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REV Responsive Element Polymorphism from Gp41 of Human Immunodeficience Virus Type 1 and Antiretroviral Susceptibility Impact in Patients from Northeast Brazil

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Abstract

Introduction: Rev Responsive Element (RRE) is a RNA molecule responsible to mRNA from HIV-1 virus nuclear transportation to cytoplasm through RRE-Rev pathway, essencial to virus replication. Enfuvirtide resistance mutations are primary located in a perimeter of 10 amino acids of HR1, a corresponded region of RRE. Charactherize RRE should provide a new approach for HIV therapy.

Objectives: Sequence and characterize RRE from gp41 to evaluate variability and correlate with laboratory parameters in sequences from HIV-1-infected patients, whitch were receiving regimens including Enfuvirtide, naïve or rescue therapy.

Methods: Sixty-two samples from HIV patients in Northeast Brazil were collected and Thirty-five RRE sequences and clinical follow-up were analyzed, distributed into three groups: N (naïve therapy), T (treated patients with rescue regimens) and F (rescue regimens containing Enfuvirtide). Sequences obtained were aligned with Los Alamos HIV sequence database by using the HIV BLAST Search.

Results: A phylogenetic analyses demonstrated higher prevalence of HIV-1 subtypes B (97.2%). An increased immunology response was observed in CD4 count higher on group T (71,5%) compared with F (2,98%). Group N most commum mutations and polymorphisms were Q32L (41.6%), N42S (8.3%), R46K (33.3%), L54M (41.6%); group T: Q32R (8.3%), R46K (25%), L54M (33.3%); and group F: Q32L (18.2%), G36D (9.1%), V38A (9.1%), N42S (27.3%), N42T (9.1%), R46K (27.3%), L54M (45.4%), K77R (54.5%). Three samples demonstrated significant resistance mutations to fusion inhibitors. Analysis of RRE nucleotide primary sites observed mutation 28A in 27.2% and 8.3% on groups F and N respectively, and 27S in 8.3% on group T. There was selective pressure on HR1 region from HIV-1 patients using antiretrovirals, independent of enfuvirtide exposure.

Conclusions: This study defined most prevalent RRE polymorphisms in Northeast Brazil and suggests highly preserved regions primary sites to Rev connection. Observed a low resistance profile to enfuvirtide in failing regimens with this drug. Selective pressure on HR1 region in failed regimens with out fusion inhibidors was detected.

Keywords: HIV-1; Rev; RRE; Enfuvirtide; Polymorphisms; Viral resistance

Introduction

Resistant HIV-1 variants are known to exist for every current therapeutic agent used to antiretroviral therapy, therefore different treatments and agents have been developed trying to avoid resistance actually known. Virus fusion to CD4 T cell is essential for HIV-1 life cycle and an important target for therapy [1]. Rev is a viral encoded protein, considered the most functionally conserved regulatory protein of lentiviruses, located at gene *env*. This molecule interacts directly with a *cis*-acting target named Rev Response Element (RRE), presented in all incompletely spliced viral mRNAs regulating Rev nuclear exportation [2,3]. Generally mRNA containing introns are retained into cell nucleous due to interaction with splicing factors (commitment factors), before been processed or degraded. RRE is composed for structure with five interlocking stem loop, which is highly conserved in viruses. Stem Loop IIB and IID from RRE have demonstrated high affinity to Rev connection site, while remaining stems and loops can provide connection to secondary sites to Rev [4-7]. Arginine-rich region, containing residues 34 to 50, is responsible for both nuclear and nucleolar importation. Studies have shown that four arginine residues (35, 39, 40 and 44) have high binding affinity to stems IIB and IID of RRE, while residues at positions 34, 38, 41-43, 46 and 48 have with sugar phosphate in this same arginine region [8].

Enfuvirtide is a fusion inhibitor peptide derived from HR2 of gp41, and binding to HR1 can blocks integration process [9-11]. Main viral resistance developed to this drug is resulted from mutations primarily located in a perimeter of 10 amino acids of HR1 and identified as Q32H, Q32R, G36D/S, I37V, V38A/M, Q39R, Q40H, N42T and N43D/K/S [12-26]. Other mutations found *in vivo* and also related with viral resistance were V38G, V38W, V38E, S42G, N42Q/H and N43Q [27-29].

Changes in gp120 and gp41 are supposed to be associated with differences in viral susceptibility to enfuvirtide. Previous study with a fusion inhibitor named C34 proposed that mutations in gp41 conferring resistance to these compounds are restricted, due to maintain a functional RRE [30]. A sequence of HR1, containing three amino acids (GIV at positions 36 to 38), is critical for inhibition of HIV-1 entrance by HXB3 T20, and efficient association between HR1 and

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peptide T20 [25]. Although GIV sequence is highly conserved among isolates of HIV-1, and primary resistance mutations at codons 36-38 were rarely found in infected and potentially long-time pre-treated patients with inhibitors protease (PR) and transcripase reverse (TR) [25,31-33]. In phenotypic studies, single mutations V38E or I37K and various combinations of two mutations as G36D/N42T, G36V/N42D, Q41R/N43D appear to be related to high resistance to fusion inhibitor [34,35]. L33S and V38E mutations, located in stem-loop IIB and in the middle of RRE stem IIB of HIV-1 respectively, were also correlated with resistance. Synonymous mutations in gp41 residues Gln41 and Leu44, which affect stem III of RRE, act as secondary mutations, increasing replication of HIV-1 primary resistance mutations associated with enfuvirtide [36].

Ceará is a Brazilian state, around 3 million inhabitants and 10,092 patients reported with HIV infection in July 2011. This data is considered a high prevalence and serious public health problem. Many patients are receiving treatment, therefore present study proposes to analyze mutational and polymorphic profile of RRE in HIV-1 infected population, comparing treatment-naïve or in use of antiretroviral therapy which could include enfuvirtide, assessing impact of this drug in preservation of RRE binding sites to Rev.

Methods

A total of 62 samples were collected between 2007 and 2008 from individuals regularly attended on HIV Clinics of Hospital Sao jose of Infectious Diseases at Fortaleza (HSJ), Ceara/Brazil. Protocol was approved by institutional Ethics Review Board and blood samples were collected after informed written consent. Medical records, laboratory tests (viral load and CD4 T-cell counts) and data on therapeutic failure were obtained by medical charts. For subtyping and phylogenetic analysis, HIV-1 RNA was extracted from plasma samples using QIAamp Viral RNA Mini Kit (Cat. No 53704 Qiagen Inc., Santa Clarita, CA). Amplification of RRE of HIV-1 was performed with two sets of primers in a two-step nested PCR strategy. Primers used during the RT-PCR were designed from template sequence of virus pNL4-3, primer 396 to 245 (nucleotide sequence of the pNL4-3 from 7485 to 8403). 396 external upstream primer to reading frame -5' TAA ACA TGT GGC AGG AAG TAG G 3' (22mer), 1438 intern upstream primer reading frame -nt 7712-7733-5' AAG GCA AAG AGA AGA GTG GTG C 3' (22mer), 245 extern downstream primer readind frame -5' GGC CTG TCG GGT CCC CTC GGG 3' (21mer), 1439 intern downstream primer reading frame-nt 8018-8039-5' GCA CAG CAG TGG TGC AAA TGA G 3' (22mer).

Sequencing of nested PCR products of RRE were realized with Bio Apply 3100 (Applied Biosystems, Foster City, CA). Subjects included (N=62) were correctly sequenced in 80% from group N (N=12, naïve patients), 41.37% in group T (N=12, using antiretroviral therapy without fusion inhibitor-HAART) and 61.1% in group F (N=11, using enfuvirtide in antiretroviral regimen). Sequences obtained were aligned with Los Alamos HIV sequence database and used HIV BLAST Search (http://www.hiv.lanl.gov).

Patients were diagnosed with HIV between July/1981 and July/2007. For comparative mutational analysis purposes, were considered to Enfuvirtide mutations in gp41 HR1 region previously isolated in studies of patients on HAART failures optimized with this drug or previous monotherapy use, defined as 36A/D/S/V/E, 37M/T/V, 38A/E/K/M/G, Q39H, Q40H/K/P/T, N42T/D/Q/H, N43D/H/S/K/Q, L44M, L45Q/M, Q32H/R, Q39R/H, Q40H, R46M and V69I (ANRS-http://www.hivfrenchresistance.org) [26,37-39].

Results

Samples analizes demonstrated 71% of male and 29% female patients, and median age of 38.9 years (variance 18-62 years). Comparing CD4 count at baseline and on sample collection date, there was an increase of 2.98% in group F, 71.5% in group T and 4.95% decrease in group N. Epidemiological data are summarized in table 1. Changes in amino acids found in region of RRE studied in Ceará are found in table 2. I69V mutational change was not considered a resistance one, since it is the correct position in majority of virus B [38-41].

There were no statistically significant differences in mutations found between groups N, T and F in RRE-Rev primary sites. We found changes in codon 27 (8.3% in group T) and 28 (8.3% in group N and 27.2% in group F) (p=0.09). Mutation at codon 28 occurred from amino acid V to A (GTA-GCA) and 27 from T to S (TCG-ACG).

Phylogenetic analysis showed that most viruses belonged to subtypes B (97.2%), except one strain subtype F1 at group N, with single mutation N42S detected.

There was a lower ratio synonymous mutations (ds)/non-synonymous mutations (dn) in groups T (0.1) and F (0.23) compared with group N (0.6) [37,38].

Amino acid changes between GIV (36 to 38 in gp41) are critical to binding N-HR and T20 *in vitro* [25,42]. Mutations 30V and 36G mantain stability in presence of RRE 37T and 37K, respectively [30]. Nucleotide substitutions in 30V, 36G, and 38T/K also encode RRE and 30A and 36D are located completely in stem IIC. Mutations 33S and 43K are located between the top of stem IIC (UUA to UCA) and stem III, indicating that changes in RRE are minimal. Mutation 42S has been reported in subtypes A, B, C and G but not F; 56R in the CRF02_AG and subtype A, subtype B 54M in CRF14_BG [37,41,43]. In all samples presence of mutations in codons 30, 33, 37, 39 or 43 were not detected.

Characterization	Group F (18)*	Group T (29)	Group N (15)
Sex	N (%)	N (%)	N (%)
Male	15 (83)	18 (62)	11 (73)
Female	3 (17)	11 (38)	4 (27)
Transmission			
Homosexual	5 (28)	7 (24)	1 (6,6)
Heterosexual	5 (28)	16 (55)	5 (33,4)
Bisexual	3 (16)	4 (14)	5 (33,4)
Drug	0	1 (3,5)	1 (6,6)
Transfusion	0	0	0
Ignored	5 (28)	1 (3,5)	3 (20)
Symptomatic	2 (11)	2 (6,9)	0
Asymptomatic	16 (89)	27 (93,1)	15
CD4 initial	N=16	N=28	N=15
Median (cel/mm^3)	393	336	644
Standard Deviation	438,45	255,9	179,4
Viral Load inicial	N=14	N=15	N=11
Median (copies/mm^3)	198.207	352.661	36.015
Standard Deviation	407.505,52	844.766	38.650,7
CD4 during test	N=18	N=29	N=15
Median (cel/mm^3)	404	577	612
Standard Deviation	284,23	255,9	233,4
Viral Load during test	N=10	N=13	N=15
Median (copies/mm^3)	41.386	6.611	52.507
Standard Deviation	46.486,342	14.370,1	127.353
Viral Load under 50 copies/mm^3	8	16	0

Group N = naïve patients, Group T = patients under antiretroviral therapy without fusion inhibitors, and Group F = patients in antiretroviral therapy with Enfuvirtide.
*Period of Enfuvirtide use: median 13 months (variance 1-34 months).

Table 1: Epidemiologic, Clinic and Imunovirologic parameters from patients submitted to Genotypic Study of RRE (N=62) in Ceará.

Sample	Sequence Substitutions	Resistance Mutations related to T20	Mutations in RRE primary sites stem loop IIA, B, C e D
N101	A1Q, V2S, G3S, InsG, M24V, T95L	-	M24V
N102	V2L, I4M, G13S, M24I, N42S, I84L	N42S	M24I
N103	InsT, I4L, L7M, M24V, R46K, L54M, K77Q, Q80R, N105K, K106L, S107C	R46K, L54M	M24V
N105	G3T, L7M, Q32L,R46K, V72I, K77R	Q32L, R46K	Q32L
N106	V2A, L7M, F8I, Q32L, R46K, K77R, N105T, K106A	Q32L, R46K	Q32L
N107	L7M, Q32L, S35T, L54M, K77G	Q32L, S35T, L54M	Q32L, S35T
N108	G3T, I4L, L7M, M24V, L54M, K77R	L54M	M24V
N109	S2L, InsT, I4M, L7M, L9I, M24V, A96N	-	M24V
N110	V2I, I4M, A14T, M24L, R46K, E49D, V69I, Q66R, I84L	R46K, E49D	M24L
N111	I4L, L7M, M24I, V28A, E49D, Q66R, R74K, K106H	E49D	M24I, V28A
N112	L7V, G13S, M24V, Q32L, Q52H, K77R	Q32L, Q52H	M24V, Q32L
N114	InsT, I4L, L7M, S23A, M24T, Q32L, L54M, K77Q, S107A	Q32L, L54M	S23A, M24T, Q32L
T205	L7M, A14T, M24L, Q66R, V69I, I84L	-	M24L
T207	L7V, M24I, L54M, K77R, T95N	L54M	M24I
T212	InsV, I4L, L7M, M24V, V69I, K77R	-	M24V
T217	L7M, L54M	L54M	-
T218	V2I, I4L, L7M, T25A, V69I, K77Q, K90R, T95L	-	T25A
T219	G3V, InsG, I4L, I84L	-	-
T220	V2I, InsT, I4L, L7M, M24L, L54M, T95L	L54M	M24L
T223	L7V, F11L, M24V, T25A, T27S	-	M24V, T25A, T27S
T226	InsT, I4M, L7M, L9I, M24V, R46K, E49D	R46K, E49D	M24V
T228	InsT, I4L, L7M, A14T, M24I, Q32R	Q32R	M24I, Q32R
T230	InsT, L7M, F8I, L9I, A14T, M24L, K77Q, Q79R	-	M24L
T231	V2I, I4L, L7V, M24I, L54M, V69I, K77Q, T95N	L54M	M24I
F301	G3A, InsA, L7M, F8I, Q32L, R46K, K77R, G83R	Q32L, R46K	Q32L
F304	A1S, V2S, InsT, L7M, M24I, Q32L, G36D, L54M, A71S, K77R, K106H, S107A	Q32L, G36D, L54M	M24I, Q32L, G36D
F306-2	L7M, M24I, V28A, L54M, G83R	L54M	M24I, V28A
F307	InsV, I4L, L7M, M24I, V28A, L54M, K77R, I84M	L54M	M24I, V28A
F308	G13S, T18A, M24I, V38A, N42T, R46K	V38A,N42T,V38A/N42T, R46K	T18A, M24I, V38A
F309	N42S, V72L, Y75F, K77R	N42S	-
F302	InsV, L7M, M24V, N42S, V72L, Y75F, K77R, S107Q	N42S	M24V
F310	I4L, L7M, M24I, V28A, N42S, L54M, K106A	N42S, L54M	M24I, V28A
F312	L7V, S23A, M24V, L54M, K77R, K106H	L54M	S23A, M24V
F313	V2A, L7V, M24V, R46K, Q52H, T95L	R46K Q52H	M24V
F317	InsG, I4L, L7M, M24V, T95N	-	M24V

N = patients naïve to antiretroviral therapy, T = patients on antiretroviral therapy without fusion inhibitors and F = patients on antiretroviral therapy containing Enfuvirtide.

Table 2: Genotypic Sequences of RRE from gp41 of HIV-1.

Association of mutations 54M/Q56K or 34M/L54M/Q56R demonstrated around five times changing in enfuvirtide sensitivity. There were no detected mutations at codon 56 and 34 in Ceará viral samples. Although, 54M mutation was found in high frequency in virus from groups N (41.6%), T (33.3%) and F (45.4%), considered as a naturally occurring polymorphism in this region. Mutation 46K was found into three groups of Ceará, with high frequency, N (33.3%), T (25%) and F (27.3%), although not correlated with resistance in any of the studies found to date [26]. Therefore, 46K seem to behave as a characteristic polymorphism of Ceará strains naïve to antiretroviral therapy.

In recent Brazilian study, 43K and 44M were considered common polymorphisms in antiretroviral treatment-naïve patients (3.8%), and identification of polymorphisms 137K, 130I, 129N and 138A, in region HR-2 [44]. Besides, substitutions were not identified at codons 43 or 44 in present study samples analyzed.

Mutations 36E, 43S and 42T were observed in patients previously treated in Brazil, two of the three samples detected in patients who had been previously exposed to enfuvirtide [45]. There was also a reduction in sensitivity of patients to other very experienced oral antiretroviral, compared with short exposure to these drugs [27]. Another study demonstrated that high resistant strains to enfuvirtide were associated with changes in positions 38A, 41R, 42D/T, 43D, 44M and 45M [22]. Group F in current study had a higher prevalence of 42T than others groups (27.3%), although not statistically significant compared with naive group therapy in Ceará.

A study conducted in Brazil, evaluating 65 samples from patients failing HAART, found a high prevalence of 36D (7.6% and 11.1% on subtype B) although it was not found in any of 1.079 samples analyzed in naive patients therapy ($p < 0.0001$) [46]. Therefore, characterizing 36D as drug-induced mutation and not naturally occurring in patients without exposure to fusion inhibitors or antiretroviral therapy. This was corroborated by appearance of this mutation only in a single sample belonging to group F in present study.

A high frequency of 32L was observed in group N (41.6%) compared with group T ($p=0.037$), which one completely disappeared. This mutation has no impact on viral resistance and also not found in non-B subtypes, suggesting that it is naturally occurring polymorphism in viruses not exposed to antiretroviral therapy from subtype B.

Mutation 77R was found in high prevalence groups sequenced in Ceará, 54.5% in group F, 16.6% in group T and 33.3% in group

N, without statistical differences. Therefore, it could be considered a characteristic polymorphism of viruses found in Brazil.

In Group F, three patients had isolated or combined mutations which could be related to viral resistance. In the first virus strain, changes were identified as 42S and 54M, with a low resistance profile. In the second isolate, presence of 32L, 36L and 54M demonstrated mostly significant mutational profile found in sequences studied, probably assigned to presence of 36D. In the third isolate, it was detected presence of mutations 38A and 42T. Previous studies differ as to impact of viral resistance in 38A, ranging change in the fold change from 16 to 1000 times [22,37,47]. Mutation 42T isolation changes by up to 4 times, and association 38A/42T up to 149 times [48,49]. Therefore, it was considered high-resistance profile in this sample sequenced. Besides 38A been located on the stem IIa and 42T in stem loop III, which are not conformational close, and this interaction may propose a somatory or protective effect regarding viral resistance.

Hypothetically synonymous mutations in gp41/RRE improve viral replication affected by primary mutations and could have an important role as secondary mutations [36]. Comparison between number of non-synonymous mutations (dn) and number of synonymous mutations may suggest that natural selection is acting to promote fixation of advantageous mutations (positive selection) or removing deleterious mutations (purifying selection) [50]. This study revealed a lower ratio ds/dn in groups T (ratio ds/dn = 0.1) and F (ratio ds/dn = 0.23), compared with group N (ratio ds/dn = 0.6).

A nucleoside substitution was detected at codon 28 GTA-GCA, but the nucleotide linking to Rev is adenine, which remained preserved. In codon 27 substitution occurred in ACG-TCG, which the nucleotide-binding guanine was also preserved. This suggests that process of multimerization of Rev-RRE may be related to links in other regions outside primary binding site in loop IIB [51,52]. Therefore, study of changes in other regions outside the primary sites for binding of Rev and their impact on Rev-RRE function need to be further studied.

Conclusion

This study identified low resistance to fusion inhibitors in all isolated samples, independent of previous exposure to antiretroviral drugs, including enfuvirtide. Reduction of synonymous mutations in RRE sequences was detected in patients previously exposed to HAART, containing or not enfuvirtide, suggesting selective pressure in HR1 region with possibility of developing resistance to this class easier than in patients naïve to antiretroviral therapy. This study also demonstrated high conserved RRE molecule in brazilian samples, which is required for normal viral cycle, besides exposure to antiretroviral therapy, pointing to a novel therapeutic target.

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