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Extended spectrum of HIV-1 reverse transcriptase mutations in patients receiving multiple nucleoside analog inhibitors

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Abstract

Objective—To characterize reverse transcriptase (RT) mutations by their association with extent of nucleoside RT inhibitor (NRTI) therapy. To identify mutational clusters in RT sequences from persons receiving multiple NRTI.

Design—A total of 1210 RT sequences from persons with known antiretroviral therapy were analyzed: 641 new sequences were performed at Stanford University Hospital; 569 were previously published.

Methods—Chi-square tests and logistic regression were done to identify associations between mutations and NRTI therapy. Correlation studies were done to identify mutational clusters. The Benjamini-Hochberg procedure was used to correct for multiple comparisons.

Results—Mutations at 26 positions were significantly associated with NRTI including 17 known resistance mutations (positions 41, 44, 62, 65, 67, 69, 70, 74, 75, 77, 116, 118, 151, 184, 210, 215, 219) and nine previously unreported mutations (positions 20, 39, 43, 203, 208, 218, 221, 223, 228). The nine new mutations correlated linearly with number of NRTI; 777 out of 817 (95%) instances occurred with known drug resistance mutations. Positions 203, 208, 218, 221, 223, and 228 were conserved in untreated persons; positions 20, 39, and 43 were polymorphic. Most NRTI-associated mutations clustered into three groups: (i) 62, 65, 75, 77, 115, 116, 151; (ii) 41, 43, 44, 118, 208, 210, 215, 223; (iii) 67, 69, 70, 218, 219, 228.

Conclusions—Mutations at nine previously unreported positions are associated with NRTI therapy. These mutations are probably accessory because they occur almost exclusively with known drug resistance mutations. Most NRTI mutations group into one of three clusters, although several (e.g., M184V) occur in multiple mutational contexts.

Keywords

HIV drug resistance; resistance mutations; reverse transcriptase inhibitors; HIV diagnostic tests; antiretroviral therapy

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Introduction

Sequencing of HIV-1 reverse transcriptase (RT) and protease is recommended to help select optimal antiretroviral therapy [1-4]. Mutations at 18 RT residues have been shown experimentally to reduce HIV-1 susceptibility to nucleoside RT inhibitors (NRTI) [1,5]. However, many other RT mutations are typically found during sequencing of clinical isolates and the effect of these other mutations on drug susceptibility is not known. Some of these may be neutral polymorphisms; others may contribute to drug resistance.

We analyzed RT sequences from more than 1100 individuals - including nearly 500 previously unreported sequences - to characterize RT mutations according to their association with NRTI or non-nucleoside RT inhibitors (NNRTI), to identify mutations that increased in proportion to the extent of NRTI therapy, and to identify statistically significant correlations between known drug resistance mutations and previously unreported drug-associated mutations.

Methods

Isolates and sequences

We analyzed HIV-1 RT sequences from individuals having both subtype B viruses and well-characterized antiretroviral treatment histories. We obtained these sequences from previously published studies (appearing in the April 15, 2002 release of the Stanford University HIV RT and Protease Sequence Database; http://hivdb.stanford.edu [6]) and from sequences performed at the Stanford University Hospital Diagnostic Virology Laboratory between 1 July 1997 and 31 December 2001 (see Appendix for GenBank accession numbers). Isolates were subtyped by comparing their sequences with reference sequences of known subtype [7,8] and by bootscanning using a window size of 200 base pairs (bp) and steps of 20 bp [9].

The treatment histories of individuals described in the literature were obtained from the primary publication and were often supplemented by clarifications obtained directly from the authors. The treatment histories of individuals undergoing sequencing at Stanford were obtained by reviewing their medical records. Isolates from individuals lacking a complete record of which NRTI and NNRTI had been received were excluded from analysis.

When an individual had multiple isolates, we included the most recent isolate obtained while the individual was receiving an RT inhibitor. We included more than one isolate from the same individual only when isolates were available both before and after beginning initial treatment with an RT inhibitor, in which case we included one pre-treatment isolate and the most recent post-treatment isolate. Only isolates that included positions 40-240 and that were sequenced by dideoxynucleotide sequencing (i.e., not by hybridization assays) were included in the analysis.

Mutations

Mutations were defined as differences from the consensus B sequence [7]. Sequences having a mixture of wild-type (consensus B sequence) and mutant residues at a single position were considered to have a mutation at that position. Mixtures were detected by clonal sequencing if at least two clones contained the same non-consensus residue. Mixtures were detected by direct PCR sequencing if a non-consensus residue was represented by an electrophoretic peak at least 20-30% of the largest peak present. Mutations at positions 41, 44, 62, 65, 67, 69, 70, 74, 75, 77, 115 116, 118, 151, 184, 210, 215, 219 were considered known NRTI resistance mutations [1]. Mutations at positions 98, 100, 101 103, 106, 108, 179, 181, 188, 190, 225, 227, 230, 236, and 238 were considered known NNRTI resistance mutations [1]. Positions with fewer than five mutations were considered invariant.

Statistical analysis

To identify positions associated with drug therapy, we performed chi-square tests of independence to determine if there was an association between drug treatment and a mutation at the given position. The chi-square statistic was based on a 2×2 contingency table containing counts of individuals with and without NRTI treatment and those with and without mutations at the given position. For most positions, we performed the analysis on the NNRTI-naive subset of patients who had not received any NNRTI therapy. For selected positions, when the number of observed mutations in this subset was too small or when we wished to study a mutation previously reported in the literature, we extended the chi-square analysis to include the total set of patients.

To investigate whether there was a linear relationship between the number of NRTI received and the prevalence of a mutation, we performed a logistic regression analysis in which the number of drugs was the independent variable and the presence or absence of mutation was the dependent variable. Patients were categorized into one of three groups according to extent of treatment: light (one to two NRTI), medium (three to four NRTI), and heavy (five or more NRTI). As with the chi-square analysis, we performed the analysis on the NNRTI-naive subset of patients, but extended the analysis to the total set of patients for selected positions.

We studied the association between mutations and NNRTI therapy using a chi-square analysis similar to our analysis for NRTI therapy. We included all patients in this analysis. We did not perform a logistic regression analysis in this case to study linear relationships, because most patients received at most one NNRTI.

For all of the above tests, we determined which results were statistically significant by applying the method of Benjamini and Hochberg [10]. This method was developed for the problem of multiple hypotheses testing when multiple significant findings are not unexpected. In contrast to the Bonferroni correction, which divides the significance cutoff by the number of hypotheses tested (n), the Benjamini-Hochberg method ranks the hypothesis by their *P* values. Each hypothesis of rank *r* is compared with a significance cutoff, now called a false discovery rate (FDR), divided by (n-r). In this study, FDR of 0.01 and 0.05 were used to determine statistical significance.

We investigated the correlation of mutations between positions induced by NRTI and NNRTI therapy, by calculating the binomial (phi) correlation coefficient for the simultaneous presence of mutations at two positions in the same isolate. We computed the correlations for the subset of patients who had received three or more NRTI and for the subset of individuals that had received an NNRTI. We further investigated the relationships among positions by performing a principal components analysis on the patients who had received three or more NRTI. We used the matrix of correlation coefficients as a measure of similarity between positions. All statistical analysis was performed using the statistical programming package Splus.

Results

Treatment histories

Table 1 groups the individuals in the study according to their treatment histories. Sequences of 1210 isolates from 1124 individuals met our study criteria. Eighty-six individuals had sequences of two isolates each, including one pre-therapy and one post-therapy isolate. Sequences of 569 (47.0%) isolates had been previously published; sequences of 641 (53.0%) isolates were performed at Stanford University Hospital between 1 July 1997 and 31 December 2001. 267 (22.1%) isolates were from previously untreated individuals; 584 (48.2%) isolates were from individuals receiving NRTI but not NNRTI; 357 (29.5%) isolates were from

individuals who received both NRTI and NNRTI; two (0.2%) isolates were from individuals who received NNRTI but not NRTI.

Number of RT mutations and extent of previous NRTI treatment

Fig. 1 shows the median number of differences from consensus B (mutations) and known drug resistance mutations per sequence as a function of the number of NRTI received. The figure shows that the number of mutations increases from four in untreated individuals to 14 in individuals receiving five or more NRTI, while the median number of known NRTI resistance mutations increased from zero in untreated individuals to six in individuals receiving five or more NRTI. The median number of known NNRTI resistance mutations increased from zero in untreated individuals to one in individuals receiving one or more NNRTI. There was a statistically significant stepwise increase in the number of mutations and NRTI resistance mutations associated with increasing NRTI exposure. This relationship was observed in the NNRTI-naive subset as well as the total set of individuals.

RT mutations and NRTI treatment

Fig. 2 shows the mutation frequency at RT positions 1-240 according to the number of NRTI received in the 851 NNRTI-naive individuals. Based on our chi-square analysis, 23 positions were correlated with exposure to NRTI including mutations at 16 known NRTI resistance positions (41, 44, 62, 67, 69, 70, 74, 75, 77, 116, 118, 151, 184, 210, 215, 219) and at seven positions that have not been known to be associated with NRTI resistance (20, 39, 43, 203, 208, 218, and 228).

Extension of our chi-square analysis to the total set of patients revealed three additional positions that correlated with NRTI: 65 (0/269 versus 25/941; uncorrected $P = 0.01$), 221 (0/269 versus 29/941; uncorrected $P = 0.007$), and 223 (0/269 versus 28/941; uncorrected $P = 0.008$). Of these positions, position 65 is a known drug resistance mutation, whereas positions 221 and 223 are novel. The previously reported NRTI resistance mutation, Y115F, occurred more commonly in treated than in untreated individuals in the total set of patients with a $P = 0.05$, but this P value was not statistically significant following the adjustment for multiple comparisons.

The nine previously unreported mutations at positions 20, 39, 43, 203, 208, 218, 221, 223, and 228 occurred almost exclusively together with known drug resistance mutations (777/817, 95%). Three of these mutations, K20R, T39A, and K43E/Q/N were polymorphic occurring in 4%, 4%, and 1% of untreated persons, respectively. The remaining six of these mutations (E203K/D, H208Y, D218E, H221Y, D223Q/E, L228H/R) were completely conserved in untreated individuals. Mutations at positions 60, 64, 104, 122, 135, 196, 200, 207, 211 were polymorphic positions that were statistically associated with drug therapy before the correction for multiple comparisons. Mutations at positions 88 (W88C/S) and 111 (V111I/L) each occurred in 10 treated and no untreated individuals but this was not statistically significant even before the correction for multiple comparisons.

RT mutations and number of NRTI

Our logistic regression analysis revealed mutations at 16 positions that had a statistically significant positive linear relationship between the number of NRTI received and the presence of a mutation in the NNRTI-naive subset of patients. These 16 positions included 10 known drug resistance loci (41, 44, 67, 69, 70, 118, 184, 210, 215, 219) and six of the nine previously unreported drug resistance loci (20, 39, 43, 208, 218, 228). The known drug resistance mutations at positions 62, 65, 74, 75, 77, 115, 116, and 151 and the previously unreported mutations at 203, 221, and 223 were not linearly associated with the number of NRTI received in this NNRTI-naive subset.

RT mutations and their association with NNRTI

In our chi-square analysis of NNRTI-associated mutations, 39 positions were significantly associated with NNRTI therapy, including 24 positions that were associated with NRTI treatment in our original analysis (including 65, 221, 223 but not 116 and 151), 11 positions at which known NNRTI resistance mutations occur (98, 100, 101, 103, 106, 108, 179, 181, 188, 190, 225), and four polymorphic positions (35, 122, 196, 207). The NNRTI resistance mutations at positions 227, 230, 236, and 238 occurred in only two, six, two, and four treated patients, respectively, and were therefore not significantly associated with NNRTI therapy.

Correlations between NRTI resistance mutations

To identify patterns of drug resistance mutations, we calculated the pairwise binary (phi) correlation coefficients among the 18 previously reported NRTI resistance mutations and the additional nine NRTI-associated mutations that we discovered in this study. Fig. 3 illustrates the correlations between each of these 27 NRTI-associated mutations using the color of connecting lines to indicate the direction of association (blue, positive; red, negative) and the thickness of connecting lines to indicate the strength of association. All statistically significant negative correlations are shown; only those positive correlations with a correlation coefficient of < 0.27 are shown.

The tightest cluster was formed by the positions associated with multi-nucleoside resistance: positions 75, 77, 116, and 151. The correlation coefficients between these positions ranged from 0.48 to 0.90. Positions 65 and 115 were also associated with this cluster (median correlation coefficients ranging from 0.27 to 0.42 and 0.36 to 0.60, respectively).

The thymidine analog mutations formed two clusters. One cluster was formed by positions 41, 210, and 215 (correlation coefficients 0.52 to 0.73). Positions 43, 44, 118, 203, 208, and 223 were also linked to this cluster. The second cluster was formed by mutations at positions 67, 70, and 219 (correlation coefficients 0.43 to 0.55). Positions 69, 218, and 228 were also linked to this cluster. Position 215 was correlated with positions 67 (0.36) and 219 (0.18) but not position 70.

Negative correlations occurred between the multi-nucleoside resistance cluster and positions 41 and 215 and between each of the two thymidine analog mutations clusters (e.g., positions 41 and 70, 210 and 70).

A principal components analysis of the relative distance between the 27 NRTI treatment-associated positions based on their degree of co-mutation in isolates from treated persons is shown in Fig. 4. Positions with similar patterns of co-mutation are generally near one another, whereas those with different patterns of comutation are further apart. The first axis, which represents the dimension with the maximum variability, shows the tight clustering of positions associated with multi-nucleoside resistance (positions 65, 75, 77, 115, 116, and 151) and their separation from the mutations selected by thymidine analogs. The second axis, which represents the dimension with the next greatest degree of variability, shows that the clustered mutations at positions 41, 43, 44, 118, 203, 208, 210, 215, and 223 are distinct from those at positions 67, 69, 70, 218, and 219.

Correlations between NNRTI resistance mutations

We repeated the above analysis on the 11 NNRTI-associated mutations that we determined to be statistically significant in this study. The strongest correlation observed was between the NNRTI resistance positions 101 and 190 (0.40); weaker correlations were observed between the NNRTI resistance positions 100 and 103 (0.25) and 181 and 190 (0.21). The only negative correlation was between 101 and 103. Mutations at the NRTI resistance locus at position 74

was positively associated with three NNRTI resistance positions (100, 181, 190); the NNRTI resistance locus at position 108 was weakly correlated with positions 184 and 228; and the NNRTI resistance locus at position 181 was weakly correlated with position 221.

Discussion

The number of RT mutations between positions 1 and 240 in subtype B virus increased from a median of four in untreated individuals to 14 in heavily treated individuals. The increase in mutations with treatment is largely explained by the 18 known NRTI-associated mutations and the 14 known NNRTI-associated mutations. However, mutations at nine additional positions (20, 39, 43, 203, 208, 218, 221, 223, 228) that were for the most part conserved in untreated persons were also strongly associated with drug therapy. Mutations at 11 additional positions were weakly associated with therapy. Sixty-eight positions were polymorphic and appeared to be neutral. One hundred and twenty positions were invariant.

The nine previously unreported mutations included K20R, T39A, K43E/Q/N, E203D/K, H208Y, D218E, H221Y, D223E/Q, L228H/R. Seven of these mutations were clearly associated with NRTI therapy alone; two (H221Y and D223E/Q) were associated with therapy only if individuals receiving both NRTI and NNRTI were included. Each of the nine new mutations was highly correlated with the number of previously received NRTI and generally occurred together with other known NRTI-associated mutations.

The fact that the newly identified mutations occur primarily in combination with previously reported drug resistance mutations suggests that they act as accessories, increasing the level of resistance to NRTI or compensating for decreases in replication associated with other mutations. This supposition should be tested experimentally by site-directed mutagenesis studies to assess the effect of these mutations on drug susceptibility and replication in a wild-type construct and a construct containing the NRTI resistance mutations with which the new mutation commonly occurs.

We performed a cross-sectional analysis of RT mutations in isolates from persons treated with a range of RT inhibitor therapy. Such data indicate the prevalence of specific mutations in individuals with varying degrees of RT inhibitor experience. Only 75 sequences were available from individuals receiving a single NRTI, predominantly zidovudine or didanosine. Longitudinal data would indicate the incidence of new mutations in patients receiving new RT inhibitors and would provide additional insight into the role played by individual mutations in the development of resistance to specific drugs.

One of the nine previously unreported mutations, H208Y, has been associated with foscarnet resistance [11], but none of the other mutations have previously been associated with antiretroviral therapy. The mutations at positions 39 and 43 are close to the thymidine analog-associated mutation at position 41 and are correlated with this mutation. The mutations at positions 218, 221, 223, and 228 are in a highly conserved part of the RT gene that is close to the known NRTI-associated mutations at positions 215 and 219. These amino acids make up parts of the conserved motifs D and E found in all retroviruses [12].

Although the associations between drug therapy and mutations at 221 and 223 were only significant when NNRTI-treated as well as NRTI-treated patients were analyzed, we suspect that these are primarily NRTI-associated mutations because these mutations also occurred in the absence of NNRTI therapy. To explore this question further, we examined the atomic coordinates of these residues within the three-dimensional structure of HIV-1 RT co-crystallized with the NNRTI nevirapine [13]. The closest molecular distances between atoms in H221 and D223 to nevirapine were 11 and 9.5 Å, respectively whereas the closest molecular

distances between the 14 known NNRTI-associated positions and nevirapine is between 2.6-8.8 Å [13].

The correlation and principal components analysis revealed several mutational clusters. The distinction between the multi-nucleoside resistance mutations [14,15] and the thymidine analog mutations defined the first principal component. The distinction between the two sets of thymidine analog mutations defined the second principal component. The strong association between multi-nucleoside resistance and mutations at positions 65 and 115 has not been previously reported. The fact that the thymidine analog associated mutations often cluster into two groups has been noted by others [16-18] and may be particularly relevant for resistance to the nucleotide RT inhibitor tenofovir in that mutations at positions 41, 210, and 215 are associated with higher levels of resistance to tenofovir than mutations at positions 67, 70, and 219 [19]. The fact that M184V was not statistically associated with any other mutation suggests that the decreased fitness associated with this mutation [20,21] does not consistently lead to the development of any other treatment-associated mutation.

Several interactions between NNRTI resistance mutations and NRTI resistance mutations have been reported. The NNRTI mutations at positions 100 and 181 have been shown to reverse T215Y-mediated zidovudine resistance mutations [22,23]. Mutations at positions 74 and 75 have been reported to develop following the development of a mutation at position 190 in isolates cultured in the presence of an experimental NNRTI compound [24]. We did not observe a negative association between mutations at positions 181 and 215. However, we did observe a positive correlation between mutations at positions 74 and 190.

The large number of mutations associated with NRTI therapy is probably a consequence of the high genetic barrier to resistance posed by most dual NRTI combinations. In patients receiving a dual NRTI combination, it is generally not sufficient for HIV-1 to develop just one or two RT inhibitor resistance mutations to escape virus suppression; rather multiple primary as well as secondary accessory mutations appear to be required. The intricate patterns of mutational clusters are further evidence of this genetic barrier to resistance. The negative interactions between certain mutations suggest that resistance does not result simply from the accumulation of any RT mutations but rather from specific combinations of mutations.

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Appendix

The nucleic acid sequence, mutations, drug treatment histories, and GenBank accession numbers can be downloaded as a PDF file from: http://hivdb.stanford.edu. Of the 641 isolates sequenced at the Stanford University Hospital between 1 July 1997 and 31 December 2001, 408 had already been submitted to GenBank [8,25,26]; 286 new sequences were submitted together with this manuscript (AF513992-AF514278).

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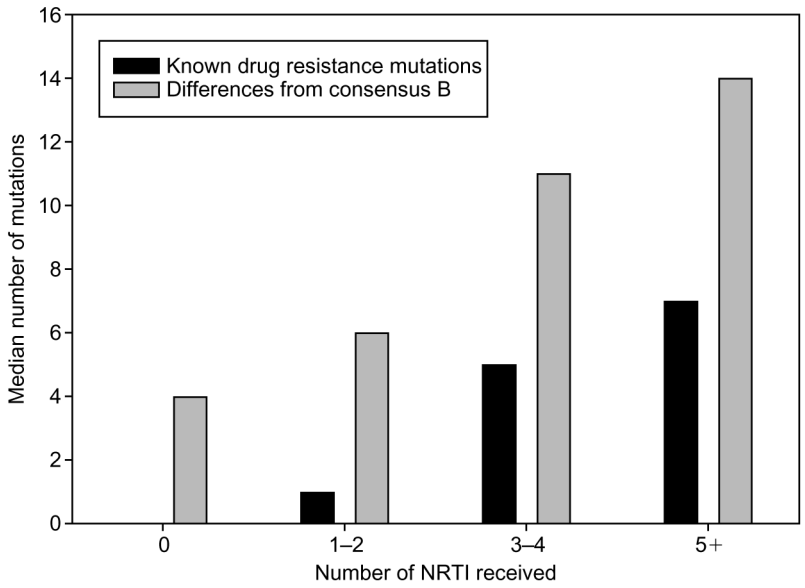

Fig. 1. Median number of differences from consensus B (mutations, grey bars) and known drug resistance mutations (black bars) according to the number of NRTI received

The patients receiving three to four and five or more NRTI were more likely to have also received NNRTI. The median number of NRTI mutations for these two groups was four and six, respectively. The median number of NNRTI mutations for both groups was one.

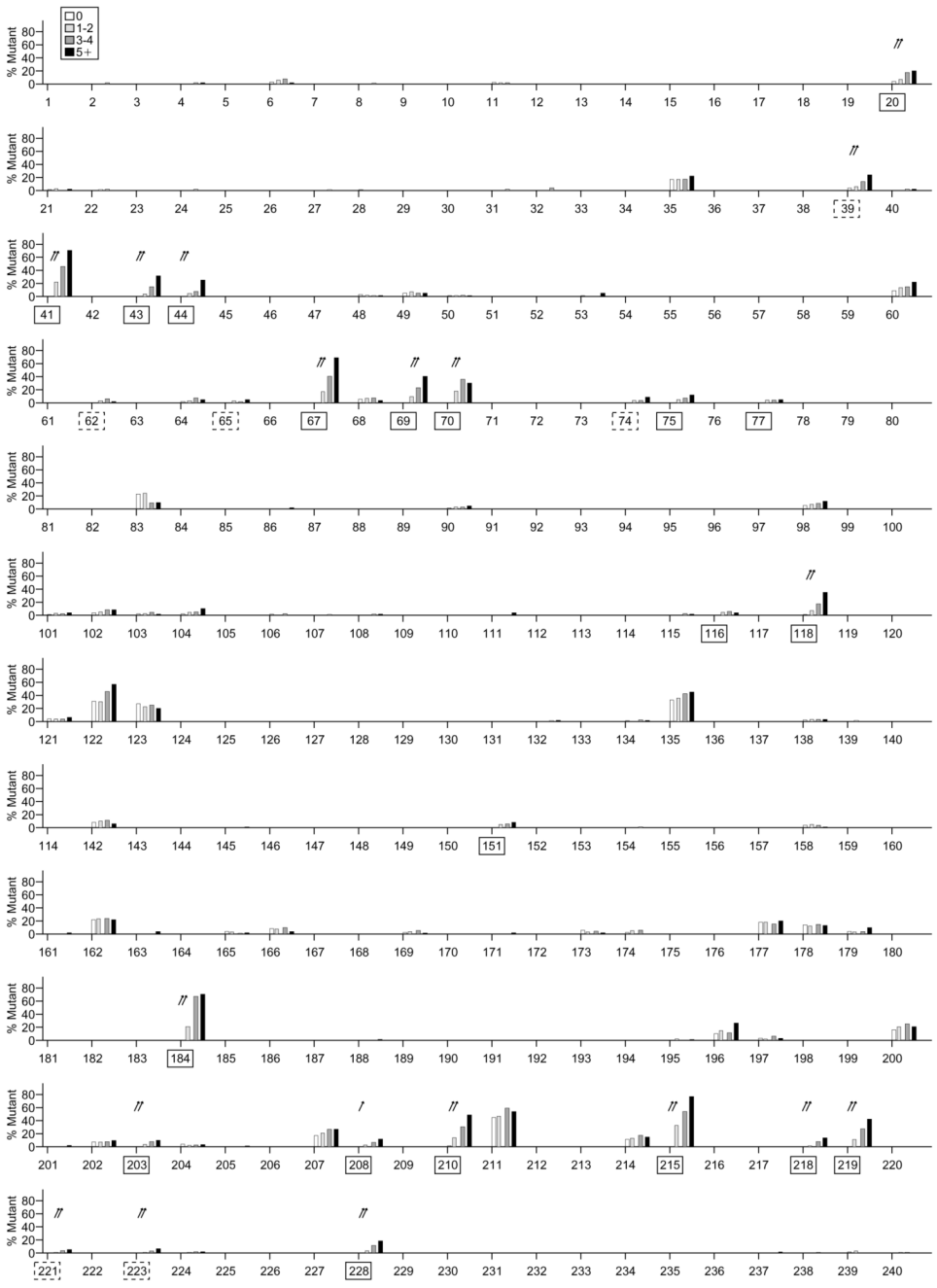

Fig. 2. Mutation frequency at RT positions 1-240 according to the number of NRTI received in NNRTI-naive patients (with the exception of positions 65, 221, and 223, which include data from all patients)

Each bar represents one of the subgroups as defined in Fig. 1, namely 0, 1-2, 3-4, and 5+ NRTI. At each position, a chi-square test of independence was performed to determine if there was an association between NRTI treatment and a mutation at the given position. Positions that were significantly associated with therapy using an FDR of 0.01 (chi-square analysis) are boxed with a solid line; those that were significant using an FDR of 0.05 are boxed with a dotted line. Positions that were significantly associated with increasing drug therapy (logistic regression)

using an FDR of 0.01 are marked with two arrows; those that were significant using an FDR of 0.05 are marked with one arrow.

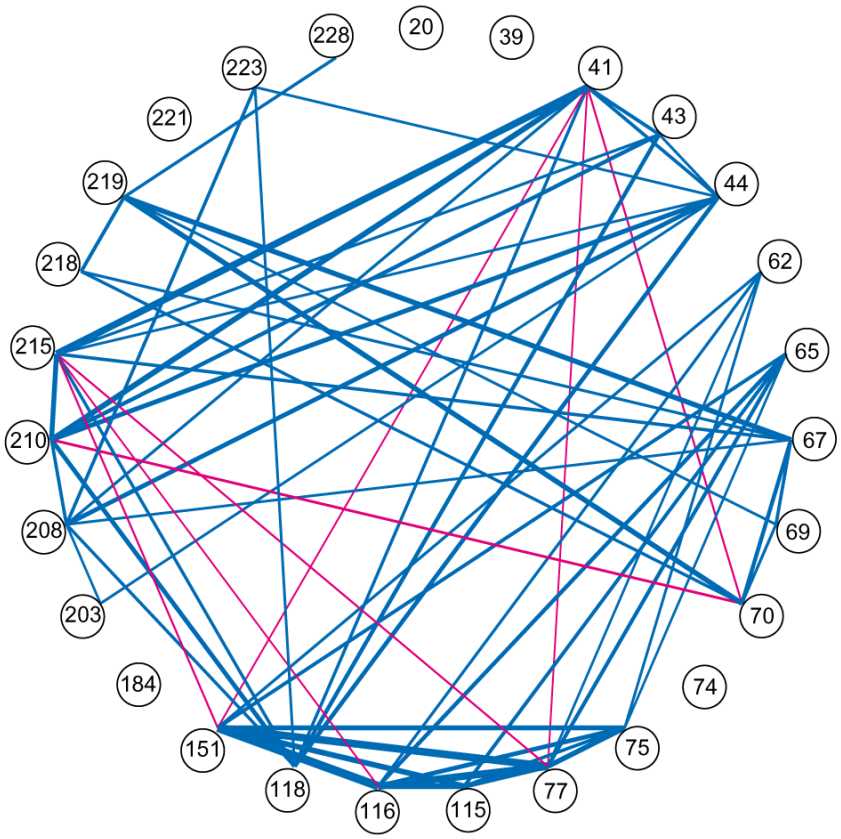

Fig. 3. Correlations among the 18 known NRTI resistance mutations as well as the nine newly identified mutations

Blue lines indicate positions with positive correlation coefficients > 0.27. Red lines indicate positions with negative correlation coefficient. The width of each line is linearly proportional to the value of the correlation coefficient. All positive correlations were statistically significant at $P = 0.05$ using a Bonferroni correction for 351 pairwise comparisons.

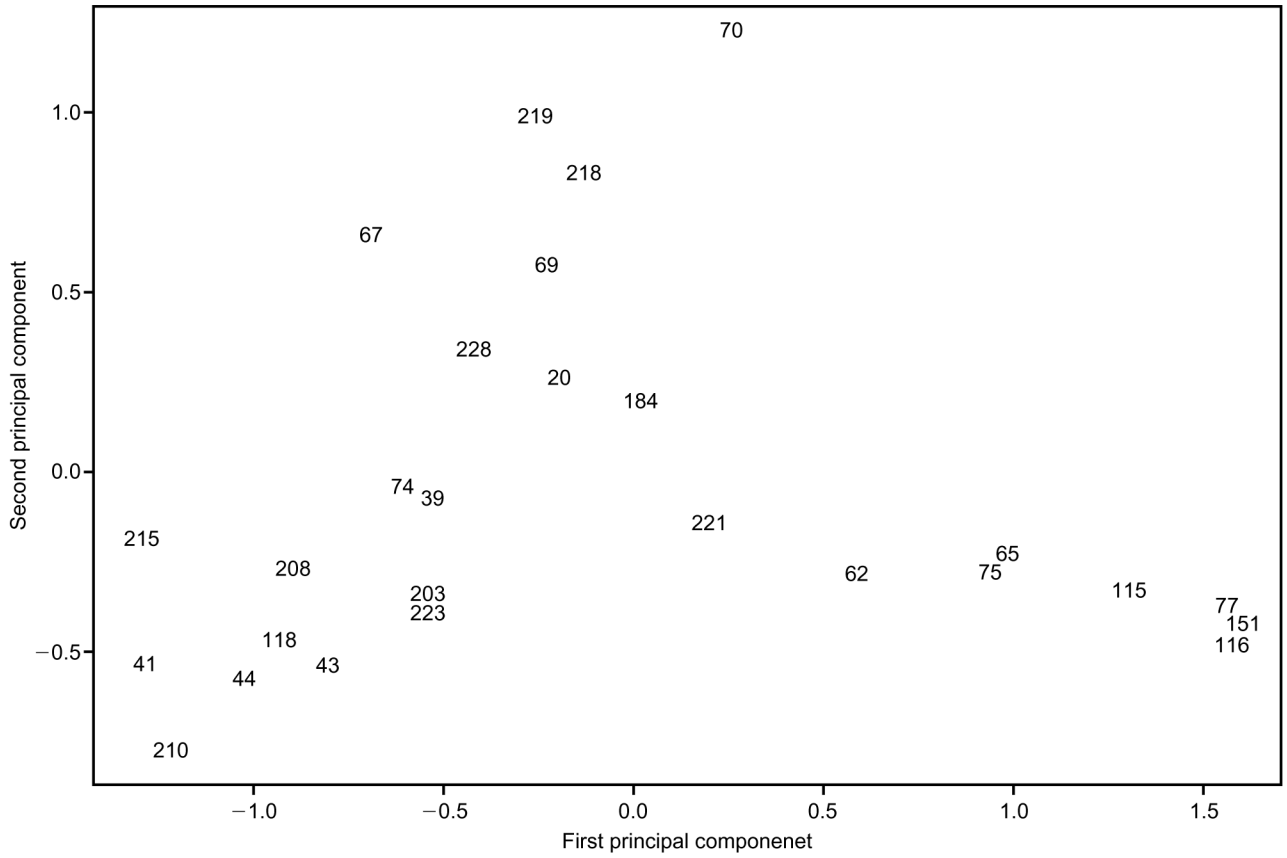

Fig. 4. Principal component analysis

This figure shows the 27 positions that have been either previously reported as associated with NRTI therapy or found to be associated in this study. The graph is a two-dimensional projection of the distances among the 27 positions, where the similarity between any two positions is measured by their binary (phi) correlation coefficient among patients who have received three or more NRTI. Positions that are close together on the graph are those with a high degree of comutation in patients, whereas positions that are far apart are those with a low or negative degree of co-mutation.

Table 1

Summary of RT inhibitor drugs received by 1124 study patients[a]

NRTI received (n)	NRTI								NNRTI		Reference source	
	3TC	ABC	ADV	AZT	d4T	ddC	ddI	TDF	Naive	Experienced	SUH	HIVDB
0	0	0	0	0	0	0	0	0	267	2	46	223
1	1	0	0	54	5	0	15	0	73	2	19	56
2	138	1	1	189	113	17	135	0	269	28	132	165
3	119	4	2	115	84	31	47	0	87	47	94	40
4	221	24	5	218	213	52	175	0	96	131	177	50
5+	206	93	43	206	207	146	204	0	59	149	173	35
Total:	685	122	51	782	622	246	576	0	851	359	641	569

[a]The table shows 1210 isolates because 86 patients had one isolate before NRTI therapy and one isolate following NRTI therapy. NRTI, Nucleoside reverse transcriptase (RT) inhibitor; NNRTI, non-nucleoside RT inhibitor; 3TC, lamivudine; ABC, abacavir; ADV, adefovir; ZDV, zidovudine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; TDF, tenofovir; SUH, sequences done at Stanford University Hospital; HIVDB, previously published sequences in HIV RT and Protease Sequence Database [6].