Identification of Novel Resistance-Related Polymorphisms in HIV-1 Subtype C RT Connection and RNase H Domains from Patients Under Virological Failure in Brazil

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Abstract

Mutations in the connection and RNase H C-terminal reverse transcriptase (RT) domains of HIV-1 have been shown to impact drug resistance to RT inhibitors. However, their impact in the context of non-B subtypes has been poorly assessed. This study aimed to characterize resistance-related mutations in the C-terminal portions of RT in treatment-failing patients from southern Brazil, a region with endemic HIV-1 subtype C (HIV-1C). Viral RNA was isolated and reverse transcribed from 280 infected subjects, and genomic regions were analyzed by polymerase chain reaction, DNA sequencing, and phylogenetic analysis. Two novel mutations, M357R and E529D, were evidenced in Brazilian HIV-1C strains from treatment-failing patients. In global viral isolates of subjects on treatment, M357R was selected in HIV-1C and CRF01_AE and E529D was selected in HIV-1 subtype B (HIV-1B). While most C-terminal RT mutations described for HIV-1B also occur in HIV-1C, this work pinpointed novel mutations that display subtype-specific predominance or occurrence.

Keywords: HIV, epidemiology, antiretroviral therapy, reverse transcription

Introduction

HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) is classified into nine pure subtypes (A–D, F–H, J, and K) and multiple unique and circulating recombinant forms.¹ These variants circulate heterogeneously worldwide.^{2,3} In Brazil, the major circulating HIV-1 subtype is B (HIV-1B), yet other subtypes such as A1, C, D, F1, BF1, and BC recombinants have also been found.^{4–7} The southern region of Brazil is unique, with an endemicity of HIV-1 subtype C (HIV-1C). Several studies have reported an increase in HIV-1C prevalence in that region.^{8–16} In the city of Rio Grande, one of the foci of this study, such an increase is clearly evident: from 22% in 2002 to 56% in 2010.^{16,17}

The introduction of highly active antiretroviral therapy (HAART) in HIV-infected patients is associated with a marked reduction in morbidity and mortality.^{18,19} Resistance to antiretroviral (ARV) drugs is a major cause of treatment failure and has been associated with higher mortality rates.^{20,21}

The ARV drugs that compose HAART target key enzymes of the virus life cycle, including reverse transcriptase (RT), protease (PR), and integrase, the first being the main target to which two classes of inhibitors have been developed, the nucleoside and the nonnucleoside RT inhibitors (NRTI and NNRTI).

HIV-1 RT is formed by two subunits; the first harbors a polymerase (POL), a connection (CN), and an RNase H (RNH) domain, whereas the latter lacks RNH. RTI act in the POL domain, where all drug resistance mutations have been initially characterized.²² However, a novel mechanism of RT resistance has been recently proposed, in which mutations in CN and RNH can decrease susceptibility to thymidine analogues by decreasing the RNH enzymatic activity.^{23,24} Currently, CN and RNH are not included in resistance genotyping assays, but their clinical impact is controversial and remains poorly characterized. Limited studies have attempted to evaluate these mutations among drug-naïve and treated subjects,^{14,25–28} and virtually all so far conducted have assessed

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them in HIV-1B, with very few reports in non-B subtypes.^{14,28} Studies carried out with treatment-experienced patients have pinpointed differences in drug resistance occurring at the RT POL domain of distinct HIV-1 subtypes. For example, HIV-1C acquires less resistance mutations than HIV-1B to PI and NRTI.²⁹ HIV-1 RT C-terminal domains may as well display differences in the context of non-B variants, a subject that requires investigation.³⁰

This study aims to characterize resistance-related mutations in the C-terminal portions of HIV-1 RT from patients under virological failure followed in southern Brazil, a region where HIV-1C prevails.

Methods

Study subjects and sample collection

A total of 287 HIV⁺ samples have been studied in this work. One hundred five HIV⁺ patients under virological failure followed at the HIV/AIDS Service of Universidade Federal de Rio Grande Hospital ($n=35$), at Porto Alegre City Health Secretary ($n=36$) and at Curitiba City Health Secretary ($n=34$), all located in southern Brazil (states of Rio Grande do Sul and Paraná), were enrolled in the study. In addition, 105 samples of treatment-naïve patients (42 from Porto Alegre and 63 from Curitiba) and 77 samples of treated patients under virological control (undetectable HIV viral load) from Hospital de Clínicas de Porto Alegre were analyzed for comparison. This study was approved by the Ethics Committee of Universidade Federal de Rio Grande University Hospital (CEPAS; no. 63/2008) and by the Curitiba City Health Secretary (no. 137/2007).

Among the patients failing therapy at the time of sample collection, the average number of different regimens to which they have been subjected to was four. Approximately 40% had been subjected to previous mono and/or dual therapy with NRTI. Of those subjected to HAART, 25% only used PI as a second class of ARV drugs (in addition to NRTI), 22% used only NNRTI, and 52% had used both PI and NNRTI classes. One percent of the patients used triple therapy based only on NRTI, being naïve to both NNRTI and PI. Among those under virological control, only 36% had switched therapy at least once (22% due to intolerance to one of the drugs in the regimen and 14% due to failure).

Viral RNA extraction, reverse transcriptase polymerase chain reaction, and DNA sequencing

Viral RNA was extracted and complementary DNA synthesis was carried out in plasma samples from Rio Grande city, while genomic DNA was extracted from samples from Porto Alegre as previously described.⁷ HIV-1 RT CN and RNH portions from all patients were amplified in seminested polymerase chain reaction (PCR), providing PCR fragments of 665 and 469 bp, respectively. Primers and PCR conditions for both reactions have been previously described.^{14,16,28} Purified PCR products were sequenced with the Prism[®] BigDye[™] Terminator Cycle Sequencing Ready Reaction Kit (Thermo Scientific, Carlsbad, USA) using the inner primers of each respective PCR reaction. DNA sequencing was carried out in an automated ABI 3130XL Genetic Analyzer (Thermo Scientific).

Sequence and phylogenetic analyses

Sequence files were manually edited and assembled using SeqMan (DNASTar, Madison, USA). For HIV-1 subtype assignment, sequences were aligned with BioEdit v.7.0³¹ together with reference sequences of each HIV-1 subtype, retrieved from the Los Alamos HIV Database (<http://hiv-web.lanl.gov>). Aligned sequences were subject to phylogenetic analysis using neighbor-joining and Kimura two-parameter implemented in MEGA 5.0.³² Clade robustness was assessed by bootstrap, using 1,000 replicates. Sequences not clustering with any subtype were further subject to recombination analysis using bootscanning in Simplot v.3.5.1.³³ All DNA sequences generated in the study have been deposited in GenBank and were assigned the accession numbers KC680774 to KC680820.

Drug resistance mutation analysis

CN and RNH sequences were aligned with HXB2 (GenBank accession no. K03455) in BioEdit and visually inspected for resistance-related mutations. Mutations considered were N348I, A360V, T369I/V, A371V, A376S, A400T, D488E, Q509L, and Q547K for their recognized phenotypic role in drug resistance.^{34–38} For the discovery of novel HIV-1C mutations associated with drug exposure, RT C-terminal sequences of this subtype were compared to global and Brazilian HIV-1C sequences from drug-naïve subjects. Global consensus was obtained from the Los Alamos database, whereas the Brazilian consensus was inferred using sequences obtained from this study and additional 91 HIV-1C sequences of CN and 115 of RNH recently described by our group.¹⁴ The patients under treatment were divided in two groups: (1) Brazilian virologic control, in which the viral load was undetectable (<50 viral RNA copies/mL of blood) and (2) Brazilian RTI resistant.

Prevalence of novel mutations in HIV-1 subtypes and recombinant forms

To verify the prevalence of novel RT C-terminal treatment-related mutations, sequences of CN and RNH of subtypes A–D, F and G, and recombinant forms CRF01_AE and CRF02_AG were obtained from the Stanford University HIV Drug Resistance Database (<http://hivdb.stanford.edu>). While the entire RT of CRF01_AE belongs to subtype A, the one of CRF02_AG has a mixed A/G CN and a subtype G RNH domain. Sequences were aligned and classified in three groups: (1) treatment-naïve subjects, (2) viral isolates from NRTI-experimented patients, and (3) viral isolates from NRTI- and NNRTI-experimented patients. Mutation analyses were performed as described above.

We have also surveyed the potential impact of specific drugs on the selection of novel mutations in different HIV-1 subtypes and CRFs in a context of monotherapy. HIV-1 sequences were retrieved from patients receiving zidovudine (AZT) or nevirapine (NVP) monotherapy in the early days of the epidemic. Only subtypes/CRFs for which five or more sequences at the studied positions were available have been analyzed in comparison with drug-naïve strains from the respective subtypes/CRF.

Statistical analyses

The significance of differences in the presence of CN and RNH resistance mutations between HIV-1 subtypes was

TABLE 1. RESISTANCE-RELATED POLYMORPHISMS IN THE REVERSE TRANSCRIPTASE C-TERMINAL DOMAINS OF HIV-1B AND C SEQUENCES DETERMINED IN THIS STUDY

Polymorphism	Total % (n)	HIV1-B % (n)	HIV-1C % (n)
N348I	11 (11/99)	8 (4/49)	14 (6/42)
T369I/V	3 (3/99)	2 (1/49)	2 (1/42)
A371V	15 (15/99)	12 (6/49)	12 (5/42)
A376S	10 (10/99)	12 (6/49)	5 (2/42)
A400T	38 (39/99)	53 (26/49)	10 (4/42)
D488E	1 (1/111)	0 (0/49)	2 (1/53)
Q509L	5 (5/111)	4 (2/49)	6 (3/53)
Q547K	5 (6/111)	4 (2/49)	8 (4/53)

assessed with Fisher's exact and chi-square tests, and a *p*-value below .05 was considered significant.

Results

One hundred fifty-five samples were successfully processed from Porto Alegre, 35 from Rio Grande, and 97 from Curitiba. Subtyping analysis inferred by phylogenetics assigned 129 (45%) viruses as HIV-1B, 111 (38.7%) as HIV-1C, one (0.3%) as HIV-1F, and 46 (16%) as BF1, BC, and BCF1 unique recombinant forms. Among the 103 CN sequences obtained from patients failing therapy, the following resistance-related mutations were found: N348I (*n* = 11), A376S (*n* = 10), A400T (*n* = 39), A371V (*n* = 15), and T369V (*n* = 3) (Table 1). A376S and A371V were found with frequencies of 10% and 15%, respectively. N348I was observed in 11 viruses (11%), being four HIV-1B, six HIV-1C, and one recombinant form. T369V/I was found in three viruses, one HIV-1B, one HIV-1C, and one HIV-1F. With respect to RNH, three resistance mutations were found: D488E, found in one HIV-1C; Q509L, present in two HIV-1B and three HIV-1C; and Q547K, present in six isolates (two HIV-1B and four HIV-1C; Table 1). These differences were not significant between subtypes, likely due to the small number of strains analyzed.

Sequences of CN and RNH were compared to global and Brazilian HIV-1C consensus, as well as to subtype B strains from drug-naïve subjects, retrieved from the Stanford HIV Drug Resistance Database (Table 2). Global subtype B and C strains differed at 12 amino acid positions in CN and RNH. Another three polymorphisms, R366 (88%), A400 (81%), and M452 (64%), were mainly found in Brazilian subtype C sequences, while the global subtype C presented K366 (85%), T400 (64%), and I452 (42%). Importantly, the drug resistance-related polymorphism 400T represented the most frequent amino acid residue in global HIV-1C (61%; 600/985), but less frequent in Brazilian HIV-1C (16%; 15/95) (*p* < .0001), and did not appear to be selected in RTI-resistant Brazilian HIV-1C isolates (15%; 2/13; data not shown).

We evaluated HIV-1C sequences for novel polymorphisms in CN and RNH putatively associated with treatment and/or failure. The selection of CN M357R in HIV-1C strains with other RTI resistance mutations was observed in a dataset of global HIV-1C strains retrieved from Stanford and a Brazilian dataset with our samples (*p* < .05 in both cases) (Fig. 1). However, while M357T was observed in one-fourth (337/1,416) of treatment-naïve global HIV-1C strains, it was rarely found in naïve HIV-1C strains from Brazil (1%; 1/93;

TABLE 2. PREVALENCE OF AMINO ACID RESIDUE POLYMORPHISMS AT REVERSE TRANSCRIPTASE C-TERMINAL POSITIONS IN GLOBAL HIV-1B AND HIV-1C AND IN BRAZILIAN HIV-1C SEQUENCES

Position	Global HIV-1B % (n)	Global HIV-1C % (n)	Brazilian HIV-1C % (n)
335	G 97 (6,539/6,748)	D 82 (1,410/1,708)	D 89 (124/139)
356	R 86 (2,390/2,773)	K 96 (1,376/1,426)	K 94 (131/139)
366	K 89 (2,483/2,797)	K 85 (1,190/1,404)	R 88 (123/139)
400	A 53 (1,142/2,139)	T 64 (800/1,240)	A 80 (111/139)
404	E 91 (1,948/2,140)	D 96 (1,146/1,192)	D 95 (132/139)
435	V 61 (1,201/1,980)	A 70 (722/1,033)	A 70 (98/139)
452	L 85 (1,656/1,951)	I 42 (404/963)	M 71 (107/151)
471	D 97 (1,976/2,033)	E 92 (895/971)	E 96 (145/151)
483	H 66 (1,274/1,944)	Q 77 (742/967)	Q 85 (129/151)
491	L 72 (1,414/1,970)	S 79 (749/945)	S 78 (118/151)
519	S 73 (1,439/1,982)	N 96 (909/951)	N 94 (142/151)
530	K 95 (1,881/1,981)	R 93 (883/945)	R 88 (131/151)
534	A 95 (1,904/2,004)	S 97 (924/952)	S 91 (138/151)

p < .001). On the other hand, the E529D mutation was unusual in both treatment-naïve viruses from the global dataset (3.2%) and Brazil (6%), but was present only in Brazilian RTI-resistant HIV-1C (21%; *p* = .002). Other changes observed were not statistically significant (not shown).

With respect to other HIV-1 subtypes and recombinant forms, the M357R mutation was rarely found in HIV-1B (1.3%) and CRF01_AE (2.2%) (Table 3). In other subtypes/recombinant forms, its prevalence ranged from 6.6% to 90%. The E529D mutation was more rarely found, ranging from 0% to 5.7% in six subtypes and recombinant forms analyzed, and more prevalent in subtype A (61%) and CRF02_AG (94%). In viral isolates of subjects under treatment, the M357R mutation was selected in HIV-1C, HIV-1F, and CRF01_AE, while the mutation E529D was selected in HIV-1B (Table 3). Of note, M357R was selected in the context of NRTI exposure in HIV-1C, but only upon NRTI+NNRTI simultaneous exposure in HIV-1F and CRF01_AE. E529D, on the other hand, was selected in both NRTI-only and NRTI+NNRTI contexts in HIV-1B (Table 3).

Attempts to analyze the association of M357R and E529D with specific classical drug resistance mutations or even mutation classes [thymidine-associated mutations (TAMs), other NRTI or NNRTI mutations] in the RT polymerase domain were not possible, because the vast majority of HIV strains containing those two C-terminal mutations also harbored multiple mutations of all classes.

Discussion

The HIV-1 RT POL domain has been uniquely focused for drug development and understanding drug resistance. However, recent studies have shown the importance of the C-terminal RT domains (CN and RNH) to drug resistance to NRTI and NNRTI.^{23–25,30,34,39–41} In this study, mutations in these portions were found in HIV-1B and C infecting patients under virological failure. Despite previous reports that have sporadically documented RT C-terminal mutations in non-B subtypes,^{30,35,36} this is the first study specifically assessing differences of mutation occurrence in non-B subtype-specific contexts under the same therapeutic setting.

We found HIV-1B and C as highly prevalent in southern Brazil, in accordance to data reported for this area.^{9,11,16,42}

FIG. 1. Prevalence of HIV-1 RT C-terminal polymorphisms M357R and E529D in different HIV-1C (Sub C) sequence groups. The numbers in parentheses at the legend on the right side represent the actual number of sequences in each category for which the 357 (left) and the 529 (right) RT positions were available. Asterisks denote Fisher's exact test comparisons with significant *p*-values at <.05 (*) and <.01 (**) levels. RTI, reverse transcriptase inhibitor.

When CN and RNH mutations were compiled according to subtypes, an uneven distribution was found. The polymorphism A400T was more frequent in Brazilian HIV-1B from treated subjects and very low in Brazilian HIV-1C counterparts, unlike that observed for global HIV-1C strains.³⁰ This prevalence was also similar to that seen in a recent study of drug-naïve subjects from the same health service.¹⁴ Delviks-Frankenberry *et al.* demonstrated that the reversion of T400 to alanine in the connection of HIV-1C reduced the resistance conferred by TAMs by 1.8×.⁴³ This could indicate that treatment-naïve isolates of Brazilian HIV-1C would take more time to acquire drug resistance to AZT-containing regimens. Notwithstanding, this polymorphism potentiates drug resistance conferred by TAMs in HIV-1B and CRF02_AG viruses,^{35,36,43,44} and further studies are warranted to understand the impact of these changes on viral fitness.

Brazil has a specific genetic strain of subtype C endemically circulating in its southern region. Our group showed that Brazilian HIV-1C carried four amino acid changes in the protease region compared to the global consensus, but no changes with respect to the RT polymerase domain.⁷ In this study, we show three amino acid residues in the RT C-terminal domains specific to the Brazilian HIV-1C compared

to the global HIV-1C, two of which are identical to HIV-1B, including the polymorphism A400.

The CN N348I and the RNH Q509L mutations, not seen in untreated subjects,¹⁴ were found in patients under virological failure, sustaining the importance of these mutations in drug resistance.^{40,41,45–47} Interestingly, both viruses carrying Q509L were HIV-1C. Q509L has been first described during *in vitro* selection of HIV-1B in the presence of AZT,⁴⁵ but studies *in vivo* have failed to find this mutation.^{48–50} The A371V mutation, rarely seen among viruses from drug-naïve subjects,¹⁴ was notably enriched in our study (23%), further confirming its role as an accessory mutation, positively correlated with the number of co-occurring TAMs.³⁴ The N348I mutation occurred in 11% of viruses in Southern Brazil and it is associated with major resistance to nevirapine, efavirenz, and rilpivirine, and as an accessory mutation for thymidine analogs.^{40,41,51,52} Of note, A376S has been associated with failure to NNRTI, particularly nevirapine-containing regimens,^{53,54} suggesting a functional link between this mutation and K103N.

Two novel C-terminal resistance-related mutations have been found in HIV-1C, M357R in CN and E529D in RNH. The former is more likely to display a distinguishable phenotype, as it substitutes a polar, charged residue (R) for a

TABLE 3. PREVALENCE OF M357R AND E529D POLYMORPHISMS IN VIRUSES FROM TREATMENT-NAÏVE (1) AND TREATED (2) AND (3) GROUPS OF DISTINCT HIV-1 SUBTYPES AND RECOMBINANT FORMS

Subtype/CRF	M357R % (n)			E529D % (n)		
	Treatment naïve (1)	Treated (2) ^a	Treated (3) ^a	Treatment naïve (1)	Treated (2)	Treated (3)
HIV-1A	17 (99/596)	14 (5/35)	19 (7/36)	61 (82/135)	—	—
HIV-1B	1.3 (36/2,763)	0.6 (4/689)	1.7 (59/3,378)	1.1 (23/2,008)	4.1 (10/241)	3.3 (4/121)
HIV-1C	24 (337/1,416)	42 (32/76)	29 (65/226)	3.2 (30/951)	—	3.8 (3/80)
HIV-1D	6.6 (21/320)	5.9 (1/17)	0 (0/23)	3.4 (1/29)	—	—
HIV-1F	54 (32/59)	—	14 (5/35)	0 (0/16)	—	—
HIV-1G	90 (130/145)	—	89 (16/18)	3.4 (1/29)	—	—
CRF01_AE	2.2 (19/861)	3.3 (2/61)	11 (4/35)	5.7 (39/687)	0 (0/18)	—
CRF02_AG	34 (216/638)	34 (10/29)	33 (49/149)	94 (81/86)	—	86 (6/7)

Numbers in *bold* are significantly different from the Treatment-naïve viruses at the *p* < .05 level.

^aTreated (2) and treated (3) refer to groups of NRTI-experienced and of NRTI+NNRTI-experienced patients, respectively, as described in the Methods section.

NRTI+NNRTI, nonnucleoside reverse transcriptase inhibitors.

nonpolar counterpart (M). To the best of our knowledge, this is the first report of an HIV-1 non-B subtype-specific mutation occurring in the RT C-terminal portions of viruses infecting patients under treatment failure. The mutation E529D also was selected among global HIV-1B strains; however, the low number of RNH sequences from drug-resistant strains of non-B subtypes did not allow us to evaluate whether the mutation could be selected in other HIV-1 subtypes and/or recombinant forms. Although one fifth of RTI-resistant Brazilian HIV-1C selected the E529D mutation, the same was not observed among global HIV-1C strains. Phenotypic studies are warranted to evaluate the detailed impact of these changes on drug resistance and RT fitness in different HIV-1 genetic forms.

We were unable to analyze associations of specific mutations with antiretroviral treatment since drug schemes were always multiple combinations, and especially with regard to nucleoside RT inhibitors, all schemes used in our analyzed patients used two drugs of that class. However, possible specific drugs that could be specifically analyzed are AZT and NVP, by surveying patients exclusively exposed to AZT monotherapy in the early days of HIV treatment and patients exposed to NVP monotherapy against mother-to-child transmission in some African countries. Those sequences and associated clinical history could be retrieved from the Los Alamos National Laboratory HIV Sequence Database. We found 78 HIV-1B-, 12 HIV-1C-, and 19 CRF01_AE-infected patients subjected to AZT monotherapy in the Los Alamos database. No significant differences between drug-naïve and AZT-experienced patients were found in the frequency of the M357R mutation, whereas very limited sequences had the 529 position available. For NVP monotherapy, over 500 HIV-1C patients were retrieved, but only 26 with position 357 were available. Most importantly, a borderline significant *p*-value (.05) was found when comparing these patients to drug-naïve HIV-1C, where the frequency of M357R decreased from 24% in the latter to 7.6% in the former. For the other subtypes (A and D), no significant differences were found between drug-naïve and NVP-exposed patients. These data suggest that, as seen in Table 3, NVP antagonizes the selection of that mutation by NRTI in HIV-1C. These findings further highlight the impact of specific drug resistance mutations on the development of resistance in distinct HIV-1 subtypes.

Despite the sample size limitation and the retrospective nature of this study, we think it brings relevant information on the development of C-terminal CN and RNH mutations in different HIV-1 subtypes, especially in HIV-1C, the most prevalent worldwide.² While most C-terminal RT mutations previously described for HIV-1B also occur in HIV-1C,³⁰ this work showed that some of them display subtype-specific predominance and others even occur in specific subtypes. Another major limitation of our study is that, we were unable to infer clinical implications of the two novel mutations identified herein. Additional research is warranted to unveil the mechanisms of action and the phenotypic impact of these mutations in drug resistance of non-B subtypes, as treatment rolls out throughout the developing world, where they predominate.

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Author Disclosure Statement

No competing financial interests exist.

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