Connection Domain Mutations During Antiretroviral Treatment Failure in Mali: Frequencies and Impact on Reverse Transcriptase Inhibitor Activity

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Abstract

Mutations in the connection domain (CD) of reverse transcriptase (RT) have been implicated in RT inhibitor (RTI) resistance, but this is controversial and little is known in non-B subtype HIV-1. We determined CD mutations prevalence in a population infected predominantly with CRF02_AG and investigated associations with phenotypic RTI resistance. Detected CD mutations were G335D (82.3%), A371V (69.8%), E399D (9.4%), N348I (5.2%), V365I (4.2), Y318F (2.1%), G333E (2.1%) and A360V (2.1%). Mutations were largely polymorphic and did not confer RTI resistance. The observed trend towards reduced likelihood of etravirine or nevirapine resistance in the presence of G335D should be investigated further.

Keywords

Connection Domain; Mali; resistance; treatment-experienced; etravirine; nevirapine

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INTRODUCTION

HIV-1 reverse transcriptase (RT) has a complex structureand encodes 560 amino acids in its N- and C-terminals.[1, 2] Inhibitors of RT are widely used as antiretroviral agents globally. Mutations in the N-terminal are well-characterized precursors of resistance to RT inhibitors.[3] Less is known about the C-terminal since standard genotyping focuses on the approximately 300 codons in the N-terminal. The connection domain (CD), codons 316–437, is the proximal part of the C-terminal and links the N-terminal to the RNaseH domain. It appears CD mutations can evolve during antiretroviral therapy (ART) or contribute to resistance to specific RT inhibitors, but this is controversial and there is no consensus that CD mutations have clinical relevance.[4 –11] Of note, studies to date, with few exceptions,[10] have focused on subtype B HIV-1. We determined the prevalence of CD mutations in ART-experienced HIV-1 patients infected with non-B subtype HIV-1 and investigated associations between CD mutations, N-terminal mutations and resistance to RT inhibitors.

METHODS

Study Population

Participants were recruited between August 2009 and February 2010 from four outpatient HIV clinics in Mali, West Africa. Inclusion criteria were: confirmed HIV-1 infection and virologic failure on nevirapine based first-line therapy or boosted protease-inhibitor (PI) based second-line therapy. Virologic failure was defined as plasma HIV-1 RNA > 1000 copies/ml after at least six months on current regimen. Patients with HIV-2 co-infection were excluded. The ethics committee of the University of Bamako School of Medicine approved the study. Each patient gave informed consent.

Procedures and definitions

CD4 + T cell (CD4) count and HIV RNA measurement were performed at the Virology Laboratory, University of Bamako, using the BD FACSCount [Becton, Dickinson and Company, San Jose, United States] and Easy Q HIV-1 v1.2 assay [EQ; BioMerieux, France], respectively.

Full-length population sequencing (RT and Protease genes) and phenotyping were performed at Janssen Diagnostics, Beerse, Belgium using frozen plasma samples. To create amplicons, RNA was extracted from plasma on an automated RNA extraction platform. The *Gag* (p7/p1-p1/p6)-Protease-Reverse Transcriptase (PR-RT) coding sequence was reverse transcribed and amplified in a one-step RT-PCR, followed by a nested PCR. For genotypic testing Dideoxy sequencing reactions were performed on the purified amplicon. Sequence data files were grouped per sample identifier and aligned against the reference HXB2 reference sequence. Procedures used to determine viral phenotype are described in detail elsewhere [12]. Phenotype was expressed as half maximal effective concentration (EC_{50}) values defined as the concentration of compound achieving 50% inhibition of the virus-induced EGFP signals as compared to the untreated virus-infected control cells. The ratio between the plasma isolate EC_{50} and the wild-type reference virus (IIIB) EC_{50} gave the fold change (FC) value. **Clade was determined using the vircoTYPE algorithm.**

Drug resistance mutations in RT were interpreted according to the International AIDS Society–USA Drug Resistance Mutations 2011 update.[3] Thymidine analogue mutations (TAMs) were defined as M41L, D67N, K70R, L210W, T215F/Y, K219E/Q. The CD mutations considered were E312Q, Y318F, G333D/E, G335C/D, N348I, A360I, A360V, V365I, A371V, A376S, and E399G.[4]

Statistical analysis

HIV-1 subtype was dichotomized as CRF02_AG (the predominant subtype in Mali) versus 'other'. Mutations detected by genotype were included in analyses only if >10% prevalent in the study cohort. HIV phenotype for each drug was classified as resistant or sensitive based on EC_{50}-fold change from wild-type virus inhibition. HIV RNA and CD4 counts were log-transformed to fit a uniform distribution. Multivariate models were chosen by step-wise removal of non-significant terms until the difference in the modified Bayesian information criterion suggested weak support for a nested model. The conservative Bonferroni correction offset the problem of multiple-comparisons.

Three logistic regression analyses were performed to determine variables associated with: connection domain mutations; N-terminal mutations; and phenotypic drug resistance. Independent variables assessed in both univariate and multivariate analyses were CD4 count, HIV RNA, first-line failure, subtype CRF02_AG, and presence of G335D, A371V or 'other CD' mutation. Multicollinearity of the independent variables was assessed by pairwise correlations and found to be minimal with a mean variance inflation factor of 1.33. The P value threshold for significance was 0.05, uncorrected. Database management and analytical testing was completed on Stata SE 10.1 (College Station, TX).

RESULTS

Study population

Ninety-six individuals (76 failing first-line ART and 20 failing second-line ART) met inclusion criteria. The first-line regimen for all 96 individuals was nevirapine (NVP) plus lamivudine (3TC) plus a thymidine analogue (zidovudine, ZDV or stavudine, d4T). Second-line therapy comprised a ritonavir boosted-PI and two or three nucleos(t)ide reverse transcriptase inhibitors (NRTI). The specific antiretroviral drugs used in second-line were ritonavir-boosted lopinavir (90%), 3TC (75%), ZDV (40%), didanosine (30%), abacavir (30%), tenofovir (20%) and d4T (10%). HIV-1 subtypes were CRF02_AG (69.8%), CRF06_cpx (16.7%), CRF09_cpx (4.2%), and other (any of subtype C, subtype A1, CRF01_AE, CRF19_cpx, subtype G, CRF05_DF = 9.4%). Median [IQR] CD4 count was similar in both failure groups (137.5 [93.0–255.5] versus 143.5 [110.5–222.5] cells/mm^3, P = 0.675). Median [IQR] HIV RNA was higher in the second-line failure group ($\log_{10}$ 4.29 [3.83–4.82] versus $\log_{10}$ 5.28 [4.75–.5.74] copies/mL, P<0.005).

Distribution of CD and N-terminal mutations

Table 1 shows prevalence of CD mutations, N-terminal mutations, resistant phenotypes, and their associations in univarate analysis. Eight CD mutations were identified: G335D (82.3%), A371V (69.8%), E399D (9.4%), N348I (5.2%), V365I (4.2), 318F (2.1%), G333E (2.1%) and A360V (2.1%). In univariate analysis, G335D (OR 19.9 [95%CI 5.1–78.2], P <0.005) and A371V (OR 10.8 [3.9–30.0], P <0.005) were significantly associated with CRF02_AG, and with each other (OR 8.75 [2.7–28.3], P <0.005). After controlling for HIV-1 subtype, their association with each other diminished (OR 3.16 [0.8–12.4], P= 0.10), thus highlighting separate independent associations of G335D and A371V with viral subtype (G335D OR 12.0 [2.8–52.6], P <0.005; A371V OR 7.0 [2.3–21.7], p<0.005). CD4 count, HIV RNA or treatment failure group did not affect the prevalence of CD mutations.

The most prevalent N-terminal mutations were M184V/I (55.2%), Y181C/I/V (29.2%), K103N (20.8%) and TAMs (19.8%). Forty-seven other mutations were present, each in less than 10% of the cohort. There were no differences in the prevalence of the N-terminal mutations by CD4 count or HIV RNA. On univariate analysis, M184V/I was associated with 'other' CD mutations (OR 2.91 [1.03–8.2], P= 0.043). The association remained significant

in a multivariate model (OR 4.1 [1.3–12.5], P= 0.014, Bonferroni adjusted significance = 0.025). Within the examined cohort, K103N mutation was only present in subtype CRF02_AG (p <0.005) and always occurred with G335D (P= 0.019). In analysis limited to only CRF02_AG (N= 67), there was no longer an association between K103N and G335D (P= 0.549, Fisher's exact). G190A (10.4% prevalence) was negatively associated with CRF02_AG (OR 0.14 [0.02–0.8], P= 0.028) amongst first-line failures (N= 76) in multivariate modeling.

Phenotypic resistance to RT inhibitors

Among patients who completed phenotypic resistance testing (N= 69), resistance irrespective of treatment experience was most prevalent against nevirapine (69.6%), 3TC (66.7%), efavirenz (62.3%), and emtricitabine (62.3%). In the multivariate model, nevirapine resistance was less likely with subtype CRF02_AG (OR 0.17 [0.03–0.90], P= 0.037) despite 100% prevalence of K103N within CRF02_AG virus. The only other prevalent NVP-specific resistance mutations were Y181C/I/V (29.0%) and G190A (7.3%), and they were always associated with phenotypic nevirapine resistance. However, K103N, Y181C/I/V mutations and G190A were not collinear and were absent in 27.1% of isolates with phenotypic nevirapine resistance. Further univariate analysis dichotomizing subtype as CRF02_AG (n=50) versus CRF06_CPX (n=12) attenuated the association of CRF02_AG with reduced likelihood of phenotypic resistance to NVP (OR 0.12 [0.02–1.24], P= 0.078, but remained significant after controlling for A371V (OR 0.50 [0.003–0.66], P= 0.023). Phenotypic etravirine resistance was present in 37.7% of isolates with no difference between first- and second-line failure groups (OR 4.3 [0.8–22.7], P= 0.091) on multivariate analysis.

On univariate analysis, G335D was associated with reduced risk of resistance to nevirapine (OR 0.12 [0.01–0.99], P= 0.049) and etravirine (OR 0.31 [0.09–1.00], P= 0.05), but not efavirenz (OR 0.34 [0.09–1.3], P= 0.121). In the multivariate model, there remained a significant association with reduced risk of resistance to etravirine (OR 0.27 [0.08–0.94], P=0.040) in the presence of G335D. The reduction of resistance to etravirine is further highlighted when controlling for Y181C/V/I mutations and presence of K103N (OR 0.10 [0.02–0.7], P= 0.020). For nevirapine, the protective effect of G335D was also evident (OR 0.09, P= 0.034), however it was difficult to separate the effect of subtype. The protection remained significant in those without K103N or Y181C/V/I mutations (OR 0.04 [0.005–0.4], P= 0.008, N=37).

DISCUSSION

We identified eight CD mutations, most commonly G335D (82.3%) and A371V (69.8%), among HIV-1 patients failing ART in Mali. Each of the other identified CD mutations (E399D, N348I, V365I, 318F, G333E, and A360V) was less than 10% prevalent. The distribution of CD mutations was similar in first-and second-line failures. In addition, the distributions of HIV-1 subtypes and CD mutations in the two failure groups in our study were similar to data from ART-naïve patients in Mali.[13] These findings suggest that the CD mutations are largely polymorphic in the study population. A high prevalence of CD mutations has been reported as well in CRF01_AE-infected patients experiencing treatment failure [10] In B subtype HIV-1, CD mutations are less common and appear to evolve during ART. This was demonstrated in the OPTIMA study (96.8% subtype B) where the frequencies of CD mutations in ART failures were A371V (21.4%), A376S (15.5%) and N348I (12.9%)[4]. CD mutations in OPTIMA were less frequent than observed in our study, but more frequent than in treatment-naïve subtype B patients. In another study, N3481, R356K, R358K, A360V and A371V were more frequently detected in ART-exposed compared with ART-naïve subtype B patients.[5]

It is uncertain whether CD mutations have clinical relevance, despite suggestions that some can influence antiretroviral susceptibility, typically when co-existent with N-terminal mutations. Illustratively, polymorphic G335D or A371V in CRF01_AE subtype did not confer resistance by themselves, but there was increased ZDV resistance when either of these mutations was present with TAMs.[10] In subtype B, CD mutations that have been associated with resistance include E312Q, G333E/D, G335D, N3481, A360I/V, V365I, T369I, A371V and A376S [6–9], but these associations are controversial; other investigators did not find a major detrimental effect of N3481, R356K, R358K, A360V or A371V on response to ART.[5] In the current study, we found an association between presence of G335D and a tendency towards reduced risk of phenotypic etravirine or nevirapine resistance. No association was detected between resistance to any NRTI and detection of CD mutation(s). Our finding should be interpreted with caution because we could not completely untangle potential confounding effects of viral subtype. Further, K103N was seen primarily in CRF02_AG subtype and always with G335D in our cohort. The K103N mutation does not cause etravirine resistance and it has been suggested that it may increase susceptibility to etravirine.[14] In contrast to our results from a non-B subtype HIV population, a previous study found that CD mutations had no impact on virologic response to etravirine, but approximately two-thirds of the patients in that study had B-subtype HIV infection.[15] Also in contrast to our results, studies with approximately 90% B subtype HIV-1 representation reported that N348I and A376S conferred varying degrees of nevirapine resistance[11], and that E399D conferred resistance to etravirine.[16]

A key strength of our study is that we assessed phenotypic resistance, thus avoided potential flaws of genotype-based resistance algorithms especially with poorly characterized CD mutations. Because we did not have pre-treatment and longitudinal samples from the patients however, we were unable to ascertain which CD mutations were present before ART exposure. Nevertheless, comparison of our result with data from treatment-naïve patients revealed striking similarities in the distribution of CD mutations, suggesting that selection of CD mutations under antiretroviral drug pressure was uncommon in our cohort. **Phylogenetic analysis suggested rare transmission events and did not alter our results (data not shown).**

In conclusion, there is a high prevalence of CD mutations in non-B subtype HIV-1 patients failing ART in Mali. Identified CD mutations are likely polymorphic and do not appear to confer NRTI or NNRTI resistance in the prevalent subtypes. Our finding of a tendency towards reduced resistance to etravirine and nevirapine in the presence of G335D should be investigated further.

Acknowledgments

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Table 1

Prevalence of N-terminal, Connection domain (CD) mutations and phenotypes

Dependent variable	Prevalence N (%)	log10 CD4 OR (p-val)	log10 VL OR (p-val)	First-line failure OR (p-val)	CRF02_AG OR (p-val)	G335D OR (p-val)	A371V OR (p-val)	otherCD OR (p-val)
CD mutations[1]								
G335D	79 (82.3)	1.41 (0.715)	0.75 (0.397)	1.21 (0.763)	**19.9 (<0.005)**	--	**8.75 (<0.005)**	0.71 (0.564)
A371V	67 (69.8)	1.03 (0.966)	0.79 (0.406)	0.72 (0.570)	**10.8 (<0.005)**	**8.75 (<0.005)**	--	0.59 (0.288)
other CD mutation	23 (24.0)	0.37 (0.247)	1.31 (0.362)	2.02 (0.299)	0.59 (0.288)	0.71 (0.563)	0.59 (0.288)	--
N-terminal mutations[2] (N=96)								
Any TAMs	19 (19.8)	0.48 (0.411)	1.49 (0.220)	0.98 (0.979)	0.52 (0.212)	1.18 (0.807)	0.92 (0.885)	2.22 (0.148)
D67N	12 (12.5)	0.54 (0.564)	1.26 (0.548)	0.76 (0.705)	0.56 (0.360)	2.59 (0.379)	2.37 (0.287)	2.62 (0.134)
V90I	12 (12.5)	0.22 (0.156)	1.31 (0.486)	1.36 (0.705)	5.5 (0.111)	pos (0.117)[3]	2.37 (0.287)	0.60 (0.531)
A98G	10 (10.4)	2.56 (0.465)	0.99 (0.981)	**0.13 (0.004)**	0.39 (0.161)	0.85 (0.841)	0.39 (0.161)	2.35 (0.219)
M184V/I	53 (55.2)	3.27 (0.123)	1.13 (0.641)	1.30 (0.599)	1.49 (0.370)	1.49 (0.458)	2.23 (0.076)	**2.91 (0.043)**
K103N	20 (20.8)	0.74 (0.731)	1.11 (0.740)	2.79 (0.195)	**∞ (<0.005)[3]**	**∞ (0.019)[3]**	2.95 (0.107)	2.71 (0.065)
G190A	10 (10.4)	0.30 (0.284)	0.79 (0.581)	pos (0.115)[3]	0.39 (0.161)	0.85 (0.841)	1.83 (0.463)	3.78 (0.053)
Y181C/I/V	28 (29.2)	0.85 (0.839)	0.91 (0.731)	2.78 (0.129)	0.44 (0.087)	0.52 (0.235)	0.56 (0.217)	1.08 (0.878)
H221Y	12 (12.5)	0.32 (0.284)	1.30 (0.497)	1.36 (0.705)	1.34 (0.675)	1.09 (0.920)	0.85 (0.801)	1.71 (0.420)
Phenotypic resistance (N=69)								
abacavir	14 (20.3)	0.32 (0.288)	1.00 (0.991)	**0.16 (0.009)**	2.68 (0.227)	1.86 (0.454)	1.43 (0.586)	0.98 (0.975)
didanosine	16 (23.2)	0.56 (0.570)	0.95 (0.883)	0.33 (0.105)	7.71 (0.057)	5.38 (0.119)	1.23 (0.735)	1.27 (0.719)
efavirenz	43 (62.3)	0.37 (0.297)	0.95 (0.863)	**7.06 (0.007)**	0.49 (0.235)	0.34 (0.121)	0.99 (0.982)	2.97 (0.121)
emtricitabine	43 (62.3)	0.88 (0.886)	1.18 (0.586)	0.80 (0.733)	1.29 (0.641)	0.79 (0.695)	2.21 (0.126)	1.89 (0.324)
etravirine	26 (37.7)	0.79 (0.790)	0.94 (0.829)	3.64 (0.115)	0.42 (0.119)	**0.31 (0.050)**	1.01 (0.982)	0.79 (0.695)
lamivudine	46 (66.7)	0.52 (0.499)	1.02 (0.927)	1.00 (1.000)	1.24 (0.703)	0.67 (0.537)	2.33 (0.111)	1.49 (0.537)
nevirapine	48 (69.6)	0.48 (0.457)	0.65 (0.204)	**4.30 (0.027)**	**0.19 (0.040)**	**0.12 (0.049)**	0.67 (0.475)	2.00 (0.327)
stavudine	10 (14.5)	**0.07 (0.045)**	**3.55 (0.018)**	0.24 (0.053)	3.95 (0.208)	1.13 (0.885)	1.29 (0.732)	1.68 (0.497)
tenofovir	12 (17.4)	0.18 (0.134)	2.06 (0.094)	0.33 (0.121)	0.71 (0.622)	0.48 (0.291)	0.46 (0.230)	2.09 (0.291)
zidovudine	24 (34.8)	0.62 (0.599)	1.07 (0.841)	0.46 (0.230)	0.88 (0.825)	0.75 (0.632)	0.83 (0.729)	0.92 (0.894)

[1] Not present: E312Q, E312Q, G333D, G335C, A360I, A376S, E399G; >0 and 5%: Y318F, G333E, A360V, V365I; >5 and 10%: N348I, E399D; -- = not analyzed

[2] Present <10%: M41L, A62V, K65R, K70R, L74V, V75I, F77L, L100I, K101E, K101P, V106A, V106I, V106M, V108I, F116Y, Q151M, Y155F, V179D, V179E, V179F, V179I, V179T, Y188C, Y188H, Y188L, G190E, G190Q, G190S, G190T, L210W, L215F, K219E, K219Q, K101H, K101I, K101R, E138A, E138G, E138R, V179L, V179M, T215Y, P225H, M230L, K238N, K238T

[3] Fisher's exact used given inestimable coefficients for binomial logistic regression; pos = positive direction of association, ∞ = asymptotically infinite