

Co-Receptor Tropism Determined by Genotypic Assay in HIV-1 Circulating in Cuba

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Abstract

Introduction: R5-tropic viruses predominated at the time of initial HIV-1 infection and a switch to X4-tropic occurs in about 50% of patients in late-stage disease.

Objective: To associate different variants of Cuban HIV-1 with the co-receptor use, and the implication for the use of co-receptor inhibitors.

Methodology: Viral HIV-1 subtype was determined in 42 Cuban individuals using COMET V.2, Rega subtyping toolV.3 algorithms and phylogenetic analysis. Co-receptor tropism was predicted using the geno2pheno [co-receptor] algorithm. Additionally, all V3 loop HIV-1 Cuban sequences deposited previously at Los Alamos database were also analyzed for comparison of subtype and co-receptor tropism.

Results: The most frequent subtypes detected were CRF20–23–24_BG (35.7%), subtypes B (33.3%) and CRF19_cpx (14.3%) when pol and V3 regions were analyzed. Overall, 61.9% of the samples were R5 viruses and 14.3% were X4. Viruses CRF19_cpx were more often R5X4/X4 (5/6 samples, p=0.009) or X4 strains (3/6 samples, p=0.019). The additional analysis of 359 Cuban env sequences demonstrated that only 29.3% were X4 viruses. Interestingly, 43.6% of the CRF19_cpx were R5X4/X4 viruses, confirming the previous association (p=0.011). Characteristic amino acids in the V3 loop (V/T12, R13, Q18, V19, G22) were identified at higher frequencies in CRF19_cpx viruses than in subtype B (p<0.0001).

Conclusion: CRF19_cpx is a genetic form with high proportion of X4-tropic viruses. This supports the increased pathogenicity of CRF19_cpx, potentially leading to rapid disease progression. The high frequency of X4 tropism in CRF19_cpx infected patients would imply that CCR5 antagonists could be ineffective in most of these patients.

Keywords: HIV; Co-receptors; X4; CRF19_cpx; Cuba

Introduction

The V3 loop is a highly variable, loop-like structure within the gp120 protein of HIV and is considered the immunodominant domain of the envelope [1] but the most relevant function is the binding to the cellular receptors CCR5 and CXCR4 during virus entry [2,3], thus defining viral tropism.

Currently, viruses that utilize CCR5 as an entry receptor are referred to as R5 viruses, while viruses that utilize CXCR4, are referred to as X4 viruses [4]. Viruses that can utilize either CCR5 or CXCR4 as an entry cofactor are referred to as dual tropic, or R5X4.

CCR5-tropism is characteristic of viral isolates that persist during asymptomatic disease, and are further thought to be the principal subset of virus responsible for new infections [5]. Over the course of HIV infection, a switch to primarily X4 or R5X4 isolates occurs in about 50% of patients, generally associated with rapid depletion of CD4+ T cells and progression to AIDS [6,7].

Furthermore, changes in V3 have been specifically associated with changes in susceptibility to entry inhibitors [8]. Maraviroc is a selective small molecule CCR5 antagonist currently being used to treat patients with resistance to multiple HIV drugs but has been also approved for first-line treatment regimens. The major concern in the therapeutic administration of co-receptor inhibitors is the possibility that resistance will manifest by a change in co-receptor tropism from CCR5 to CXCR4, or that an outgrowth of an X4-tropic virus subset

will come to dominate the intra-patient virus population. In Cuba, Maraviroc has never been used so far [9].

By the end of 2015 more than 24 000 individuals had been diagnosed with HIV in Cuba and more than 70% were under antiretroviral therapy (National Registry of HIV, Ministry of Health, Cuba). Despite the low prevalence of HIV-1 (0.2%), the epidemic in Cuba is characterized by an unusually high viral diversity of HIV-1, in contrast to the rest of the Caribbean region, where subtype B predominates [10-12].

The genetic forms reported include subtypes B, C, G, H, BG circulating recombinant forms (CRF20_BG, CRF23_BG and CRF24_BG) and two complex CRFs, CRF18_cpx and CRF19_cpx, which are also known as Cuban CRFs [13-16]. Subtype B represents only a third of the Cuban epidemic and BG recombinants and various CRFs in Cuba are currently expanding [17-19]. Furthermore, a recent report

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Received June 03, 2016; Accepted June 15, 2016; Published June 22, 2016

Citation: Kourí V, Alemán Y, Díaz D, Pérez L, Limia CM, et al. (2016) Co-Receptor Tropism Determined by Genotypic Assay in HIV-1 Circulating in Cuba. J AIDS Clin Res 7: 592. doi:10.4172/2155-6113.1000592

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showed that the viral variant CRF19_cpx was exclusively associated with rapid progression to AIDS in Cuban individuals [20].

The mechanisms and consequences of co-receptor switching are poorly understood at this time. It is unclear what factors drive this switch, or why switching is generally limited to late phases of disease progression. Indeed, it is not clear whether a switch from R5 to X4 tropism is a cause or consequence of disease progression. There are few reports in Cuba regarding the molecular characterization and genetic variability of the V3 loop region of HIV-1 [21]. Additionally, the association of specific Cuban genetic variants with viral tropism has not been described in details.

Objective

In the present research we aimed to study the V3 loop of HIV-1 sequences obtained from Cuban individuals and its association with co-receptor tropism and other epidemiological characteristics, as well as the implication for the use of co-receptor inhibitors.

Methodology

Study populations

In order to genetically characterize the V3 loop of gp120 of HIV-1 sequences from Cuban individuals, a cross sectional study was performed. The study population included plasma samples from Cuban HIV-1 seropositive individuals received in the Sexually Transmitted Infection Laboratory at IPK for genotypic resistance test during the period of January 2014 to January 2015. Patients under antiretroviral therapy were tested because they were failing to therapy; therapy naïve patients were studied for surveillance of transmitted drug resistance. The inclusion criteria for the study were: samples that had been successfully amplified during the procedure for genotypic resistance test; a total of 42 plasma samples were analyzed. Clinical (AIDS CDC classification, therapy status), and epidemiological data (age, place of residence, gender, sexual orientation, date of diagnosis and infection) were collected from a questionnaire at the time of sample collection.

Patients were classified as recent diagnosis if the date of diagnosis occurred within one year from the date of sampling. Recent infection was considered in those patients that were diagnosed, as maximum, one year from the date of infection. Date of infection was considered as the midpoint time between the date of last HIV negative test and date of diagnosis. This information was not available in five patients.

Ethics statement

The study protocol was designed in accordance with the Helsinki Declaration and approved by the ethics committee of the Tropical Medicine Institute "Pedro Kourí" (IPK) in Havana. All participants provided written informed consent.

Measurement of immunological markers and viral load

CD4 cell counts were determined by FACScan (Becton Dickinson, USA). Plasma HIV-1 viral loads were determined using Nuclisens Easy Q HIV-1 kit, version 2.0 (Biomérieux, France).

HIV sequencing and subtyping

Amplification and population-based bi-directional Sanger sequencing of *env* fragment was performed as described previously [22]. The sequenced region spanned in average 369 NT for partial-C2-V3-C3-partial region of gp 120. Results of HIV-1 Subtype in *pol* region was obtained from the genotypic analysis of sequences performed as

routine in our lab for resistance test and subtype, covering a fragment of 1300 bp that overlaps with codons 1–99 of protease and 1–335 of reverse transcriptase [23].

COMET version 2 (available at (<http://comet.retrovirology.lu>) [24], and REGA version 3 (available at <http://bioafrica.mrc.ac.za:8080/rega-genotype-3.0.2/hiv/typingtool/>) [25,26] were used to do an initial subtype classification of the sequences. Since the Cuban epidemic is characterized by a high number of circulating recombinant forms (CRFs) and unique recombinant forms (URFs) [17], all assignments were confirmed with manual phylogenetic analysis. Sequences were aligned with MUSCLE, minimally edited with Mega 5 [27]. A ML phylogenetic tree was constructed using Mega 5, using the Jukes-Cantor substitution model and 1000 replicates of bootstrap. 30 reference sequences for the different HIV-1 subtypes and the 42 Cuban sequences obtained in this study were compared (average of 350 nucleotides).

Because of similar breakpoints in the *pol* region and lack of breakpoints in the *env* region, sequences initially assigned to CRF20_BG, CRF23_BG or CRF24_BG were called CRFs BG [15].

Viral tropism and Co-receptor use

Viral tropism was predicted using the genotypic tool geno2pheno [co-receptor] (G2P) version 2.5 (<http://coreceptor.bioinf.mpi-inf.mpg.de>). Based on German guidelines, tropisms were divided into three groups according to the FPR (the likelihood of incorrectly identifying an R5 virus as X4). A false-positive rate (FPR) <5% are mainly X4 and ≥ 20% are mainly R5 variants. Therefore, we classified V3 loop sequences with FPR ≥ 20% as R5 viruses, with FPR ≥ 5% and <20% as dual-tropic viruses (R5X4) and with FPR <5% as X4 viruses as described previously [20,28,29].

Mutations that confer change in viral tropism were manually analyzed. V3 net charge was calculated by subtracting negatively charged residues [aspartic acid (D) and glutamic acid (E)] from positively charged ones [arginine (R) and lysine (K)] in the V3 loop. A V3 net positive charge ≥ 5 was considered to be predictive of X4 tropism, while a positive charge below 5 was predictive of R5 tropism. Furthermore, we also performed the analysis according to the 11/25 rule, where the presence of positively charged amino acids (R or K) at positions 11 or 25 of the V3 loop is considered to be predictive of X4 tropism [30].

Additionally, we retrieved all V3 HIV-1 Cuban sequences deposited previously by our laboratory and by others, at Los Alamos database. We determined the subtype and the co-receptor use in 317 sequences using the same programs described above. We then added the 42 Cuban samples sequenced in this study in order to analyze the association of subtype with the co-receptor use for all the Cuban HIV-1 V3 loop sequences available.

Statistical analysis

Mean and standard deviation (SD), median and interquartile range (IQR), and frequencies (%) were used to describe patients' characteristics. The χ^2 test and Fisher were used to compare categorical and continuous variables. The odds ratio (OR) and its 95% confidence interval (CI) were estimated. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using the SPSS statistical software version 18 (SPSS Inc., Chicago, IL).

Results

Descriptive characteristics of study population

The patients were predominantly men who have sex with men (MSM) from the western region of the country (including Havana) and with a median age at sampling of 41.0 years. The median (IQR) CD4 cell count and HIV-1 RNA viral load were 306.5 (151.3-548.0) cells/mm³ and 32 200 (7152.0-97548.5) copies/mL, respectively. Sixty four percent of patients had more than 10 000 copies/mL of viral load at sampling, while 21.4% had the CD4 count below 200 cells/mm³. Twenty two (52.4%) patients were under ARV therapy, 40.5% had been recently diagnosed at the time of the study and already 19% of them had developed AIDS (Table 1).

Subtype analysis

For the 42 patients, both partial *pol* and partial *env* regions sequences were available. In the *env* region, the most frequent viral variants were CRF20–23–24_BG, subtype B and CRF19_cpx (38%, 35.7%, 14.3%, respectively). Other subtypes were less frequent (Table 2 and Figure 1). Analyzing the HIV-1 subtypes in both regions together, 14 patients (33.3%) were infected with subtype B, 15 (35.7%) with CRF20–23–24_BG, 6 with CRF19_cpx, 2 with CRF18_cpx, one with C and H each, and 3 with URFs (CRF24_BG/CRF18_cpx, CRF06_cpx/CRF18_cpx, B/CRF12_BF) (Table 2). Greater than 80% agreement

	N	%
Patients	42	100.0
Age, median (IQR)	41.0 (31.3-51.8)	
Sex		
Male	36	85.7
Region of residence		
Havana	20	47.6
West (excluding Havana)	7	16.7
Center	11	26.2
East	4	9.5
Route of transmission		
MSM	33	78.6
Viral load (median copies/mL, IQR)	32 200 (7152.0-97548.5)	
<1000	4	9.5
1000-10 000	9	21.4
>10 000	27	64.3
unknown	2	4.8
CD4 ⁺ count, median cells/mm ³ (IQR)	306.5 (151.3-548.0)	
<200	9	21.4
200-500	15	35.7
>500	12	28.6
unknown	6	14.3
ARV Treated patients	22	52.4
Recent diagnosis	17	40.5
Recent infection	8	19.0
AIDS stage	14	33.3
Developed AIDS during the first year after diagnosis	8	19.0

Abbreviations: MSM: Men who have Sex with Men; IQR: Interquartile Range; ARV: Antiretroviral

Table 1: Epidemiological, clinical and immunological characteristics of the patients analyzed.

HIV-1 Subtype	V3 loop <i>env</i> (%)	<i>pol</i> (%)	Overall subtype (%)
B	15 (35.7)	14 (33.3)	14 (33.3)
C	1 (2.4)	1 (2.4)	1 (2.4)
CRF06_cpx	1 (2.4)	0 (0.0)	0 (0.0)
CRF18_cpx	2 (4.8)	4 (9.5)	2 (4.8)
CRF19_cpx	6 (14.3)	6 (14.3)	6 (14.3)
CRF20–23–24_BG	16 (38.0)	14 (33.3)	15 (35.7)
G	0 (0.0)	1 (2.4)	0 (0.0)
H	1 (2.4)	1 (2.4)	1 (2.4)
CRF12_BF	0 (0.0)	1 (2.4)	0 (0.0)
URF	0 (0.0)	0 (0.0)	3 (7.1)
Total	42 (100.0)	42 (100.0)	42 (100.0)

Table 2: HIV-1 Subtype detected in two partial sequences from the samples analyzed.

between subtyping based on the V3 loop and subtyping based on *pol* was found.

Prediction of co-receptor use and subtype

Overall, 61.9% of the 42 HIV-1 samples studied were R5 viruses. However, when the different HIV-1 subtypes (considering the subtype in *env*) were compared regarding the co-receptor use prediction, we found that CRF19_cpx were more often R5X4/X4 strains ($p=0.009$; OR:13 CI:1.346-125.520) or X4 strains ($p=0.019$; OR:11.00, CI:1.504-80.425) (Table 3). Furthermore, the analysis of the 359 Cuban *env* sequences (317 retrieved from Los Alamos database and the 42 generated in the present study) demonstrated that only 29.3% of them were able to use the CXCR4 co-receptor. Interestingly, 43.6% of the CRF19_cpx strains analyzed were R5X4/X4 viruses, confirming the above mentioned association ($p=0.011$; OR:2.131, CI:1.181-3.847), conversely, CRF18_cpx were more likely R5 strains ($p=0.000$; OR:0.098, CI:0.023-0.413) (Table 4).

As it is shown in Table 5, no significant differences were observed between age, gender, sexual orientation, clinical status and therapy history between patients infected with CCR5 and CXCR4-using viruses. However, patients with recent diagnosis (12/17, 70.6%) and recent infection (6/8, 75%) were more likely to harbor R5 virus. None of the six patients infected with X4 virus were recently infected and five (83.3%) were under ARV therapy.

Mutations in gp120 V3 region of Cuban HIV-1 sequences.

Many differences were observed between the 42 samples analyzed when compared with the consensus B sequence and among subtypes in the V3 loop (Figure 2). Overall, high concordance was observed for the six samples predicted to be X4 tropics using G2P, when compared either with the simple rule 11/25 (66.7%, 4/6 samples) or the net charge analysis (83.3%, 5/6 samples). The same was observed for samples predicted to be R5 tropic with G2P, all of them were identified as R5 by the other two analyses. However, this correlation failed in samples considered by G2P as dual tropic or R5/X4 strains (FPR \geq 5% and <20%) (Figure 2).

A comparison in V3 loop amino acid sequences showed that threonine (T) and histidine (H) at position 13 and arginine (R) at position 18 were more frequently detected in Subtype B, compared to CRFs CRF20–23–24_BG and CRF19_cpx that in these positions had mainly R and glutamine (Q), respectively. Additionally, all CRF19_cpx samples had valine (V) or T at position 12, V at position 19 and glycine (G) at position 22, in contrast to the isoleucine (I), alanine (A) and T or

	N	%	R5	%	R5X4/X4	%	X4	%	Subtype B	%	CRF19_cpx	%	CRF20–23–24_BG	%
Patients	42	100.0	26	100.0	16	100.0	6	100.0	14	100.0	6	100.0	15	100.0
Age (in years)														
<25	5	11.9	3	11.5	2	12.5	1	16.7	1	7.1	2	33.3	2	13.3
25-45	21	50.0	12	46.2	9	56.3	2	33.3	8	57.1	2	33.3	6	40.0
>45	16	38.1	11	42.3	5	31.3	3	50.0	5	35.7	2	33.3	9	60.0
Sex														
Male	36	85.7	23	88.5	13	81.3	5	83.3	12	85.7	5	83.3	13	86.7
Region of residence														
Havana	20	47.6	11	42.3	9	56.3	6	100.0	6	42.9	4	66.7	7	46.7
West (excluding Havana)	7	16.7	5	19.2	2	12.5	0	0.0	2	14.3	1	16.7	3	20.0
Center	11	26.2	6	23.1	5	31.3	0	0.0	4	28.6	1	16.7	4	26.7
East	4	9.5	3	11.5	1	6.3	0	0.0	2	14.3	0	0.0	1	6.7
Sexual orientation														
MSM	33	78.6	19	73.1	14	87.5	5	83.3	11	78.6	4	66.7	12	80.0
Viral load (median copies/m)														
<1000	4	9.5	2	7.7	2	12.5	1	16.7	1	7.1	0	0.0	2	13.3
1000-10000	9	21.4	4	15.4	5	31.3	2	33.3	3	21.4	2	33.3	4	26.7
>10000	27	64.3	19	73.1	8	50.0	3	50.0	9	64.3	4	66.7	8	53.3
unknown	2	4.8	1	3.8	1	6.3	0	0.0	1	7.1	0	0.0	1	6.7
CD4 ⁺ count, median cells/mm ³														
<200	9	21.4	5	19.2	4	25.0	2	33.3	2	14.3	2	33.3	4	26.7
200-500	15	35.7	9	34.6	6	37.5	3	50.0	3	21.4	2	33.3	6	40.0
>500	12	28.6	7	26.9	5	31.3	1	16.7	5	35.7	2	33.3	4	26.7
unknown	6	14.3	5	19.2	1	6.3			4	28.6	0	0.0	1	6.7
ARV Treated patients	22	52.4	11	42.3	11	68.8	5	83.3	4	28.6	4	66.7	12	80.0
Recent diagnosis	17	40.5	12	46.2	5	31.3	2	33.3	7	50.0	4	66.7	5	33.3
Recent infection	8	19.0	6	23.1	2	12.5	0	0.0	2	14.3	0	0.0	2	13.3
AIDS stage	14	33.3	8	30.8	6	37.5	2	33.3	2	14.3	2	33.3	8	53.3
Developed AIDS during the first year after diagnosis	8	19.0	5	19.2	3	18.8	1	16.7	2	14.3	0	0.0	6	40.0

Table 5: Comparison of the epidemiological, clinical and immunological characteristics of the 42 patients analyzed with the prediction of co-receptor and the major subtypes detected.

genetic diversity was observed, a characteristic that has been previously reported in the Cuban HIV-1 epidemic, probably due to its contacts with Central-Africa [17,19,31]

At the FPR cut off set to <20% by G2P tool, we found that 38.1% of patients harbored viruses with reduced susceptibility to use CCR5 co-receptor. Furthermore, the predicted X4 phenotype was detected in 14.3% of the 42 sequences analyzed and associated to the presence of the viral variant CRF19_cpx. This finding was confirmed also when all the Cuban V3 sequences available at Los Alamos database were analyzed.

It has been hypothesized if inter-subtype recombination may give rise to more pathogenic strains if genomic fragments from different subtypes join together in a better replicating virus, but no direct evidence for this scenario had been found. However, a recent report from our group found that CRF19_cpx was associated with rapid progression to AIDS and one of the hypotheses for explaining this rapid progression was the finding of higher levels of RANTES and significantly more frequent proportion of X4 virus among the patients infected with this viral variant [20].

CRF19_cpx is a recombinant complex composed of a mixture of A/D/G subtypes (14). Currently, it has become the third HIV-1 variant in frequency to be circulating in Cuba (between 17-19%) [18,19], despite of the evidenced central African ancestry [12,14].

Previous studies in Western countries, where HIV-1 subtype B

predominates, reported that 80–90% of untreated HIV-1 infected patients [32,33] and 50–60% of those exposed to ART [34] harbored R5 strains.

Nevertheless, other reports on non-B subtypes have found associations of specific viral variants with the use of CXCR4 co-receptors. The probability of having a CXCR4-using virus is higher in subtype D than in subtype A infections in non-AIDS clinical status in Uganda [35,36]. A high prevalence of X4 tropism among CRF14_BG-infected IDUs was also recently reported in Spain [37] as well as the association of CRF01_AE with the predicted X4 phenotype in Thailand [38].

In the present study, 70.6% and 75% of patients with recent diagnosis and recent infection, respectively, were more likely to harbor R5 virus, although the difference was not significant. Furthermore, none of the six patients infected with X4 virus was recently infected and 83.3% were under ARV therapy. However, not significant differences were observed between age, gender, sexual orientation, clinical status and therapy history between patients infected with CCR5 and CXCR4-using viruses. It is known that more than 80% of patients are initially infected with HIV-1 viruses that are solely R5 tropic and that X4 tropic viruses usually emerge in later stages of HIV-1 infection. However, when this phenotype emerges, it is present in low proportions relative to R5 tropic viruses [7]. The difference between these proportions may be influenced by the different characteristics of the populations studied, the methodology used for tropism determination or the inclusion of late-diagnosed individuals in the antiretroviral drug-naive cohorts.

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