptase open reading frame) were full-length sequenced in sense and antisense orientations by an automated sequencer (ABI 3100) by using seven different overlapping sequence-specific primers (6, 29). Sequences having a mixture of wild-type and mutant residues at single positions were considered to have the mutant(s) at that position. The isolates were subtyped by comparing them to reference sequences of known subtype (http://hivdb.stanford.edu). The majority of nucleotide sequences from drug-naïve and NRTI-treated patients treated with at least one protease inhibitor within the highly active antiretroviral therapy regimen have already been submitted to GenBank (6, 29, 41), and more recently 622 sequences from NRTI-treated and drug-naïve patients have been submitted (see below).

Statistical analysis. (i) Mutation prevalence. To assess the association of reverse transcriptase mutations with NRTI treatment, we calculated their respective frequencies in isolates from 551 drug-naïve patients, 865 patients failing their last antiretroviral regimen containing NRTIs but not NNRTIs, and 490 patients failing their last antiretroviral regimen containing NRTIs plus NNRTIs. We then performed chi-squared tests of independence (based on a 2×2 contingency table) to verify statistically significant differences in frequency between the following groups of patients: (i) drug-naïve patients versus NRTI- but not NNRTI-treated patients, (ii) drug-naïve patients versus NRTI-plus-NNRTI-treated patients, and (iii) NRTI- but not NNRTI-treated patients versus NRTI-plus-NNRTI-treated patients.

We focused our attention on 16 mutations (K20R, V35I, V35M, T39A, K43E, K43N, K43Q, I50V, R83K, K122E, G196E, E203D, E203K, H208Y, F214L, and D218E), to which we refer as novel mutations henceforth, as they have not yet been reported to be associated with NRTI resistance by the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) and by the International AIDS Society (20).

To assess the association of each novel mutation with treatment with a specific NRTI, we compared the rate of occurrence of each novel mutation in the subpopulation that underwent treatment with a specific NRTI with that in the subpopulation that did not undergo treatment with that NRTI; we then performed chi-squared tests of independence to verify statistically significant differences ($P < 0.05$).

In our analysis, the Cochran rule, which is a conventional criterion for the chi-squared test to be valid, was fully satisfied. In fact, in each contingency table performed with our data set, 80% of the expected frequencies exceed 5, and all the expected frequencies exceed 1. In addition, in those few cases where the expected frequency in a single cell of the contingency table was less than 5, the significance was also confirmed by using the Monte Carlo significance test procedure (17).

We used the Benjamini-Hochberg method (5) to identify results that were statistically significant in the presence of multiple-hypothesis testing. A false discovery rate of 0.05 was used to determine statistical significance. This analysis was performed on the subset of 865 patients failing an antiretroviral regimen containing NRTIs but not NNRTIs.

To assess the association of novel reverse transcriptase mutations with viremia and CD4 cell count, we compared viremia and CD4 cell count values of patients harboring isolates with a specific novel mutation with those of patients harboring isolates without that novel mutation. Viremia and CD4 cell count values analyzed were concomitant with the genotype resistance testing ($\pm$30 days).

To verify statistically significant differences, the median test was performed, and the Benjamini-Hochberg method was used to correct for multiple-hypothesis testing. A false discovery rate of 0.05 was used to determine statistical significance.

(ii) Mutation covariation. In the set of 1,355 NRTI-treated patients, we exhaustively analyzed patterns of pairwise interactions among mutations associated with NRTI treatment, including the 16 novel mutations. Specifically, for each pair of mutations and corresponding wild-type residues, Fisher's exact test was performed to assess whether co-occurrence of the mutated residues differed significantly from what would be expected under an independence assumption. Again, the Benjamini-Hochberg method was used to correct for multiple testing, here at a false discovery rate of 0.01. Samples having a mixture of two or more

Downloaded from jvi.asm.org by on December 4, 2009

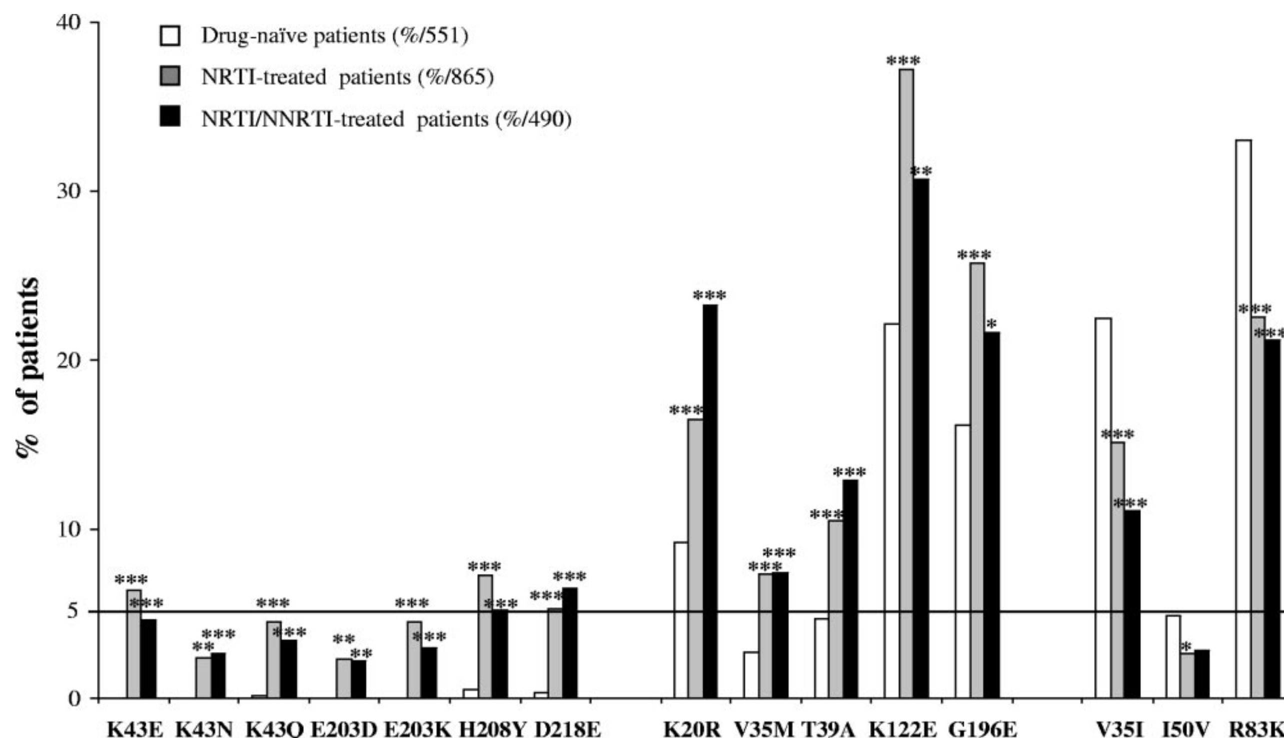

FIG. 1. Frequency of novel reverse transcriptase mutations in isolates from drug-naïve patients and in isolates from patients failing their last antiretroviral regimen containing an NRTI but not an NNRTI and from patients failing their last antiretroviral regimen containing an NRTI plus an NNRTI. Statistically significant differences were assessed by chi-squared tests of independence. P values were significant at a false discovery rate of 0.05 following correction for multiple comparison. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mutations at a given pair of positions were ignored in calculating the covariation, since it is not possible to identify whether these mutations are indeed located in the same viral genome.

(iii) Cluster analysis. To analyze the covariation structure of mutations in more detail, we performed average linkage hierarchical agglomerative clustering, as described elsewhere (38).

Hierarchical clustering methods, which under different names are also widely used in phylogenetic tree building, rely on a matrix of pairwise dissimilarities between entities, based on which groups are associated into hierarchical clusters of decreasingly strong association. As such, it is in the first instance an explorative and not a predictive tool. Briefly, in average linkage clustering, clusters of increasing size are formed starting from one-element groups by iteratively joining two clusters with minimum average intercluster distances between pairs of mutations. The distance between a pair of mutations was derived from the phi correlation coefficient, which is a measure of the association between two binary random variables, with 1 and −1 representing maximal positive and negative association, respectively. This similarity measure was transformed into a distance by mapping phi = 1 to distance 0 and phi = −1 to distance 1, with linear interpolation in between. The distance between different mutations at a single position was left undefined, as such pairs never co-occur in a single sequence (except from mixtures) and would lead to distorted dendrograms owing to their great distance. The resulting partial distance matrix was then used as input for the clustering algorithm, ignoring undefined distances in computing averages. To assess the stability of the resulting dendrogram, confidence values for all subtrees in the dendrogram were computed by 100 replications of the clustering procedure on sequence sets bootstrapped from the original 1,355 sequences (38). For instance, a bootstrap value of 1 simply means that out of 100 runs, all 100 had these two mutations (or groups of mutations) most closely linked.

Association with NRTI susceptibility. We analyzed genotype-phenotype correlations from the HIV Stanford Drug Resistance Database (http://hivdb.stanford.edu) to assess the association of mutations with NRTI susceptibility. In particular, for each NRTI we compared the median changes in resistance (n-fold) in relation to sequences with or without specific novel mutations. The change in resistance (n-fold) was measured by Virco's Antivirogram assay.

Structural analysis. The X-ray crystallographic coordinates of HIV-1 reverse transcriptase deposited in the Protein Data Bank (http://www.rcsb.org/PDB/) with code 1rtd were used for the structural analysis. The feasibility of direct side chain-side chain interaction between the mutated amino acids (both intrasubunit and intersubunit) was assessed by means of molecular graphic visualization using the public domain program PyMol (http://pymol.sourceforge.net/).

Nucleotide sequence accession numbers. The GenBank accession numbers for 622 sequences of NRTI-treated and drug-naïve patients are as follows: DQ345123 to DQ345278, DQ347967 to DQ348070, DQ369047 to DQ369180, DQ369219 to DQ369262, and DQ370181 to DQ370306.

RESULTS

Novel reverse transcriptase mutations and their association with treatment. By evaluating the first 335 amino acids in reverse transcriptase sequences derived from 551 drug-naïve and 1,355 NRTI-treated patients (865 failing their last antiretroviral regimen containing NRTIs but not NNRTIs and 490 failing their last antiretroviral regimen containing NRTIs plus NNRTIs), we identified and characterized 16 novel reverse transcriptase mutations.

Fifteen novel mutations were significantly associated with treatment with NRTIs, based on the assumption that these mutations occurred with different frequencies in treatment-naïve subjects and in patients failing NRTI therapy, respectively (38; F. Ceccherini-Silberstein, V. Svicher, T. Sing, M. Santoro, N. Beerenwinkel, F. Gago, A. Bertoli, F. Forbici, M. C. Bellocchi, P. Narciso, A. d'Arminio Monforte, A. Antinori, and C. F. Perno, Abstr. 14th Int. HIV Drug Resist.

Downloaded from jvi.asm.org by on December 4, 2009

TABLE 1. Association of novel reverse transcriptase mutations with NRTI treatment

Novel mutation	Frequency (%) of mutations			Association(s) with NRTIs[b]
	Naïve patients (n = 551)	NRTI-experienced patients[a]		
		3 or fewer (n = 448)	4 or more (n = 417)	
K43E	0	**4.7**	**8.4**	ddC (P = 0.011)
K43N	0	**1.3**	**3.6**	
K43Q	0.2	**2.7**	**6.5***	
E203D	0	**1.6**	**3.1**	
E203K	0	**3.1**	**6.0**	
H208Y	0.5	**3.6**	**11.3***	ABC (P = 0.0001); ddI (P = 0.005); **d4T** (P = 0.008)
D218E	0.4	**2.2**	**8.6***	**ABC** (P = 0.001); ddC (P = 0.002); ZDV (P = 0.008); ddI (P = 0.011)
K20R	9.3	12.9	**19.9***	
V35M	2.7	4.7	**10.3***	ddC (P = 0.0001); **ABC** (P = 0.0001); **TDF** (P = 0.009)
T39A	4.7	**8.3**	**12.9***	ddC (P = 0.002)
K122E	22.1	**32.8**	**42.2***	d4T (P = 0.001); **TDF** (P = 0.01)
G196E	16.2	**23.0**	**28.8**	
V35I	22.5	**14.2**	**14.2**	
I50V	4.9	3.8	**1.4**	
R83K	33.0	**25.4**	**19.7**	**d4T**[c] (P = 0.0001); **ddI**[c] (P = 0.005)

[a] The prevalence (percentage) of each novel reverse transcriptase mutation was determined according to the total number of NRTIs to which the 865 patients failing their last antiretroviral regimen containing NRTIs without NNRTIs had been exposed. Values in boldface indicate statistically significant differences, corrected for multiple-hypothesis testing by the Benjamini-Hochberg method at a false discovery rate of 0.05, with respect to the results for isolates from drug-naïve patients. Asterisks indicate statistically significant differences, corrected for multiple-hypothesis testing by the Benjamini-Hochberg method at a false discovery rate of 0.05, between isolates from patients experienced with three or fewer NRTIs and isolates from patients experienced with four or more NRTIs.

[b] Statistically significant associations with the use of specific NRTIs are reported. All P values were significant at a false discovery rate of 0.05 following correction for multiple-hypothesis testing. Associations with the use of specific NRTIs at the time of genotypic resistance testing are in boldface. Abbreviations: ddC, zalcitabine; ABC, abacavir; ddI, didanosine; d4T, stavudine; ZDV, zidovudine; TDF, tenofovir.

[c] R83K is negatively associated with the use of stavudine and didanosine. All the other associations of novel mutations with NRTIs are positive.

Workshop, abstr. 96, 2005). These mutations were grouped into three classes, based on their prevalence in isolates from treatment-naïve patients (Fig. 1).

Class I included seven mutations (K43E, K43Q, K43N, E203D, E203K, H208Y, and D218E) that were completely absent or occurred with a frequency of <0.5% in isolates from drug-naïve patients and showed a significant increase (P = 0.05 to <0.001) in isolates from patients failing NRTIs (with and without NNRTIs). Significant frequency differences between the two groups of patients treated with NRTIs with and without NNRTIs were not observed. However, K43E and H208Y exceeded 5% in frequency only in isolates from patients failing NRTIs without NNRTIs (7.3% and 6.5%, respectively), while D218E reached 6.5% prevalence only in isolates from patients failing NRTIs with NNRTIs.

Class II included five mutations (K20R, V35M, T39A, K122E, and G196E) already present in isolates from drug-naïve patients at a frequency of >2.5% but with a significant increase (P = 0.05 to <0.001) in isolates from both groups of NRTI-treated patients. In particular, K122E occurred at a frequency of 22.1% in isolates from drug-naïve patients and reached 30.7% and 37.2% prevalence in patients failing NRTIs with and without NNRTIs, respectively. Similarly to class I, statistically significant differences in frequency were not observed between the two groups of NRTI-treated patients.

Interestingly, and unlike the other classes, class III included three mutations (V35I, I50V, and R83K) that showed a significant frequency decrease in isolates from NRTI-treated patients compared to drug-naïve patients. Specifically, I50V significantly decreased (P = 0.038) from 4.9% to 2.6% and 2.8% in isolates of patients failing NRTIs without and with NNRTIs, while V35I and R83K decreased from 22.5% to 15.1% and 11.1% and from 33% to 22.6% and 21.2% in isolates from patients failing NRTIs without and with NNRTIs, respectively (P < 0.001).

We also included in our study the common polymorphism F214L, whose frequency remained stable (around 18%) in isolates from drug-naïve patients and in both groups of NRTI-treated patients (data not shown).

All class I and class II mutations showed a remarkable increase in frequency in isolates from patients who had experienced more than four NRTIs (Table 1). In particular, for K20R, V35M, T39A, K43Q, K122E, H208Y, and D218E, the increase in frequency observed in isolates from patients who had experienced more than four NRTIs was statistically significant (P < 0.05) not only compared to isolates from drug-naïve patients but also compared to isolates from patients who had experienced fewer than three NRTIs. On the other hand, class III mutations showed a progressive decrease in prevalence irrespective of an increasing number of NRTIs (Table 1).

Most class I and II mutations were also significantly associated (P < 0.05) with the use of specific NRTIs (Table 1). In detail, association with the use of one or more specific NRTIs in the last regimen was found for H208Y (stavudine), D218E (abacavir), V35M (abacavir and tenofovir), and K122E (tenofovir). In addition, H208Y was also associated with a previous use of abacavir and didanosine; D218E with a previous use of zidovudine, zalcitabine, and didanosine; V35M with a previous use of zalcitabine; and K122E with a previous use of stavudine, while T39A and K43E were specifically associated with previous use of zalcitabine. In class III, R83K was negatively associated with either stavudine or didanosine treatment (Table 1). None of the other mutations was significantly associated with treatment with any specific NRTI in our analysis.

Downloaded from jvi.asm.org by on December 4, 2009

TABLE 2. Significantly correlated pairs of mutations

Novel mutation	Frequency no. (%)[a]	Correlated mutation[b]	Frequency no. (%)[a]	Covariation frequency (%)[c]	Phi	P value[d]
T39A	155 (11.4)	M41L	488 (36.0)	110 (70.9)	0.25	4.9e-19
		L210W	297 (21.9)	63 (40.6)	0.19	1.1e-10
		T215Y	463 (34.2)	92 (59.3)	0.18	1.7e-10
K43E	79 (5.8)	E44A	16 (1.2)	10 (12.6)	0.34	1.5e-11
		L210W	297 (21.9)	62 (78.5)	0.33	3.4e-25
		V118I	230 (16.9)	44 (55.7)	0.26	5.1e-15
		M41L	488 (36.0)	70 (88.6)	0.25	9.3e-20
		T215Y	463 (34.2)	68 (86.1)	0.24	2.6e-17
		H208Y	89 (6.6)	17 (19.1)	0.17	1.9e-6
		V75M	25 (1.8)	8 (10.1)	0.17	2.1e-5
K43N	34 (3.2)	L210W	297 (21.9)	27 (79)	0.20	5.2e-10
		M41L	488 (36.0)	30 (88.2)	0.18	1.9e-10
		V118I	230 (16.9)	17 (50.0)	0.17	3.2e-7
		T215Y	463 (34.2)	29 (85.3)	0.16	2.6e-8
K43Q	56 (4.1)	L210W	297 (21.9)	43 (76.8)	0.28	1.7e-18
		V118I	230 (16.9)	30 (53.6)	0.24	2.0e-12
		E44D	123 (9.1)	24 (42.9)	0.24	1.8e-10
		M41L	488 (36.0)	49 (87.5)	0.22	6.2e-16
		T215Y	463 (34.2)	48 (85.7)	0.22	1.6e-15
		H208Y	89 (6.6)	18 (32.1)	0.21	5.6e-8
		K219R	37 (2.7)	10 (17.9)	0.19	4.6e-6
		D67N	444 (32.8)	40 (71.4)	0.17	5.0e-9
		T69D	98 (7.2)	15 (26.8)	0.16	6.0e-6
K122E	475 (35.1)	V118I	230 (16.9)	132 (27.8)	0.23	1.7e-16
		L210W	297 (21.9)	161 (33.9)	0.22	5.5e-15
		M41L	488 (36.0)	228 (48)	0.18	4.9e-11
		T69D	98 (7.2)	58 (12.2)	0.16	1.3e-8
		E44D	123 (9)	71 (14.9)	0.15	1.0e-7
G196E	330 (24.3)	H208Y	89 (6.6)	39 (11.8)	0.16	8.3e-8
		D67N	444 (32.8)	148 (44.8)	0.15	3.1e-8
		E44D	123 (9)	56 (17.0)	0.15	2.7e-7
E203D	31 (2.3)	H208Y	89 (6.6)	11 (35.5)	0.16	2.2e-5
E203K	54 (4.0)	H208Y	89 (6.6)	21 (38.9)	0.32	1.9e-14
		E44D	123 (9.1)	24 (44.4)	0.27	2.1e-12
		V118I	230 (16.9)	30 (55.5)	0.22	1.0e-10
		L210W	297 (21.9)	35 (64.8)	0.21	1.3e-11
		K219R	37 (2.7)	10 (18.5)	0.20	2.1e-6
		M41L	488 (36.0)	40 (74.1)	0.16	5.9e-9
		K219N	30 (2.2)	7 (13.0)	0.15	2.8e-4
H208Y	89 (6.6)	E44D	123 (9.1)	38 (42.7)	0.35	2.2e-20
		L210W	297 (21.9)	71 (79.8)	0.34	2.6e-27
		E203K	54 (4.0)	21 (23.6)	0.32	1.8e-14
		V118I	230 (16.9)	50 (56.2)	0.31	6.3e-20
		M41L	488 (36)	83 (93.3)	0.28	2.4e-24
		D67N	444 (32.8)	66 (74.2)	0.25	2.1e-18
		T69D	98 (7.2)	24 (27)	0.23	1.9e-10
		T215Y	463 (34.2)	71 (79.8)	0.21	1.5e-13
		K43Q	56 (4.1)	18 (20.2)	0.21	5.6e-8
		K219R	37 (2.7)	12 (13.5)	0.19	1.0 e-6
		K43E	79 (5.8)	18 (20.2)	0.17	1.9e-6
		E203D	31 (2.3)	11 (12.4)	0.16	2.2e-5
		L74I	36 (2.6)	7 (7.9)	0.15	1.1e-4
D218E	78 (5.8)	K219Q	207 (15.3)	53 (68)	0.34	6.0e-23
		D67N	444 (32.8)	64 (82)	0.27	3.4e-21
		K70R	325 (24.0)	56 (71.8)	0.23	2.0e-13
		L74I	36 (2.6)	55 (70.5)	0.23	5.9e-8
		T215F	134 (9.9)	25 (32.0)	0.18	1.7e-7
V35I	186 (13.7)	R83K	301 (22.2)	69 (37.1)	0.17	1.2e-08
I50V	37 (2.7)	M184V	794 (58.6)	14 (37.8)	−0.07	6.6e-3
R83K	301 (22.2)	D67N	444 (32.8)	43 (14.3)	−0.18	2.7e-12
		K219Q	207 (15.3)	11 (3.6)	−0.15	4.1e-10
		K70R	325 (24.0)	33 (11.0)	−0.14	2.9e-8
		V118I	230 (16.9)	28 (9.3)	−0.11	4.1e-5
		F214L	249 (18.4)	32 (10.6)	−0.10	5.5e-5
		M41L	488 (36.0)	85 (28.2)	−0.10	2.2e-4

Continued on facing page

Downloaded from jvi.asm.org by on December 4, 2009

TABLE 2—*Continued*

Novel mutation	Frequency no. (%)[a]	Correlated mutation[b]	Frequency no. (%)[a]	Covariation frequency (%)[c]	Phi	P value[d]
F214L	249 (18.4)	K219Q	207 (15.3)	83 (33.3)	0.25	4.0e-17
		K70R	325 (24.0)	117 (47)	0.24	1.6e-16
		T215Y	463 (34.2)	9 (1.9)	−0.27	1.0e-30
		M41L	488 (36.0)	24 (9.6)	−0.23	2.9e-20
		L210W	297 (21.9)	1 (0.4)	−0.21	1.9e-22
		E44D	123 (9.1)	3 (1.2)	−0.10	2.3e-5
		R83K	301 (22.2)	32 (12.8)	−0.10	5.5e-5

[a] The frequency was determined in 1,355 isolates from NRTI-treated patients.
[b] Positive correlations and negative correlations with phi > 0.15 and phi < −0.07 are shown, respectively.
[c] Percentages were calculated for patients containing each specific novel mutation.
[d] All P values for covariation were significant at a false discovery rate of 0.01 following correction for multiple-hypothesis testing (Benjamini-Hochberg method).

Associations among reverse transcriptase mutations. Another goal of our study was to assess the association of novel reverse transcriptase mutations with other mutations observed in the 1,355 NRTI-treated patients, focusing our attention on those involved in NRTI resistance.

To identify significant patterns of pairwise correlations between novel mutations and specific reverse transcriptase mutations observed in isolates from RT inhibitor-treated patients, we calculated the binomial correlation coefficient (phi) and its statistical significance for each pair of mutations (Table 2).

(i) Novel reverse transcriptase mutations involved in positive correlations with NRTI resistance mutations. Class I and class II mutations were positively (phi > 0, $P < 0.0001$) correlated as pairs with either NAMs 1 (M41L, L210W, and T215Y) or NAMs 2 (D67N, K70R, and K219Q), both clinically relevant for their ability to confer cross-resistance to NRTIs (19) (Table 2). Seven mutations (T39A, K43E, K43N, K43Q, K122E, E203K, and H208Y) were preferentially correlated as pairs with several NAMs 1 (Table 2). In particular, the most frequently selected NAM 1, M41L (prevalence, 36.0%), was correlated with H208Y (phi = 0.28), T39A (phi = 0.25), K43E (phi = 0.25), and K43Q (phi = 0.22), while L210W (prevalence, 21.9%) showed strong pairwise correlations with H208Y (phi = 0.34), K43E (phi = 0.33), K43Q (phi = 0.28), K122E (phi = 0.22), E203K (phi = 0.21), and K43N (phi = 0.20). T215Y (prevalence, 34.2%) was correlated with K43E (phi = 0.24), K43N (phi = 0.22), and H208Y (phi = 0.21). The seven mutations that correlated with NAMs 1 were also correlated as pairs with other NRTI resistance mutations known to occur at high frequency in combinations with NAMs 1, such as E44D, V118I, K219N, and K219R (15, 31, 44). In particular, E44D (prevalence, 9.1%) was strongly correlated with H208Y (phi = 0.35) and E203K (phi = 0.27), while V118I (prevalence, 16.9%) was strongly correlated with H208Y (phi = 0.28), K43E (phi = 0.26), K43Q (phi = 0.24), K122E (phi = 0.23), and E203K (phi = 0.22) (Table 2).

On the other hand, D218E was preferentially correlated with NAMs 2: K219Q (phi = 0.34), D67N (phi = 0.27), and K70R (phi = 0.23). In addition, D218E was significantly correlated with L74I (phi = 0.23) and T215F (phi = 0.18), a mutation at position 215 that occurs preferentially with NAMs 2. Within class II mutations, also K20R showed significant pairwise correlations with NAMs 2 and T215F, although less strongly than those observed with D218E (0.09 ≤ phi ≤ 0.13; 5.8e-6 ≤ P ≤ 1.4e-3) (data not shown).

None of the class I and class II mutations was significantly correlated with either lamivudine-selected mutation M184V or tenofovir-selected mutation K65R, thus confirming the low genetic barrier and the specific pathway towards resistance against these two drugs. Similarly, no significant associations were found with the relatively rare multi-NRTI resistance 151 complex (A62V, V75I, F77L, F116Y, and Q151M) or the 69 insertion.

(ii) Novel reverse transcriptase mutations involved in negative correlations with NRTI resistance mutations. Class III mutations did not show any positive correlations with NRTI resistance mutations. In contrast, I50V and R83K showed significant negative correlations (phi < 0 and $P < 0.01$) with specific NRTI resistance mutations. In particular, I50V was negatively associated with the lamivudine-selected mutation M184V (phi = −0.07) and R83K showed negative correlations with NAMs 2 D67N (phi = −0.18), K219Q (phi = −0.15), and K70R (phi = −0.14) (Table 2).

(iii) The polymorphism F214L was involved in either positive or negative correlations with different NRTI resistance mutations. Even if the frequency of F214L remained stable (around 18%) in isolates from drug-naïve patients and in both groups of NRTI-treated patients, however, the F214L mutation showed significant positive correlations with some NAMs 2, such as K219Q (phi = 0.25) and K70R (phi = 0.24), but no significant correlation with the NAM 2 D67N (phi = 0.04, $P >$ 0.05). In contrast, F214L showed strong negative correlations with the NAMs 1 T215Y (phi = −0.27), M41L (phi = −0.23), and L210W (phi = −0.21) and also with E44D (phi = −0.10) (Table 2). Such negative correlation was very strong in all isolates containing the complete NAM 1 cluster M41L/L210W/T215Y (phi = −0.23) (data not shown). In fact, F214L and the NAM 1 cluster were observed in combination less frequently than expected under the independence assumption (0.07% versus 3.7%, $P < 0.001$) (data not shown). On the other hand, F214L showed positive correlation with the overall NAM 2 cluster D67N/K70R/K219Q (phi = 0.18), and the co-occurrence was observed significantly more often than expected by chance (4.5% versus 2.2%, $P < 0.001$, data not shown).

Clusters of correlated mutations. Because pairwise analysis suggested that most novel mutations are associated with specific evolutionary pathways of known resistance-conferring mutations, we performed average linkage hierarchical agglomerative cluster analysis (38) to investigate this hypothesis in more detail.

Downloaded from jvi.asm.org by on December 4, 2009

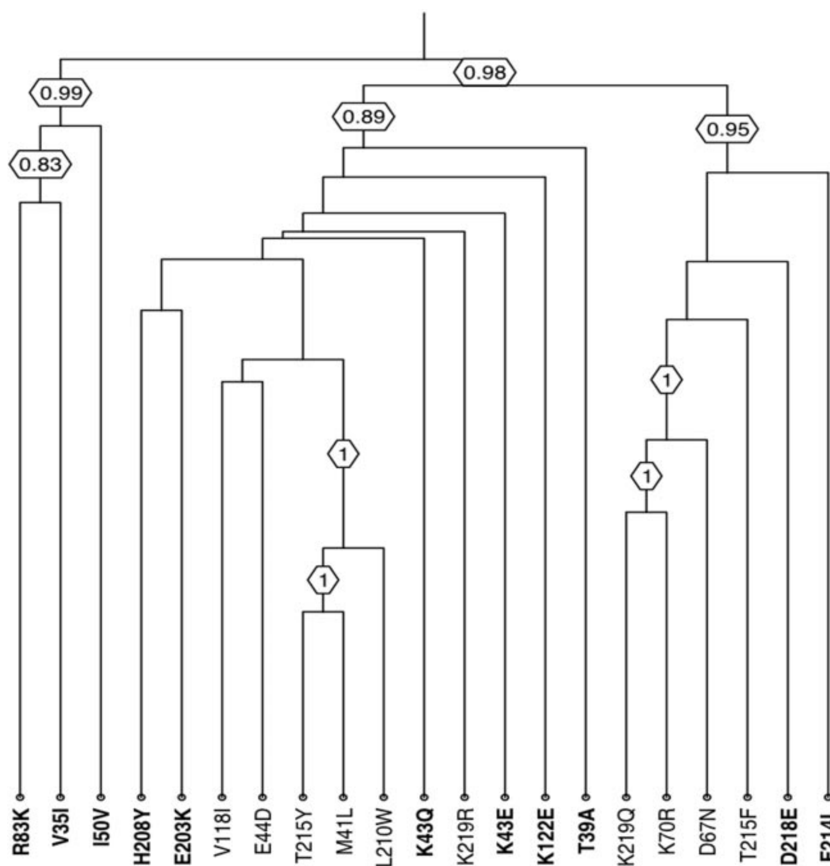

FIG. 2. Dendrogram obtained from average linkage hierarchical agglomerative clustering, showing clusters of known NRTI resistance mutations and novel mutations. The length of branches reflects distances between mutations in the original distance matrix. Bootstrap values, indicating the significance of clusters, are reported in the boxes. Novel mutations are shown in boldface.

The dendrogram in Fig. 2 shows that six novel mutations (T39A, K43E, K43Q, K122E, E203K, and H208Y) grouped together within a large cluster involving the strong NAM 1 pathway M41L/L210W/T215Y (bootstrap value = 1.0). As a whole, this cluster was highly significant (bootstrap value = 0.89), even though the exact linkage order of these novel mutations is associated with reduced bootstrap values, probably due to considerable flexibility in the order of accumulation. Likewise, a strong cluster was formed by NAMs 2 D67N, K70R, and K219Q (bootstrap value = 1.0), to which the already known mutation T215F and the novel mutations D218E and F214L were linked as well (bootstrap value = 0.95).

Similarly to D218E, K20R also clustered with NAMs 2. The introduction of K20R did not cause any changes in the topology of dendrograms but resulted in reduced confidence values. In particular, the confidence values for the NAM 1 and NAM 2 clusters decreased from 0.89 to 0.86 and from 0.95 to 0.86, respectively (data not shown). This drop can be attributed to the fact that K20R occurred in our data set with both NAMs 1 and NAMs 2 even if much more frequently so in combination with NAMs 2.

Interestingly, the absence of positive association among class III mutations with NRTI resistance mutations is also confirmed by the cluster analysis. In fact, the topology of the dendrogram shows that V35I, I50V, and R83K represent an outgroup (bootstrap value = 0.99) distinct from the other novel reverse transcriptase mutations that cluster with NAMs 1 and NAMs 2 (Fig. 2). In addition, in our data set we could observe the antagonism of R83K with the complete NAM 2 cluster D67N/K70R/K219Q (phi = −0.16), with the co-occurrence frequency being significantly lower than that expected by chance (0.4% versus 2.6%, $P < 0.001$) (data not shown).

Association of novel reverse transcriptase mutations with viral load and CD4 cell count. A further step in our study was to assess the association of novel reverse transcriptase mutations with the virologic and immunologic values at the time of therapeutic failure and genotype test (Table 3). Among all mutations analyzed, four novel mutations (V35I, K43E, K122E, and H208Y) were significantly associated with a worse virologic and immunologic outcome under NRTI failure. In fact, the presence of at least one of these four mutations in isolates from NRTI-treated patients was significantly associated with higher values of viremia and lower CD4 cell counts (Table 3). In addition, the copresence of K43E and H208Y in the NAM 1 cluster was also associated at therapeutic failure with a 3.7-fold increase in viremia ($P > 0.05$; data not shown). For V35I and K122E this association was also confirmed in drug-naïve patients (Table 3).

Association of novel mutations with reduced NRTI susceptibility. We showed that some novel mutations (K43E, K122E,

Downloaded from jvi.asm.org by on December 4, 2009

TABLE 3. Association of novel reverse transcriptase mutations with viremia and CD4 cell count

Patient group	Mutation		Frequency[a], n (%)	Viremia (median)[b], copies/ml	Ratio	P[c]	CD4 cell count[b] (median), cells/μl	Ratio	P[c]
	Position	Residue							
NRTI treated	35	V[d]	942 (69.5)	8,202			340		
		I	186 (13.7)	16,094	1.9	7.0e-4	278	0.8	9.2e-3
	43	K[d]	1,188 (87.7)	8,599			332		
		E	79 (5.8)	27,496	3.2	1.6e-2	307	0.9	>0.05
	122	K[d]	829 (61.2)	8,286			345		
		E	475 (35.1)	14,340	1.7	2.1e-2*	297	0.8	5.2e-3
	208	H[d]	1,261 (93.1)	8,414			336		
		Y	89 (6.6)	38,280	4.5	3.7e-6	202	0.6	1.7e-3
Drug naïve	35	V[d]	371 (67.3)	56,290			318		
		I	124 (22.5)	89,000	1.6	2.0e-2*	183	0.6	4.0e-3
	122	K[d]	401 (72.8)	53,650			317		
		E	122 (22.1)	78,420	1.5	2.6e-2*	199	0.6	3.5e-2*

[a] The frequency was determined in 1,355 NRTI-treated patients and 551 drug-naïve patients.
[b] Viremia and CD4 cell count values analyzed are concomitant with the genotype resistance testing (±30 days) and were available for >90% of samples.
[c] P values were determined by median test and were corrected for multiple comparison (Benjamini-Hochberg method at a false discovery rate of 0.05). Asterisks indicate P values not significant at a false discovery rate of 0.05.
[d] The wild-type amino acid in consensus B.

H208Y, and D218E) were strongly associated and clustered with known NRTI resistance mutations on divergent evolutionary pathways (NAMs 1 versus NAMs 2). Analyzing sequences with known NRTI susceptibility (measured by Virco's Antivirogram assay) from the Stanford HIV Drug Resistance Database, we found that the presence of these mutations at therapeutic failure was also significantly associated with higher zidovudine and stavudine resistance (Table 4). In particular, the presence, individually or combined, of K43E, K122E, and H208Y with the NAM 1 cluster (M41L/L210W/T215Y) was associated with a high increase in zidovudine resistance and also a less extensive increase of stavudine resistance. On the other hand, the presence of D218E with T215F and the NAM 2 cluster (D67N/K70R/K219Q), with which it has been associated, determined a 2.5-fold increase of zidovudine resistance (Table 4).

In contrast, I50V and R83K, which were negatively associated with NRTI treatment and NRTI resistance mutations, were associated with increased susceptibility to specific NRTIs (Table 5). In particular, I50V, which showed antagonism with the lamivudine-selected mutation M184V, when present together with M184V determined a 3.5-fold decrease in M184V/lamivudine resistance. Finally, R83K, which showed antagonism with both NAM 1 and NAM 2 clusters, was associated with a 1.6- to 3.5-fold decrease in both zidovudine and stavudine resistance when present with these mutations.

Location of novel mutations in the three-dimensional structure of reverse transcriptase. The distribution of novel mutations was found to be widespread in the structure of the enzyme involving residues from different subdomains (Fig. 3) such as finger (amino acids 39, 43, 50, 83, and 122), palm (196, 203, 208, 214, and 218), and template grip (83). Moreover, these mutated amino acids were generally found at positions spatially rather distant from NAM residues. In fact, out of the 70 significantly correlated pairs of residues, only 13 (17.3%) were within 10 Å of each other in the p66 subunit of reverse

Downloaded from jvi.asm.org by on December 4, 2009

TABLE 4. Novel reverse transcriptase mutations associated with a decreased NRTI susceptibility

Pattern of mutations	No. of sequences[a]	Fold increase in resistance[b] (median; IQR)						
		ZDV	d4T	3TC	ddI	ddC	ABC	TDF
NAM 1	37	18.0; 7.6–57.3	1.6; 0.7–3.2	3.5; 2.1–8.2	1.4; 1.0–2.4	1.8; 0.8–2.9	3.1; 1.5–6.3	2.1; 1.2–4.2
NAM 1 + 43E	4	32.6; 13.1–62.9	3.5; 1.9–7.1	3.8; 3.8–3.8	1.0; 0.7–1.2	2.1; 1.1–4.7	3.0; 2.3–3.8	1.8; 1.5–3.2
NAM 1 + 122E	15	45.0; 20.5–49.0	5.3; 3.1–9.2	9.5; 4.0–16.3	5.5; 3.4–6.6	4.2; 2.7–5.3	10.4; 6.6–12.0	3.3; 3.3–3.3
NAM 1 + 208Y	4	36.8; 26.5–47.1	3.9; 2.5–6.8	4.0; 3.7–5.5	1.5; 0.9–1.9	0.8; 0.5–1.2	6.4; 6.4–6.4	2.7; 2.1–3.4
NAM 1 + 43E + 122E + 208Y[c]	3	886.3; 648.1–1,077.7	3.5; 3.1–4.6	9.9; 9.9–9.9	2.1; 1.5–2.9	1.3; 1.1–2.4	3.8; 3.3–4.8	NA
NAM 2	11	5.6; 3.2–8.2	1.0; 0.6–1.2	4.4; 4.4–4.4	1.4; 0.9–2.1	2.1; 1.8–2.8	2.3; 1.2–3.7	1.2; 0.8–1.8
NAM 2 + 215F	8	12.5; 8.4–13.8	1.2; 0.7–1.3	2.2; 1.2–3.3	0.9; 0.7–1.6	0.8; 0.5–1.9	2.7; 1.6–5.7	1.6; 1.4–2.3
NAM 2 + 218E	9	4.5; 1.8–13.4	1.2; 1.0–2.6	1.2; 1.0–19.8	1.0; 0.7–1.4	1.0; 0.8–3.6	2.8; 1.9–3.	2.7; 0.7–3.1
NAM 2 + 214L	2	8.5; 7.8–9.3	0.7; 0.6–1.1	NA	0.8; 0.7–0.9	0.5; 0.5–0.7	1.6; 1.2–2.0	1.5; 1.2–1.8
NAM 2 + 215F + 218E[d]	3	30.9; 16.2–75.9	1.9; 0.4–3.4	13.2; 13.2–13.2	1.4; 0.8–1.9	2.1; 1.7–2.2	5.5; 1.3–10.9	0.7; 0.6–5.9

[a] Sequences were collected from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu).
[b] The values for increases in resistance (n-fold) are derived from the real phenotypic data measured by Virco's Antivirogram assay. Abbreviations: ZDV, zidovudine; d4T, stavudine; 3TC, lamivudine; ddI, didanosine; ddC, zalcitabine; ABC, abacavir; TDF, tenofovir; IQR, interquartile range; NA, not available.
[c] Present also with D67N+E44D+V118I+K219N and E203K and R211K.
[d] Present also with M41L and K43E.

TABLE 5. Novel reverse transcriptase mutations associated with an increased NRTI susceptibility[a]

Drug and pattern of mutations	No. of sequences[b]	Median fold increase in resistance[c] (IQR)	Ratio
ZDV			
NAM 1 + 83R[d]	101	15.5 (4.1–48)	
NAM 1 + 83K	19	9.6 (4.3–35.5)	1.6
NAM 2 + 83R[d,f]	61	5.6 (2.1–12)	
NAM 2 + 83K[f]	5	1.6 (1.5–3.7)	3.5
D4T			
NAM 1 + 83R[d]	83	1.7 (0.9–3.9)	
NAM 1 + 83K	19	0.8 (0.8–1.2)	2.1[e]
NAM 2 + 83R[d,f]	60	1.2 (0.7–2.1)	
NAM 2 + 83K[f]	6	0.7 (0.5–0.9)	1.7
3TC			
M184V + 50I[d]	101	81.7 (43.3–90.5)	
M184V + 50V	1	23.6	3.5

[a] Abbreviations: ZDV, zidovudine; d4T, stavudine; 3TC, lamivudine; IQR, interquartile range.
[b] Sequences were collected from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu).
[c] The values for increase in resistance are derived from the real phenotypic data measured by Virco's Antivirogram assay.
[d] Indicates the wild-type amino acid in consensus B.
[e] The difference in the increase in stavudine resistance is statistically significant ($P = 0.018$; median test).
[f] For the absence of sequences with 83K and NAMs 2 (D67N/K70R/K219Q), the sequences selected contained at least one NAM 2.

transcriptase, three of which were contiguous in primary sequence. Nonetheless, only for one of these (43-44) was it possible to establish the possibility of a direct interaction between their side chains (data not shown).

DISCUSSION

Our study suggests that other HIV-1 reverse transcriptase mutations, beyond the currently known NRTI resistance mutations, may contribute to the evolution of NRTI resistance in vivo. In fact, 16 novel mutations have been identified, characterized, and grouped into distinct classes.

Class I mutations (K43E/N/Q, E203D/K, H208Y, and D218E) were rare or completely absent in isolates from drug-naïve patients. In contrast, their frequency significantly increased in isolates from patients failing an antiretroviral regimen containing at least one NRTI, thus suggesting that these mutations may require the NRTI selective pressure for their emergence at virological failure. Moreover, class I mutations occurred principally in combination with several NRTI resistance mutations, suggesting that they emerge after a prolonged NRTI exposure when the virus has already accumulated a large number of NRTI resistance mutations. As a confirmation of this hypothesis, a previous study already demonstrated that the emergence of H208Y mutation may require as a prerequisite the presence of at least M41L and T215Y (40).

Class II mutations (K20R, V35M, T39A, K122E, and G196E) were already present in isolates from drug-naïve patients, but their frequency significantly increased in isolates from patients failing an antiretroviral regimen containing at least one NRTI, thus suggesting a positive association of these mutations with NRTI treatment.

These results are consistent with recent analyses conducted with a large number of reverse transcriptase sequences (13, 30, 32), which provide strong evidence for an "extended spectrum" of reverse transcriptase mutations associated with multi-NRTI treatment. The few discordances in the number and prevalence of new treatment-associated mutated positions that we observed, compared with the studies mentioned above, may probably be related to the different number of patients analyzed, the patients' characteristics, and the therapeutic regimens used.

Interestingly, two mutations (T39A and K122E) were also associated with drug resistance and/or positive selection pressure in another study (8), based on ratios of nonsynonymous to synonymous mutations (*Ka*/*Ks*) of >1 similar to those observed for the already-known RTI resistance mutations.

Moreover, all novel mutations, with the exception of one (R83K), correspond with changes in known HLA-restricted cytotoxic T-lymphocyte epitopes in reverse transcriptase (HIV Molecular Immunology Database, http://www.hiv.lanl.gov). Further studies should examine the interactions between these novel mutations (together with the other NRTI mutations) and putative cytotoxic T-lymphocyte escape mutations to better understand synergistic and/or antagonistic interactions between immune pressure and drug pressure.

Furthermore, class I and class II mutations established agonistic interactions with the currently known NRTI resistance mutations (both in pairs and in clusters). In particular, for the mutations T39A, K43E/Q, E203K, K122E, and H208Y the

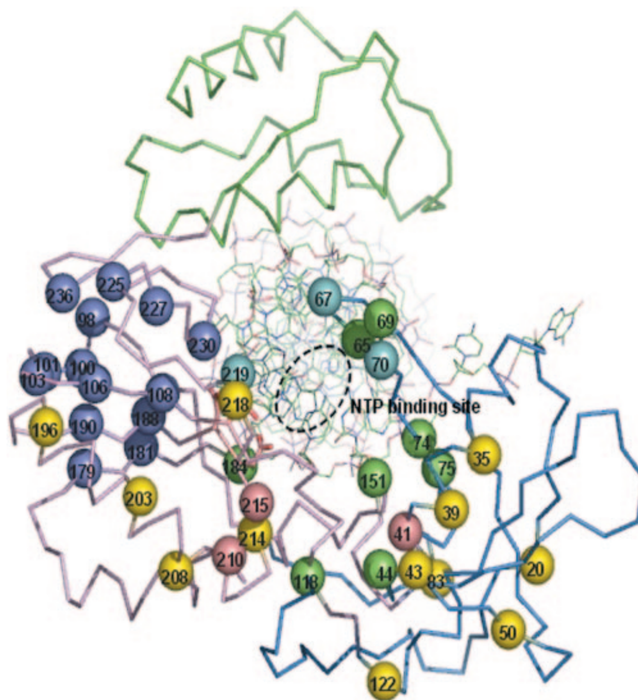

FIG. 3. Localization of novel mutations in the structure of HIV-1 RT. The structure of HIV-1 RT in the region near the polymerase active site is shown. The subdomains are color coded (palm, magenta; thumb, green; fingers, blue). NAMs 1 are in salmon and NAMs 2 in cyan; NNRTI resistance mutations are in blue and novel mutations in yellow; other RT inhibitor resistance mutations are in green.

Downloaded from jvi.asm.org by on December 4, 2009

agonistic interactions principally involved the NAM 1 pathway (M41L, L210W, T215Y, E44D, V118I, and K219R), thus suggesting that these mutations may contribute to NAM 1-mediated resistance to NRTIs. The strong agonistic interactions that we observed between the NAM 1 M41L and either T39A or K43E/Q, as well as that between the NAM 1 L210W and H208Y, could be related to the fact that these residues are located at the C terminus of an alpha-helix so that the correlated mutation probably contributes to a greater stabilization of this secondary structure element. This can be important for enzymatic activity as all of these residues are located close to the binding site of the incoming nucleotide.

On the other hand, two mutations (D218E and K20R) were associated with the NAM 2 pathway (D67N, K70R, K219Q, and T215F). As D218E was significantly associated with the use of zidovudine, and its presence in the NAM 2 cluster determined a 2.5-fold increase in zidovudine resistance, we suggest that this mutation may contribute to NAM 2-mediated resistance to zidovudine. This hypothesis is consistent with results from a recent study which showed that a prolonged exposure to zidovudine is associated with an increased probability of observing the NAM 2 profile (A. Cozzi-Lepri, L. Ruiz, C. Loveday, A. N. Phillips, B. Clotet, P. Reiss, and J. D. Lundgren, Abstr. 12th Conf. Retrovir. Opportunistic Infect., abstr. 708, 2005).

While our results provide strong evidence that additional reverse transcriptase mutations are preferentially associated with one of the classical NAM pathways, we emphasize that this association is not exclusive, not even among the core patterns M41L+L210W+Y215Y and D67N+K70R+K219Q. In fact, a recent study (D. T. Dunn, Abstr. 14th HIV Drug Resist. Int. Workshop, abstr. 130, 2005) has found 116 distinct NAM patterns in a set of 2,379 sequences, and 40% of these sequences contained mutations from both pathways, with D67N accounting for most of the "crossover" between the groups. These "fuzzy," rather than rigid, covariation patterns can be summarized and visualized via principal component analysis (13) or multidimensional scaling (38), for example.

In the light of the observed NAM pattern diversity, it is reasonable to expect that the frequency of a given pattern is correlated with replication parameters associated with the pattern. Indeed, as an example, the antagonistic covariation between NAM 1 M41L and NAM 2 K70R is paralleled by a marked replicative defect characterizing viral strains with those two mutations (15, 18, 44).

The fact that the copresence of K43E, K122E, or H208Y alone or as a cluster with the NAMs 1 was associated with higher viremia and lower CD4 cell count at therapeutic failure may suggest a compensatory role of these mutations, leading to improved viral replication, especially if the response is compromised by the presence of other NRTI resistance mutations, such as NAMs 1.

It is also conceivable that class I and class II mutations contribute to a further increase in the level of resistance. Phenotypic resistance data in the Stanford HIV Drug Resistance Database support this hypothesis. In particular, we observed that some class I and II mutations at therapeutic failure were able to affect NRTI susceptibility in vitro (Stanford HIV Drug Resistance Database, http://hivdb.Stanford.edu). The presence—individually or combined—of K43E, K122E and H208Y, with and without NAMs 1, was associated with a high increase in zidovudine resistance. In addition, on an independent data set, feature ranking based on support vector machines and matched genotype-phenotype pairs indicated that six novel mutations (H208Y, K122E, T39A, K43E, E203K, and D218E) are prominently (within the top 20 mutations) involved in determining zidovudine resistance, ranking even above several of the classical zidovudine mutations (H208Y, K122E, T39A, K43E > T215F, K219Q, and K70R) (38).

The H208Y mutation, frequently selected in combination with the NAM pathway under combined therapy with zidovudine and lamivudine, was recently shown to be associated with an increase in the level of zidovudine resistance (32, 39, 40). It is known that the lamivudine-selected mutation M184V decreases the ability of HIV-1 reverse transcriptase to carry out ATP-mediated removal of zidovudine or stavudine monophosphate from the terminated cDNA chain; thus, it was supposed that the appearance of H208Y, proximal to the ATP binding site, may influence the geometry of the ATP binding site, maintaining the efficiency of the excision reaction even in the presence of M184V (34).

Moreover, the H208Y mutation was recently associated with resistance to foscarnet, a phosphonoformate that competes with the pyrophosphates (PP_i) for the hydrolytic removal of the chain-terminating NRTI (23). Overall, our data support the idea that H208Y may play a role in mechanisms that regulate NRTI resistance, presumably by promoting the primer rescue.

Interestingly, our covariation analysis showed that different mutations at the same position showed differential clustering. In fact, both hierarchical clustering (Fig. 2) and a multidimensional scaling analysis (38) are consistent with previous studies of the differential behavior of mutation T215F versus T215Y and K219Q versus K219R. In fact, mutagenesis studies in combination with growth competition assays showed that viral strains with the mutation T215F had a lower replicative capacity than the T215Y variant in the presence of M41L and L210W (Z. X. Hu, P. Reid, H. Hatano, J. Lu, and Kuritzkes, Abstr. 13th Int. HIV Drug Resist. Workshop, abstr. 59, 2004). Thus, the phenotypic impact of T215F, which is positive in the presence of NAMs 2 and negative in the presence of NAMs 1, seems to be dependent on the genomic background in which the mutation occurs. This may have important implications for resistance prediction systems. In fact, prediction systems based on linear models cannot accommodate situations such as these, in which the effect of a mutation or mutational pattern is dependent on the genomic background in which it occurs, in contrast to nonlinear methods (12, 46) or tree- or rules-based approaches (4, 37).

Taken together, our data suggest that class I and class II mutations may actively participate in the regulation of NRTI resistance. Moreover, our data also indicate that despite an obvious preference for certain mutational patterns, the accumulation order of the novel mutations is also characterized by considerable flexibility (T. Sing, V. Svicher, N. Beerenwinkel, F. Ceccherini-Silberstein, I. Savenkov, K. Korn, C. F. Perno, H. Walter, and T. Lengauer, Abstr. 14th Int. HIV Drug Resist. Workshop, abstr. 50, 2005).

Differently from the other mutations, class III mutations (I35V, I50V, and R83K) are polymorphisms in isolates from drug-naïve patients, and their frequency decreased in isolates

Downloaded from jvi.asm.org by on December 4, 2009

from patients who failed an antiretroviral regimen containing at least one NRTI. Moreover, class III mutations were rarely found in the presence of NRTI resistance mutations and were never positively associated with any NRTI resistance mutations, thus suggesting the negative association of these mutations with NRTI treatment and failure. If anything, I50V and R83K were involved in antagonistic interactions with NRTI resistance mutations (M184V and NAMs, respectively) and were then associated, when rarely present with M184V or NAMs, respectively, with increased susceptibility to lamivudine and thymidine analogues, respectively. It is conceivable that these mutations are selectively neutral in wild-type strains but weakly deleterious in terms of viral replication in the presence of NRTI resistance mutations, thus contributing to increases in the level of the genetic barrier to NRTI resistance. However, this hypothesis needs confirmation from both in vitro studies and clinical practice.

We also analyzed the F214L polymorphism that is characterized by an inconspicuous prevalence behavior, with virtually constant frequency in naïve and NRTI-failing patients.

Surprisingly, our covariation analysis identified an agonistic interaction of the L variant with the NAM 2 pathway, whereas the more common F variant established an agonistic interaction with the NAM 1 cluster. These results are consistent with recent studies in which it was supposed that the F variant, specifically associated with the NAM 1 profile (40), may improve the efficacy of the ATP-mediated removal of the zidovudine or stavudine monophosphate from the terminated cDNA chain (34). It is conceivable that position 214 remains stable during NRTI exposure and that the particular residue found in the dominant quasispecies at position 214 before therapy onset may represent one of the determinants for NAM pathway choice. Thus, we suggest that NAM pathway choice is determined not only by chance effects, or host factors such as HLA genotype (19), but also by the genomic background of the treatment-naïve viral population. Together with the observation that the NAM 1 group exerts stronger zidovudine resistance (38) and clinical cross-resistance to tenofovir than NAMs 2 (25), baseline genotypic testing and subsequent therapy choice might benefit from taking into account positions such as 214 as possible determinants of the future course of resistance evolution.

It should be noted that all patients analyzed in this study carried the HIV-1 subtype B. Further studies should also investigate the prevalence and the role of the novel mutations in other non-B subtypes. In fact, it is known that pathways of viral evolution toward drug resistance may proceed through distinct steps and at different rates among different HIV-1 subtypes (45). Moreover, we cannot exclude the possibility that the observed patterns of correlated mutations may be the result of pharmacological pressure imposed by the drug regimens that were used by our cohort, while other treatment regimens may lead to the development of pathways which are partly different from those that we observed. Further analyses of expanded databases (with genotypic and clinical and/or phenotypic data) complemented by specific experimental verification will provide insights regarding these important open questions and will give more information on the impact of these mutations on virus replication and drug resistance. In particular, further studies using sequences of the entire reverse transcriptase/RNase H are also necessary. In fact, the exclusion of the RNase H domain from our study represents a limitation, since recent data show that mutations in RNase H were associated with the presence of NRTI mutations and may also enhance resistance to NRTIs in vitro (27; A. G. Marcelin, B. Roquebert, I. Malet, M. Wirden, A. Simon, C. Katlama, and V. Calvez, Abstr. 14th Int. HIV Drug Resist. Workshop, abstr. 50, 2005).

In conclusion, our study reinforces the complexity of NRTI resistance and contributes to a better definition of the reverse transcriptase mutational patterns involved in regulating resistance to NRTIs. It suggests that other mutations beyond those currently known to confer resistance may regulate, not only positively but also negatively, this highly complex network. On this basis, novel mutations should be taken into account to define improved algorithms for predicting phenotypic resistance or clinical response to antiretroviral drugs.

ACKNOWLEDGMENTS

This work was financially supported by grants from the Italian National Institute of Health, the Ministry of University and Scientific Research, Current and Finalized Research of the Italian Ministry of Health, and the European Community (QLK2-CT-2000-00291 and Descartes Award). The research at the Max Planck Institute for Informatics was performed in the context of the EU Network of Excellence BioSapiens (EU grant no. LSHG-CT-2003-503265). The ICONA network is supported by unrestricted educational grants from Glaxo-Smith-Kline, Italy.

We thank Alessandra Cenci, Roberta D'Arrigo, Caterina Gori, Fabio Continenza, Daniele Pizzi, Andrea Biddittu, and Sandro Bonfigli for sequencing and data management and all the ICONA study group participants and members.

Members of the ICONA study group in Italy by city are as follows: Ancona, M. Montroni, G. Scalise, A. Zoli, and M. S. Del Prete; Aviano (PN), U. Tirelli and G. Di Gennaro; Bari, G. Pastore, N. Ladisa, and G. Minafra; Bergamo, F. Suter and C. Arici; Bologna, F. Chiodo, V. Colangeli, C. Fiorini, and O. Coronado; Brescia, G. Carosi, G. P. Cadeo, F. Castelli, C. Minardi, and D. Vangi; Busto Arsizio, G. Rizzardini and G. Migliorino; Cagliari, P. E. Manconi and P. Piano; Catanzaro, T. Ferraro and A. Scerbo; Chieti, E. Pizzigallo and M. D'Alessandro; Como, D. Santoro and L. Pusterla; Cremona, G. Carnevale and D. Galloni; Cuggiono, P. Viganò and M. Mena; Ferrara, F. Ghinelli and L. Sighinolfi; Firenze, F. Leoncini, F. Mazzotta, M. Pozzi, and S. Lo Caputo; Foggia, G. Angarano, B. Grisorio, and S. Ferrara; Galatina (LE), P. Grima and P. Tundo; Genova, G. Pagano, N. Piersantelli, A. Alessandrini, and R. Piscopo; Grosseto, M. Toti and S. Chigiotti; Latina, F. Soscia and L. Tacconi; Lecco, A. Orani and P. Perini; Lucca, A. Scasso and A. Vincenti; Macerata, F. Chiodera and P. Castelli; Mantova, A. Scalzini and G. Fibbia; Milano, M. Moroni, A. Lazzarin, A. Cargnel, G. M. Vigevani, L. Caggese, A. d'Arminio Monforte, D. Repetto, R. Novati, A. Galli, S. Merli, C. Pastecchia, and M. C. Moioli; Modena, R. Esposito and C. Mussini; Napoli, N. Abrescia, A. Chirianni, C. Izzo, M. Piazza, M. De Marco, V. Montesarchio, E. Manzillo, and M. Graf; Palermo, A. Colomba, V. Abbadessa, T. Prestileo, and S. Mancuso; Parma, C. Ferrari and P. Pizzaferri; Pavia, G. Filice, L. Minoli, R. Bruno, and S. Novati; Perugia, F. Balzelli and K. Loso; Pesaro, E. Petrelli and A. Cioppi; Piacenza, F. Alberici and A Ruggieri; Pisa, F. Menichetti and C. Martinelli; Potenza, C. De Stefano and A. La Gala; Ravenna, G. Ballardini and E. Briganti; Reggio Emilia, G. Magnani and M. A. Ursitti; Rimini, M. Arlotti and P. Ortolani; Roma, L. Ortona, F. Dianzani, G. Ippolito, A. Antinori, G. Antonucci, S. D'Elia, P. Narciso, N. Petrosillo, V. Vullo, A. De Luca, L. Del Forno, M. Zaccarelli, R. Acinapura, P. De Longis, M. Ciardi, G. D'Offizi, M. P. Trotta, P. Noto, M. Lichtner, M. R. Capobianchi, E. Girardi, P. Pezzotti, and G. Rezza; Sassari, M. S. Mura and M. Mannazzu; Torino, P. Caramello, A. Sinicco, M. L. Soranzo, L. Gennero, M. Sciandra, and M. Bonasso; Varese, P. A. Grossi and C. Basilico; Verbania, A. Poggio and G. Bottari; Venezia, E. Raise and S. Pasquinucci; Vicenza, F. De Lalla and G. Tositti; and Taranto, F.

Downloaded from jvi.asm.org by on December 4, 2009

Resta and A. Chimienti. A. Cozzi-Lepri in London, United Kingdom, is also a member.

REFERENCES

1. **Arion, D., N. Kaushik, S. McCormick, G. Borkow, and M. A. Parniak.** 1998. Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry **37:**15908–15917.
2. **Balzarini, J.** 2004. Current status of the non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. Curr. Top. Med. Chem. **4:**921–944.
3. **Balzarini, J.** 1999. Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. Biochem. Pharmacol. **58:**1–27.
4. **Beerenwinkel, N., B. Schmidt, H. Walter, R. Kaiser, T. Lengauer, D. Hoffmann, K. Korn, and J. Selbig.** 2002. Diversity and complexity of HIV-1 drug resistance: a bioinformatics approach to predicting phenotype from genotype. Proc. Natl. Acad. Sci. USA **99:**8271–8276.
5. **Benjamini, Y., and Hochberg, Y.** 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B **57:**289–300.
6. **Ceccherini-Silberstein, F., F. Erba, F. Gago, A. Bertoli, F. Forbici, M. C. Bellocchi, C. Gori, R. D'Arrigo, L. Marcon, C. Balotta, A. Antinori, A. d'Arminio Monforte, and C. F. Perno.** 2004. Identification of the minimal conserved structure of HIV-1 protease in the presence or absence of drug pressure. AIDS **18:**11–19.
7. **Ceccherini-Silberstein, F., F. Gago, M. Santoro, C. Gori, V. Svicher, F. Rodriguez-Barrios, R. d'Arrigo, M. Ciccozzi, A. Bertoli, A. d'Arminio Monforte, J. Balzarini, A. Antinori, and C. F. Perno.** 2005. High sequence conservation of human immunodeficiency virus type 1 reverse transcriptase under drug pressure despite the continuous appearance of mutations. J. Virol. **79:**10718–10729.
8. **Chen, L., A. Perlina, and C. J. Lee.** 2004. Positive selection detection in 40,000 human immunodeficiency virus (HIV) type 1 sequences automatically identifies drug resistance and positive fitness mutations in HIV protease and reverse transcriptase. J. Virol. **78:**3722–3732.
9. **Clavel, F., and A. J. Hance.** 2004. HIV drug resistance. N. Engl. J. Med. **350:**1023–1035.
10. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
11. **De Clercq, E.** 2004. Antivirals and antiviral strategies. Nat. Rev. Microbiol. **2:**704–720.
12. **Draghici, S., and R. B. Potter.** 2003. Predicting HIV drug resistance with neural networks. Bioinformatics **19:**98–107.
13. **Gonzales, M. J., T. D. Wu, J. Taylor, I. Belitskaya, R. Kantor, D. Israelski, S. Chou, A. R. Zolopa, W. J. Fessel, and R. W. Shafer.** 2003. Extended spectrum of HIV-1 reverse transcriptase mutations in patients receiving multiple nucleoside analog inhibitors. AIDS **17:**791–799.
14. **Gotte, M., X. Li, and M. A. Wainberg.** 1999. HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in the initiation of the reaction. Arch. Biochem. Biophys. **365:**199–210.
15. **Hanna, G. J., V. A. Johnson, D. R. Kuritzkes, D. D. Richman, J. Martinez-Picado, L. Sutton, J. D. Hazelwood, and R. T. D'Aquila.** 2000. Comparison of sequencing by hybridization and cycle sequencing for genotyping of human immunodeficiency virus type 1 reverse transcriptase. J. Clin. Microbiol. **38:**2715–2721.
16. **Hertogs, K., S. Bloor, S. D. Kemp, C. Van den Eynde, T. M. Alcorn, R. Pauwels, M. Van Houtte, S. Staszewski, V. Miller, and B. A. Larder.** 2000. Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples. AIDS **14:**1203–1210.
17. **Hope, A. C.** 1968. A simplified Monte Carlo significance test procedure. J. R. Stat. Soc. B **30:**582–598.
18. **Jeeninga, R. E., W. Keulen, C. Boucher, R. W. Sanders, and B. Berkhout.** 2001. Evolution of AZT resistance in HIV-1: the 41–70 intermediate that is not observed in vivo has a replication defect. Virology **283:**294–305.
19. **John, M., C. B. Moore, I. R. James, and S. A. Mallal.** 2005. Interactive selective pressures of HLA-restricted immune responses and antiretroviral drugs on HIV-1. Antivir. Ther. **10:**551–555.
20. **Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman.** 2005. Update of the drug resistance mutations in HIV-1: fall 2005. Top. HIV Med. **13:**125–131.
21. **Mansky, L. M.** 1998. Retrovirus mutation rates and their role in genetic variation. J. Gen. Virol. **79:**1337–1345.
22. **Meyer, P. R., S. E. Matsuura, A. G. So, and W. A. Scott.** 1998. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc. Natl. Acad. Sci. USA **95:**13471–13476.
23. **Meyer, P. R., S. E. Matsuura, D. Zonarich, R. R. Chopra, E. Pendarvis, H. Z. Bazmi, J. W. Mellors, and W. A. Scott.** 2003. Relationship between 3′-azido-3′-deoxythymidine resistance and primer unblocking activity in foscarnet-resistant mutants of human immunodeficiency virus type 1 reverse transcriptase. J. Virol. **77:**6127–6137.
24. **Meyer, P. R., S. E. Matsuura, R. F. Schinazi, A. G. So, and W. A. Scott.** 2000. Differential removal of thymidine nucleotide analogues from blocked DNA chains by human immunodeficiency virus reverse transcriptase in the presence of physiological concentrations of 2′-deoxynucleoside triphosphates. Antimicrob. Agents Chemother. **44:**3465–34672.
25. **Miller, V., and B. A. Larder.** 2001. Mutational patterns in the HIV genome and cross-resistance following nucleoside and nucleotide analogue drug exposure. Antivir. Ther. **6**(Suppl. 3)**:**25–44.
26. **Naeger, L. K., and M. D. Miller.** 2001. Mechanisms of HIV-1 nucleoside reverse transcriptase inhibitor resistance: is it all figured out? Curr. Opin. Investig. Drugs **2:**335–339.
27. **Nikolenko, G. N., S. Palmer, F. Maldarelli, J. W. Mellors, J. M. Coffin, and V. K. Pathak.** 2005. Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. Proc. Natl. Acad. Sci. USA **102:**2093–2098.
28. **Parniak, M. A., and N. Sluis-Cremer.** 2000. Inhibitors of HIV-1 reverse transcriptase. Adv. Pharmacol. **49:**67–109.
29. **Perno, C. F., A. Cozzi-Lepri, C. Balotta, F. Forbici, M. Violin, A. Bertoli, G. Facchi, P. Pezzoti, G. Cadeo, G. Tositti, S. Pasquinucci, S. Pauluzzi, A. Scalzini, B. Salassa, A. Vincenti, A. N. Phillips, F. Dianzani, A. Apice, G. Angarano, L. Monno, G. Ippolito, M. Moroni, and A. d'Arminio Monforte.** 2001. Secondary mutations in the protease region of human immunodeficiency virus and virological failure in drug-naïve patients treated with protease inhibitor based therapy. J. Infect. Dis. **184:**983–991.
30. **Rhee, S. Y., W. J. Fessel, A. R. Zolopa, L. Hurley, T. Liu, J. Taylor, D. P. Nguyen, S. Slome, D. Klein, M. Horberg, J. Flamm, S. Follansbee, J. M. Schapiro, and R. W. Shafer.** 2005. HIV-1 protease and reverse-transcriptase mutations: correlations with antiretroviral therapy in subtype B isolates and implications for drug-resistance surveillance. J. Infect. Dis. **192:**456–465.
31. **Romano, L., G. Venturi, S. Bloor, R. Harrigan, B. A. Larder, J. C. Major, and M. Zazzi.** 2002. Broad nucleoside-analogue resistance implications for human immunodeficiency virus type 1 reverse-transcriptase mutations at codons 44 and 118. J. Infect. Dis. **185:**898–904.
32. **Saracino, A., L. Monno, L. Scudeller, D. C. Cibelli, A. Tartaglia, G. Punzi, C. Torti, S. Lo Caputo, F. Mazzotta, G. Scotto, G. Carosi, and G. Angarano.** 2005. Impact of unreported HIV-1 reverse transcriptase mutations on phenotypic resistance to nucleoside and non-nucleoside inhibitors. J. Med. Virol. **78:**9–17.
33. **Sarafianos, S. G., K. Das, J. Ding, P. L. Boyer, S. H. Hughes, and E. Arnold.** 1999. Touching the heart of HIV-1 drug resistance: the fingers close down on the dNTP at the polymerase active site. Chem. Biol. **6:**137–146.
34. **Sarafianos, S. G., K. Das, S. H. Hughes, and E. Arnold.** 2004. Taking aim at a moving target: designing drugs to inhibit drug-resistant HIV-1 reverse transcriptases. Curr. Opin. Struct. Biol. **14:**716–730.
35. **Schinazi, R. F., B. A. Larder, and J. Mellors.** 2000. Mutations in retroviral genes associated to drug resistance: update 2000–2001. Int. Antivir. News **8:**65–91.
36. **Shafer, R. W.** 2002. Genotypic testing for human immunodeficiency virus type 1 drug resistance. Clin. Microbiol. Rev. **15:**247–277.
37. **Sing, T., N. Beerenwinkel, and T. Lengauer.** 2004. Learning mixtures of localized rules by maximizing the area under the ROC curves, p. 89–96. *In* J. Hernandez-Orallo, C. Ferri, N. Lachiche, and P. A. Flach (ed.), ROC analysis in artificial intelligence. Proceedings of the 1st International Workshop ROCAI-2004, Valencia, Spain.
38. **Sing, T., V. Svicher, N. Beerenwinkel, F. Ceccherini-Silberstein, M. Daumer, R. Kaiser, H. Walter, K. Korn, D. Hoffmann, M. Oette, J. K. Rockstroh, G. Fatkenheuer, C. F. Perno, and T. Lengauer.** 2005. Characterization of novel HIV drug resistance mutations using clustering, multidimensional scaling and SVM-based feature ranking, p. 285–296. *In* A. Jorge, L. Torgo, P. Brazdil, R. Camacho, and J. Gama (ed.), Knowledge discovery in databases: PKDD 2005. Springer-Verlag, New York, N.Y.
39. **Stoeckli, T. C., S. MaWhinney, J. Uy, C. Duan, J. Lu, D. Shugarts, and D. R. Kuritzkes.** 2002. Phenotypic and genotypic analysis of biologically cloned human immunodeficiency virus type 1 isolates from patients treated with zidovudine and lamivudine. Antimicrob. Agents Chemother. **46:**4000–4003.
40. **Sturmer, M., S. Staszewski, H. W. Doerr, B. A. Larder, S. Bloor, and K. Hertogs.** 2003. Correlation of phenotypic zidovudine resistance with mutational patterns in the reverse transcriptase of human immunodeficiency virus type 1: interpretation of established mutations and characterization of new polymorphisms at codons 208, 211, and 214. Antimicrob. Agents Chemother. **47:**54–61.
41. **Svicher, V., F. Ceccherini-Silberstein, F. Erba, M. Santoro, C. Gori, M. C. Bellocchi, S. Giannella, M. P. Trotta, A. d'Arminio Monforte, A. Antinori, and C. F. Perno.** 2005. Novel human immunodeficiency virus type 1 protease mutations potentially involved in resistance to protease inhibitors. Antimicrob. Agents Chemother. **49:**2015–2025.
42. **Tantillo, C., J. Ding, A. Jacobo-Molina, R. G. Nanni, P. L. Boyer, S. H.**

Downloaded from jvi.asm.org by on December 4, 2009

Hughes, R. Pauwels, K. Andries, P. A. Janssen, and E. Arnold. 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. **243:**369–387.

43. **Telesnitsky, A., and S. P. Goff.** 1997. Reverse transcriptase and the generation of retroviral DNA, p. 121–160. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Yahi, N., C. Tamalet, C. Tourres, N. Tivoli, F. Ariasi, F. Volot, J. A. Gastaut, H. Gallais, J. Moreau, and J. Fantini.** 1999. Mutation patterns of the reverse transcriptase and protease genes in human immunodeficiency virus type 1-infected patients undergoing combination therapy: survey of 787 sequences. J. Clin. Microbiol. **37:**4099–4106.
45. **Wainberg, M. A.** 2004. HIV-1 subtype distribution and the problem of drug resistance. AIDS **18**(Suppl. 3)**:**63–68.
46. **Wang, D., and B. A. Larder.** 2003. Enhanced prediction of lopinavir resistance from genotype by use of artificial neural networks. J. Infect. Dis. **188:**653–660.

Downloaded from jvi.asm.org by on December 4, 2009