MAJOR ARTICLE

HIV-1 Reverse Transcriptase Connection Domain Mutations: Dynamics of Emergence and Implications for Success of Combination Antiretroviral Therapy

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Background. Factors promoting the emergence of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) connection domain mutations and their effect on antiretroviral therapy (ART) are still largely undetermined. We investigated this matter by analyzing genotypic resistance tests covering 400 amino acid positions in the RT of HIV-1 subtype B viruses and corresponding treatment histories and laboratory measurements.

Methods. The emergence of connection domain mutations was studied in 334 patients receiving monotherapy or dual therapy with thymidine analogues at the time of the genotypic resistance test. Response to subsequent combination ART (cART) was analyzed using Cox regression for 291 patients receiving unboosted protease inhibitors. Response was defined by ever reaching an HIV RNA level <50 copies/mL during the first cART.

Results. The connection domain mutations N348I, R356K, R358K, A360V, and A371V were more frequently observed in ART-exposed than ART-naive patients, of which only N348I and A360V were nonpolymorphic (with a prevalence of <1.5% in untreated patients). N348I correlated with M184V and predominantly occurred in patients receiving lamivudine and zidovudine concomitantly. A360V was not associated with specific drug combinations and was found to emerge later than M184V or thymidine analogue mutations. Nonpolymorphic connection domain mutations were rarely detected in the absence of established drug resistance mutations in ART-exposed individuals (prevalence, <1%). None of the 5 connection domain mutations associated with treatment showed a statistically significant effect on response to cART.

Conclusions. Despite their frequent emergence, connection domain mutations did not show large detrimental effects on response to cART. Currently, routine implementation of connection domain sequencing seems unnecessary for developed health care settings.

All currently approved drugs can lose their efficacy when human immunodeficiency virus type 1 (HIV-1) becomes resistant to antiretroviral therapy (ART) [1, 2]. Many mutations that confer resistance to specific antiretroviral compounds have been characterized [3]. Because of the mode of action of nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs), these amino acid changes are mostly located in the N-terminal region of the p66 subunit of the HIV reverse transcriptase (RT) (amino acid residues 1–321). Additionally, it has been shown that residues in the connection domain (322–440) and RNase H region (441–560) of RT may also confer resistance to antiretroviral drugs, and mul-

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tiple in vitro studies have explored the possible mechanisms of resistance [4–11]. In contrast, relatively little is known about the dynamics and factors promoting the emergence of connection domain mutations in vivo. Analyses of genotypic sequences from ART-naive and ART-exposed HIV-infected individuals have identified several amino acid changes in the connection domain that are significantly associated with any exposure to ART [9, 12–15], but drug-specific studies are rare. Through transfection experiments, Brehm et al [8] have investigated the emergence of connection domain mutations under selective pressure of zidovudine and found that A371V rapidly followed the established thymidine analogue mutations (TAMs) K70R and D67N. Yap et al [9] have studied the dynamics of the emergence of N348I, one of the few mutations consistently identified as treatment associated in prevalence studies. By analyzing genotypic tests from routine clinical care, they observed that N348I emerged at about the same time as M184V in the course of treatment but considerably earlier than TAMs. The selection of N348I was primarily associated with zidovudine use, and the risk was further enhanced through the concomitant administration of the NNRTI nevirapine. Hachiya et al [13] further speculated about a role for didanosine in the N348I selection, although on the basis of limited observational data. Recently, we have found evidence for a strong association between N348I and M184V, reflected by a 5-fold higher rate of N348I emergence in patients receiving zidovudine and lamivudine together, compared with that in patients receiving zidovudine alone [16].

To date, evidence for an effect of connection domain mutations on the response to ART is very sparse. Yap et al [9] observed an increase in plasma HIV RNA level after the emergence of N348I. In contrast, Hachiya et al [17] noted no difference in initial HIV RNA decline on ART in 21 individuals for whom connection domain mutations (other than N348I) were present in baseline samples, compared with that in a control group for whom connection domain mutations were not present. Brehm et al [18] investigated the role played by connection domain mutations in 60 patients newly initiating efavirenz and 2 NRTIs, who later experienced virological rebound during therapy within the setting of the AIDS Clinical Trials Group (ACTG) 5142 trial. Their matched comparison including baseline and failure samples yielded no connection domain mutations that were significantly associated with rebound. Paredes et al [19] studied the effect of connection domain mutations on the response to NNRTI-based combination therapy in 287 patients from EuroSIDA. The only mutation associated with a higher risk of virological failure was A376S in a subset of 115 patients receiving nevirapine. In a subanalysis of the OPTIMA (Options in Management with Antiretrovirals) trial, Dau et al [20] reported an association between the presence of at least 1 of a set of 13 connection domain mutations and a lack of a decrease in HIV RNA level by 1 $\log_{10}$ copies per milliliter after 24 weeks of therapy.

In the present study, we aimed to investigate the in vivo dynamics of the emergence of connection domain mutations in patients who received monotherapy or dual therapy with NRTIs, including the thymidine analogues zidovudine or stavudine. Moreover, we set out to assess the effect of connection domain mutations on the response to the first combination ART (cART) consisting of 2 NRTIs and either an unboosted protease inhibitor (PI) or an NNRTI.

METHODS

Viral sequences spanning the full protease and the first 400 amino acids of the RT from 3 different studies were pooled for this analysis: 351 sequences from the ACTG 320 trial (http://hivdb.stanford.edu/pages/clinicalStudyData/ACTG320.html) [21, 22], 1188 sequences from the Swiss HIV Cohort Study (SHCS) (GenBank accession nos. of sequences used for the present study: GQ848100–GQ848156) [23, 24], and 11 sequences from a lamivudine monotherapy trial (GenBank accession nos. GU301078–GU301088) [25]. From this data set, we selected subtype B sequences that were obtained while patients were receiving ART with thymidine analogue treatment (with or without lamivudine or didanosine), provided that the patient had never taken NNRTI or PI drugs at the time of genotypic testing ($n = 334$). Twenty-five patients with exposure to both didanosine and lamivudine were excluded. As a sensitivity analysis, we repeated all statistical calculations on a data set restricted to tests performed while patients were receiving zidovudine, zidovudine-lamivudine, or zidovudine-didanosine as the first ART (first-line sample; $n = 71$). These sensitivity analyses generally did not change point estimates but often no longer returned statistically significant P values. Therefore, we only report results from the larger sample because of the greater statistical power.

To study the dynamics of the emergence of connection domain mutations, we first screened the RT region for mutations, which were enriched in patients with exposure to NRTI monotherapy or dual therapy. Drug resistance mutations were defined according to the International AIDS Society–USA (IAS-USA) list of December 2008 [3]. For the present analysis, we complemented our data with 521 subtype B sequences generated before any ART exposure. The prevalence of mutations was then compared between NRTI-experienced and ART-naive patients by the Fisher exact test. After adjustment for multiple testing, only mutations with a significantly higher prevalence among treated compared with therapy-naive patients were considered for the following analyses. Next, we tested whether any of the mutations in the set of therapy-associated amino acid changes were associated with exposure to a specific NRTI drug, using the Fisher exact test. We further investigated patterns of

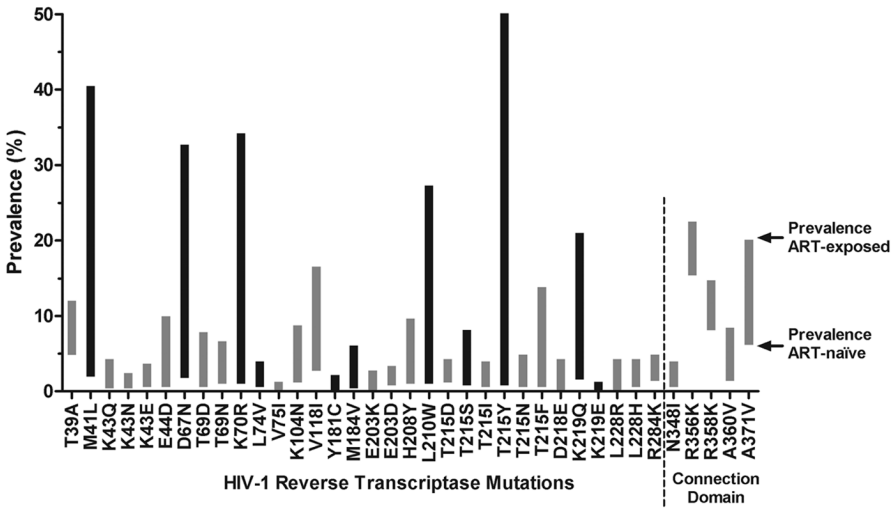

Figure 1. Prevalence of mutations associated with exposure to antiretroviral therapy (ART). Shown is the prevalence of 37 reverse transcriptase mutations with a statistically significant higher prevalence (after correction for multiple testing) among samples from 334 patients who received nucleoside reverse transcriptase inhibitor monotherapy or dual therapy, compared with that among 521 samples from ART-naive individuals (see Table 1). The lower and upper ends of bars represent the observed prevalence in untreated and treated human immunodeficiency virus (HIV) infected individuals, respectively. Black bars indicate International AIDS Society–USA drug resistance mutations (December 2008 list).

covariation between mutations by performing a hierarchical clustering analysis and by plotting cluster trees. Similarity scores between pairs of mutations (ie, the probabilities for co-occurrence of mutations) for clustering analyses were calculated on the basis of the Jaccard index, which is the proportion of sequences with a co-occurrence of 2 specific mutations, divided over all sequences with at least 1 of the mutations from that pair [26]. Robustness of results was tested by repeating the analysis on resampled data sets (bootstrapping) [27]. Complementary to the clustering analysis, we used Bayesian networks to investigate dependencies between established resistance mutations with connection domain mutations [28].

The clinical relevance of connection domain mutations in patients receiving the first cART consisting of 2 drug classes was evaluated using 2 separate analyses: the first assessed virological failure, defined as either nonresponse (>500 HIV RNA copies/mL at week 24) or a later rebound of viremia to levels >500 copies/mL, and the second assessed response to treatment, defined as achieving a viral load <50 copies/mL at least once. The failure analysis included 331 individuals initiating the first treatment with no-longer-recommended combination therapy with 2 NRTIs and an unboosted PI [29], of whom 85 individuals who were not included in the dynamics study because of concomitant exposure to didanosine and lamivudine or exposure to NNRTIs before genotypic testing. The response analysis was restricted to a subset of these 331 patients with HIV RNA quantifications determined using a detection threshold of 50 copies/mL ($n = 291$). In addition, we analyzed 40 patients who received the first NNRTI treatment and for whom a genotypic resistance test result obtained before cART initiation was available. Because the timing of HIV RNA quantification was strictly controlled within the ACTG 320 study protocol but not within the SHCS, we performed a discrete time survival analysis, whereby failure or response to cART was not evaluated at the exact time of detection but only to a level of predefined 12-week intervals [30]. Follow-up was censored after 48 weeks of therapy or if a patient changed cART before week 48 without having experienced an event. Study baseline was set at the time of cART initiation. Multivariable Cox regression was used to adjust for baseline differences in the genotypic sensitivity score of cART (as determined by the Stanford algorithm [31]), HIV RNA level, and CD4 cell count and to assess the effect of connection domain mutations on end points.

Statistical analyses were performed using Stata 11 SE (StataCorp). Bayesian network analysis was conducted using Bcourse [32]. The level of significance was set at 5%, and all *P* values are 2-sided. The Benjamini-Hochberg correction with a false-discovery rate of 5% was applied to all *P* values, to restrict the proportion of false-positive associations resulting from multiple testing [33].

RESULTS

Association between connection domain mutations and NRTI monotherapy or dual therapy. We discovered a total of 37 mutations that were significantly more prevalent in patients with exposure to ART (Figure 1 and Table 1), of which 11 (black bars in Figure 1) are listed as drug resistance mutations by the IAS-USA [3]. In addition, a number of treatment-associated mutations not considered to be primary resistance

Table 1. Associations between Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mutations and Antiretroviral Therapy

Mutation	Treatment naive (n = 521)	Treatment exposed (n = 334)	P[a]	TA only (n = 113)	TA 3TC (n = 20)	TA ddI (n = 201)	P[b]
T39A	24 (4.6)	41 (12.3)	<.001	8 (7.1)	3 (15.0)	30 (14.9)	NS
M41L	9 (1.7)	136 (40.7)	<.001	32 (28.3)	7 (35.0)	97 (48.3)	.002
K43E	2 (0.4)	13 (3.9)	<.001	2 (1.8)	0 (0)	11 (5.5)	NS
K43Q	1 (0.2)	15 (4.5)	<.001	4 (3.5)	1 (5.0)	10 (5.0)	NS
K43N	1 (0.2)	9 (2.7)	.001	0 (0)	0 (0)	9 (4.5)	NS
E44D	2 (0.4)	34 (10.2)	<.001	3 (2.7)	2 (10.0)	29 (14.4)	.002
D67N	8 (1.5)	110 (32.9)	<.001	30 (26.5)	2 (10.0)	78 (38.8)	.005
T69N	4 (0.8)	23 (6.9)	<.001	9 (8.0)	1 (5.0)	13 (6.5)	NS
T69D	2 (0.4)	27 (8.1)	<.001	8 (7.1)	0 (0)	19 (9.4)	NS
K70R	4 (0.8)	115 (34.4)	<.001	40 (35.4)	3 (15.0)	72 (35.8)	NS
L74V	2 (0.4)	14 (4.2)	<.001	0 (0)	0 (0)	14 (7.0)	.004
V75I	0 (0)	5 (1.5)	.009	0 (0)	0 (0)	5 (2.5)	NS
K104N	5 (1.0)	30 (9.0)	<.001	11 (9.7)	0 (0)	19 (9.4)	NS
V118I	13 (2.5)	56 (16.8)	<.001	7 (6.2)	4 (20.0)	45 (22.4)	<.001
Y181C	0 (0)	8 (2.4)	.001	0 (0)	0 (0)	8 (4.0)	NS
M184V	1 (0.2)	21 (6.3)	<.001	2 (1.8)	17 (85.0)	2 (1.0)	<.001
E203K	0 (0)	10 (3.0)	<.001	0 (0)	2 (10.0)	8 (4.0)	NS
E203D	3 (0.6)	12 (3.6)	.002	3 (2.7)	1 (5.0)	8 (4.0)	NS
H208Y	4 (0.8)	33 (9.9)	<.001	7 (6.2)	2 (10.0)	24 (11.9)	NS
L210W	4 (0.8)	92 (27.5)	<.001	15 (13.3)	3 (15.0)	74 (36.8)	<.001
T215S	3 (0.6)	28 (8.4)	<.001	11 (9.7)	2 (10.0)	15 (7.5)	NS
T215N	2 (0.4)	17 (5.1)	<.001	9 (8.0)	1 (5.0)	7 (3.5)	NS
T215D	5 (1.0)	15 (4.5)	.002	2 (1.8)	0 (0)	13 (6.5)	NS
T215I	2 (0.4)	14 (4.2)	<.001	2 (1.8)	1 (5.0)	11 (5.5)	NS
T215Y	3 (0.6)	173 (51.8)	<.001	51 (45.1)	6 (30.0)	116 (57.7)	NS
T215F	2 (0.4)	47 (14.1)	<.001	10 (8.9)	1 (5.0)	36 (17.9)	NS
D218E	0 (0)	15 (4.5)	<.001	1 (0.9)	0 (0)	14 (7.0)	NS
K219E	0 (0)	5 (1.5)	.009	0 (0)	0 (0)	5 (2.5)	NS
K219Q	7 (1.3)	71 (21.3)	<.001	18 (15.9)	1 (5.0)	52 (25.9)	NS
L228R	0 (0)	15 (4.5)	<.001	0 (0)	0 (0)	15 (7.5)	.004
L228H	2 (0.4)	15 (4.5)	<.001	2 (1.8)	1 (5.0)	12 (6.0)	NS
R284K	6 (1.1)	17 (5.1)	.001	4 (3.5)	1 (5.0)	12 (6.0)	NS
E312Q	4 (0.8)	3 (0.9)	NS	1 (0.9)	1 (5.0)	1 (0.5)	NS
G335C	10 (1.9)	2 (0.6)	NS	0 (0)	0 (0)	2 (1.0)	NS
G335D	15 (2.9)	2 (0.6)	NS	1 (0.9)	1 (5.0)	0 (0)	NS
N348I	2 (0.4)	14 (4.2)	<.001	4 (3.5)	7 (35.0)	3 (1.5)	<.001
R356K	79 (15.2)	76 (22.8)	.006	27 (23.9)	5 (25.0)	44 (21.9)	NS
R358K	41 (7.9)	50 (15.0)	.001	15 (13.3)	3 (15.0)	32 (15.9)	NS
A360V	6 (1.1)	29 (8.7)	<.001	11 (9.7)	2 (10.0)	16 (8.0)	NS
A360I	1 (0.2)	2 (0.6)	NS	1 (0.9)	0 (0)	1 (0.5)	NS
V365I	19 (3.7)	18 (5.4)	NS	5 (4.4)	2 (10.0)	11 (5.5)	NS
T369I	1 (0.2)	1 (0.3)	NS	1 (0.9)	0 (0)	0 (0)	NS
A371V	31 (5.9)	68 (20.4)	<.001	20 (17.7)	5 (25.0)	43 (21.4)	NS
A376S	48 (9.2)	28 (8.4)	NS	9 (8.0)	2 (10.0)	17 (8.5)	NS
E399D	72 (13.8)	27 (8.1)	NS	8 (7.1)	2 (10.0)	17 (8.5)	NS

NOTE. Data are no. (%) of patients, unless otherwise indicated. 3TC, lamivudine; ddI, didanosine; NS, not statistically significant; TA, thymidine analogue.

[a] P values are for the difference in prevalence between treatment naive and treatment exposed (Fisher exact test). Only P values that remained statistically significant after adjustment for multiple testing are shown.

[b] P values are for the difference in prevalence across the type of TA treatment (Fisher exact test). Only P values that remained statistically significant after adjustment for multiple testing are shown.

mutations by the IAS-USA were observed [27, 34]. A total of 5 mutations located in the C-terminus of the RT were found to be significantly associated with therapy, of which N348I, A360V, and A371V have previously been described as possibly conferring drug resistance. The mutations R356K, R358K, and A371V were quite common in therapy-naive patients (prevalence, >5%) and are most likely polymorphic. As a control, we examined the prevalence of these 5 and other previously described connection domain mutations (E312Q, G335C/D, A360I, V365I, T369I, A376S, and E399D) in patients exposed to lamivudine monotherapy. For the 11 patients for whom baseline and during-treatment genotypic sequences were available, we did not find amino acid changes in the connection domain, with the exception of mutations R356K (3/11 [27.3%]) and R358K (2/11 [18.2%]) (data not shown).

Several of the mutations selected in the above analysis also showed statistically significant heterogeneity across the 3 treatment groups zidovudine, zidovudine-lamivudine, and zidovudine-didanosine (Table 1). As expected, the drug-specific mutations M184V and L74V were almost exclusively observed among patients with the appropriate exposures (lamivudine and didanosine, respectively). The TAMs M41L and L210W were more frequently seen in patients exposed to zidovudine-didanosine [35]. The only connection domain mutation significantly linked to a specific treatment was N348I, which was more frequently selected in patients receiving zidovudine-lamivudine (Table 1 and [16]). The remaining connection domain mutations could not be attributed to a specific zidovudine-containing therapy.

Dynamics and covariation of connection domain mutations. When assessing covariation of mutations (Figure 2), only a few discernible patterns emerged. Aside from the expected clustering of mutations belonging to the TAM pathways [36], the only robust pattern observed was the linkage of the connection domain mutation N348I with M184V. No further patterns became apparent when we repeated the clustering analysis for each treatment group separately. Next, we explored dependencies in the appearance of the selected mutations using Bayesian network analysis (Figure 3). TAM clusters were observed in treatment-experienced patients, as seen with the hierarchical clustering analysis shown in Figure 2. A linked pathway was observed between mutations M184V and N348I, restricted to exposure to both lamivudine and zidovudine. The appearance of the mutation A360V toward the end of pathways suggested a later emergence relative to the IAS-USA mutations. On the basis of these observations, we speculated that the emergence of selected mutations follows the hierarchical order M184V < N348I < TAMs < A360V. Genotypic test results were assigned to 4 groups on the basis of the latest mutation present according to the proposed ordering (eg, a genotypic test result indicating the presence of M184V and TAMs would be attributed to the TAM group). We then compared the duration of exposure to thymidine analogues at the time of genotypic testing with respect to the 4 mutation groups and found that M184V had the shortest duration (median, 252.5 days; interquartile range [IQR], 161–344 days; $n = 2$), followed by N348I (median, 577 days; IQR, 478–894 days; $n = 5$), TAMs (median, 994 days; IQR, 492–1772.5 days; $n = 228$), and A360V (median, 1285 days; IQR, 336–1758 days; $n = 29$); the difference reached marginal statistical significance ($P = .077$, Jonckheere-Terpstra test for ordered alternatives).

Taken together, the data on the dynamics of mutation emer-

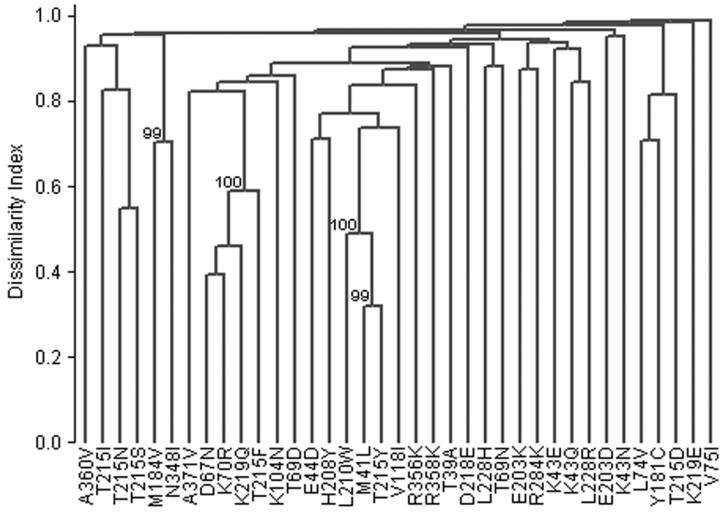

Figure 2. Clustering of connection domain and drug resistance mutations. Shown are the results of hierarchical clustering analysis of 37 mutations significantly associated with exposure to antiretroviral therapy from 334 subtype B sequences. Only bootstrap values >90% (out of 100 replications) are shown.

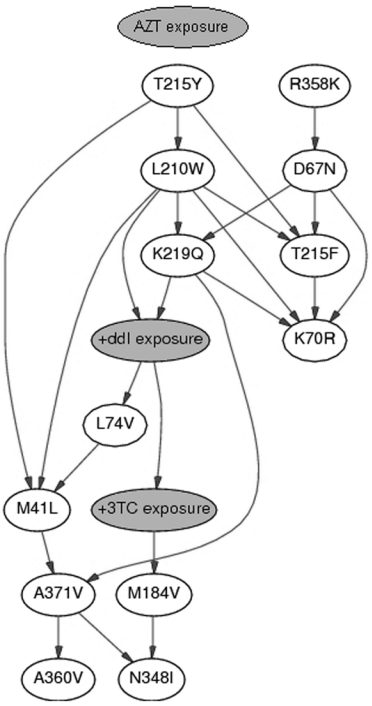

Figure 3. Bayesian network showing dependencies in the emergence of resistance mutations. All patients ($n = 334$) received zidovudine either alone or in combination with didanosine or lamivudine. A directed arrow between 2 mutations (eg, M184V and N348I) or between an antiviral drug and a mutation (eg, lamivudine and M184V) suggests a direct influence. For example, interpretations for the arrows connecting lamivudine exposure, M184V, and N348I are that M184V is selected by the antiretroviral drug lamivudine and that N348I predominantly occurs when M184V is already present. Note that the mutation R356K is not shown because it is independent from all other mutations. Moreover, the arrow from didanosine exposure to lamivudine exposure is an artefact from the analysis, because patients were selected such that they could have only received either lamivudine or didanosine. 3TC, lamivudine; AZT, zidovudine; ddI, didanosine.

gence imply that the appearance of nonpolymorphic connection domain mutations N348I and A360V does not precede currently known drug resistance mutations and that these mutations are associated with other known IAS-USA drug resistance mutations. To further test this hypothesis, we screened the full SHCS drug resistance database of 1188 HIV sequences covering the first 400 amino acids of the RT for the presence of connection domain mutations. This analysis showed that nonpolymorphic connection domain mutations (N348I and A360V, in particular) were only rarely found in the absence of other drug resistance mutations (in <1% of genotypic tests for ART-exposed patients) and at a prevalence similar to that in ART-naive patients (data not shown; all $P > .16$, Fisher exact test). This suggests that if no nucleoside analogue resistance mutations are detected in samples from ART-exposed patients, an extended search for nonpolymorphic connection domain mutations would most likely return negative results.

Effect of the presence of connection domain mutations on the outcome of cART. We proceeded to assess therapy outcomes of cART for individuals with prior thymidine analogue exposure and who for the first time started cART with 2 NRTIs and an unboosted PI ($n = 331$) or an NNRTI ($n = 40$, of whom 37 received efavirenz). Patients receiving boosted PI were not considered because of low numbers.

In addition to the connection domain mutations significantly associated with ART, we included the mutation A376S in the analysis [19]. As shown in Table 2, the proportion of patients with a virological response <50 copies/mL to combination therapy with a first-generation unboosted PI was 46% and increased to 65% in patients receiving an NNRTI. After adjustment for the genotypic sensitivity score of combination therapy and the HIV RNA level and CD4 cell count at the start of cART, none of the tested connection domain mutations reached statistical significance after correction for multiple testing in this set of highly ART-exposed individuals. The Cox models with virological failure as the end point also showed no significant associations between connection domain mutations and treatment response. Although these negative results could have been caused by insufficient statistical power, given the observed effect sizes, a post-hoc simulation showed that larger effects on treatment response (ie, response rates of <18% for the mutations N348I and A360V, which had the smallest group sizes) could have been detected with >80% power by a univariable Fisher exact test.

DISCUSSION

This data set of 334 subtype B sequences provided a unique opportunity to study the dynamics of drug resistance emergence, because all patients included in this analysis were receiving nonsuppressive NRTI treatments until the time of genotypic testing. We observed 5 mutations located in the HIV RT connection domain that were significantly more frequent in NRTI-exposed than NRTI-naive patients. Of these mutations, only N348I and A360V were nonpolymorphic. The analysis of covariation of mutations confirmed a previous finding that N348I often co-occurred with M184V and was predominantly selected during therapy containing thymidine analogues and lamivudine. Moreover, our Bayesian network analysis suggested a late occurrence of A360V relative to IAS-USA mutations. Despite their relatively frequent emergence, connection domain mutations did not seem to have a major effect on the response to cART in this population with high exposure to zidovudine monotherapy or dual therapy. Because these mutations mainly increase resistance against thymidine analogues and leave PIs and lamivudine unaffected, this was to be expected for the patients starting treatment with unboosted PIs. Because of limited sample size, no firm conclusions could be reached with regard to the effect of connection domain mu-

Table 2. Response to First Combination Antiretroviral Therapy (cART)

Parameter	Virological failure[a]		Virological response[a]	
	Proportion (%)	HR (95% CI)	Proportion (%)	HR (95% CI)
cART with unboosted PI				
Overall				
Drug naive at baseline[b]	16/90 (17.8)		53/77 (68.8)	
Drug exposed at baseline	105/331 (31.7)		135/291 (46.4)[c]	
Drug exposed, by mutation				
N348I	4/15 (26.7)	0.79 (0.26–2.47)	7/11 (63.6)	1.78 (0.72–4.39)
R356K	29/79 (36.7)	1.07 (0.70–1.64)	30/72 (41.7)	0.98 (0.65–1.49)
R358K	22/47 (46.8)	1.47 (0.88–2.46)	15/38 (39.5)	0.84 (0.49–1.44)
A360V	10/28 (35.7)	1.50 (0.72–3.13)	14/27 (51.9)	1.02 (0.59–1.76)
A371V	23/69 (33.3)	1.08 (0.68–1.72)	25/60 (41.7)	0.84 (0.54–1.32)
A376S	7/23 (30.4)	1.04 (0.53–2.03)	9/19 (47.4)	1.27 (0.75–2.16)
M184V	11/31 (35.5)	0.76 (0.34–1.70)	5/13 (38.5)	0.76 (0.21–2.72)
cART with NNRTI				
Overall				
Drug naive at baseline[d]	4/133 (3.0)		116/133 (87.2)	
Drug exposed at baseline	8/40 (20.0)		26/40 (65.0)	
Drug exposed, by mutation				
N348I	1/4 (25.0)	1.20 (0.02–66.72)	2/4 (50.0)	0.46 (0.07–3.23)
R356K	3/5 (60.0)	4.31 (0.51–36.68)	2/5 (40.0)	1.56 (0.22–10.91)
R358K	2/7 (28.6)	0.84 (0.12–6.05)	3/7 (42.9)	0.62 (0.17–2.18)
A360V	0/3 (0)	NC	3/3 (100)	NC
A371V	3/11 (27.3)	0.71 (0.18–2.73)	6/11 (54.6)	1.86 (0.85–4.05)
A376S	2/6 (33.3)	0.94 (0.12–7.25)	3/6 (50.0)	0.81 (0.31–2.18)
M184V	6/27 (22.2)	2.36 (0.25–22.21)	17/27 (63.0)	1.45 (0.48–4.34)
Pooled analysis				
Overall				
Drug naive at baseline	20/223 (9.0)		169/210 (80.5)	
Drug exposed at baseline	113/371 (30.5)		161/331 (48.6)	
Drug exposed, by mutation				
N348I	5/19 (26.3)	0.95 (0.30–2.95)	9/15 (60.0)	1.23 (0.56–2.70)
R356K	32/84 (38.1)	1.16 (0.76–1.78)	32/77 (41.6)	0.95 (0.64–1.42)
R358K	24/54 (44.4)	1.38 (0.83–2.28)	18/45 (40.0)	0.80 (0.47–1.35)
A360V	10/31 (32.3)	1.42 (0.69–2.92)	17/30 (56.7)	1.22 (0.75–1.98)
A371V	26/80 (32.5)	1.10 (0.72–1.69)	31/71 (43.7)	0.87 (0.58–1.30)
A376S	9/29 (31.0)	1.10 (0.60–2.00)	12/25 (48.0)	1.21 (0.74–1.98)
M184V	17/58 (29.3)	0.89 (0.44–1.78)	22/40 (55.0)	1.30 (0.65–2.59)

NOTE. CI, confidence interval; HR, hazard ratio; NC, not computable; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

[a] Models are adjusted for baseline CD4 cell count, baseline HIV RNA level, and genotypic sensitivity score of ART.

[b] Connection domain mutations present at cART initiation were R356K ($n = 17$), R358K ($n = 7$), A360V ($n = 2$), A371V ($n = 3$), and A376S ($n = 5$), but no association with therapy failure or response was observed in adjusted Cox regression models (data not shown).

[c] Only includes individuals for whom all HIV RNA quantifications were performed with a lower threshold of detection of 50 copies/mL.

[d] Connection domain mutations present at cART initiation were R356K ($n = 19$), R358K ($n = 10$), A360V ($n = 1$), A371V ($n = 6$), and A376S ($n = 14$), but no association with therapy failure or response was observed in adjusted Cox regression models (data not shown).

tations on combination therapy with NNRTIs. However, patients receiving efavirenz or nevirapine exhibited somewhat higher response rates than did patients receiving unboosted PIs, despite the presence of connection domain mutations (Table 2). It is worth noting that the majority of the NNRTI-treated patients received efavirenz. Considering that both N348I and A376S mutations have been associated with nevirapine resistance, a larger data set is necessary to determine the effect of these mutations on virological failure in nevirapine-experienced patients.

What are the implications of these data for clinical practice and HIV drug resistance testing? Many standard genotypic resistance assays do not cover RT regions beyond amino acid residue 240, and hence the connection domain or RNase H are

not included. The issue of whether to include the RT C-terminus genetic region in genotypic sequencing for routine clinical care is debated, especially in light of the expected additional cost [37, 38]. The clinical and virological effect of these mutations are not well understood in vivo. However, they point to an at-most subtle effect of N348I, A371V, and A360V on virologic response to treatment [9, 17–20]. Moreover, the connection domain mutations studied here were rarely observed in the absence of other IAS-USA mutations in patients exposed to nucleoside analogue inhibitors. Therefore, the danger of misinterpreting genotypic test results as being fully sensitive to all drugs despite the presence of connection domain mutations is diminishingly small. Thus, we believe that a pragmatic approach is required with regard to the sequencing of the connection domain region. As our data clearly demonstrate, the risk of the emergence of certain connection domain mutations (such as N348I, A360V, or A371V) is fairly high after prolonged exposure to virologically failing treatment containing zidovudine (or stavudine). Because certain mutations (such as N348I and A376S) also affect susceptibility to nevirapine [9], the inclusion of connection domain mutations in resistance testing may be beneficial if a salvage regimen including NNRTIs is initiated. This reasoning may be of special relevance for Africa, where generic fixed-dose combinations of stavudine, lamivudine, and nevirapine are commonly used, which may lead to an increased prevalence—and possibly also transmission—of connection domain mutations [39]. Thus, although routine implementation of connection domain and RNase H sequencing in developed health care settings currently seems unnecessary, further research is warranted to study the effect of these mutations in more specific resource-limited settings.

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