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HIV gp120 sequence variability associated with HAND in Hispanic Women

Krystal Colón¹, Fabián Vázquez-Santiago², Vanessa Rivera-Amill², Gisela Delgado³, Steven E. Massey³, Valerie Wojna^{4,5}, Richard J. Noel Jr.⁶, and Loyda M. Meléndez^{1,*}

¹Department of Microbiology and Medical Zoology, University of Puerto Rico Medical Sciences Campus, San Juan, PR

²Department of Basic Sciences, Microbiology Division, Ponce Health Sciences University-School of Medicine, Ponce Research Institute, Ponce PR

³Department of Biology, Río Piedras Campus, San Juan, PR

⁴Specialized NeuroAIDS Program, University of Puerto Rico-Medical Sciences Campus, San Juan, PR, 00936, USA

⁵Department of Internal Medicine, Neurology Division, University of Puerto Rico-Medical Sciences Campus, San Juan, PR 00936, USA

⁶Department of Basic Sciences, Biochemistry Division, Ponce Health Sciences University-School of Medicine, Ponce Research Institute, Ponce PR

Abstract

Objective—HIV-1 variants with different tropisms are associated with various neuropathologies. This study intends to determine if this correlation is determined by unique viral *env* sequences. We hypothesize that HIV-1 envelope gene sequence changes are associated with cognition status

Methods—Viral RNA was extracted from peripheral blood mononuclear cells (PBMCs) co-cultures derived from HIV-1 infected Hispanic women that had been characterized for HIV associated neurocognitive disorders (HAND).

Results—Analyses of the C2V4 region of HIV gp120 demonstrated that increased sequence diversity correlates with cognition status as sequences derived from subjects with normal cognition exhibited less diversity than sequences derived from subjects with cognitive impairment. In addition, differences in V3 and V4 loop charges were also noted as well as differences in the N-glycosylation of the V4 region.

Conclusions—Our data suggest that the genetic signature within the C2V4 region may contribute to the pathogenesis of HAND. HIV *env* sequence characteristics for the isolates grouped in milder forms of HAND can provide insightful information of prognostic value to

*Corresponding author: Loyda M. Meléndez, PhD, Professor, Department of Microbiology, School of Medicine, Medical Sciences Campus, San Juan, Puerto Rico 00935, Phone: 787-777-0079, Fax: 787-777-0078, loyda.melendez@upr.edu.

CONFLICT OF INTEREST

The authors declare that they do not have conflict of interest.

assess neurocognitive status in HIV⁺ subjects, particularly during the era of highly prevalent milder forms of HAND.

Keywords

HIV; HIV-associated neurocognitive disorders; HAND; gp120; monocyte-derived macrophages; CXCR4; CCR5

Introduction

The pandemic of infection with the human immunodeficiency virus, type 1 (HIV-1), continues to significantly affect the lives of millions (CDC Report, 2013). Some HIV⁺ subjects manifest neurocognitive comorbidities termed as HIV-associated neurocognitive disorders (HAND). HAND is clinically categorized as three subsyndromic comorbidities: 1) asymptomatic neurocognitive impairment (ANI), 2) mild motor cognitive disorders (MC/MD), and 3) HIV associated dementia (HAD) [1]. The use of combined antiretroviral therapy (cART) has attenuated neurocognitive comorbidities since its introduction in 1996 [2, 3]. However, at least 50% of HIV diagnoses who are taking cART manifest neurocognitive impairments (NCI), in which the most prevalent are the asymptomatic cases [4]. ANI is prevalent in 33% of cases, whereas MCMD and HAD are 12 and 2%, respectively [2]. HAND may result from the multifactorial interplay between host responses and viral factors [4]. HIV⁺ subjects often display parenchymal brain pathology [5], early loss of neuronal markers [6, 7], dysregulation in neurotransmitter balance [8, 9], and both impaired immune function [10] and elevated inflammatory cytokines in the central nervous system (CNS) [11, 12].

In addition, some HIV strains can cause greater neurotoxicity than do others as a consequence of viral gene heterogeneity. For instance, HIV-infection with clade B mediates a worse HIV-induced neuropathology when compared to clade C infection [13]. The HIV-1 *envelope* (*env*) gene plays an important role for mediating viral pathogenesis and influencing neurocognitive disease outcome in HIV⁺ patients [14, 15]. The *env* gene product gp120 binds to the membrane-bound CD4 receptor and to either chemokine receptors CCR5 or CXCR4 to mediate cellular fusion and viral entry [16]. The coreceptors CCR5 and CXCR4 are expressed in CD4⁺ immune cells such as monocytes-derived macrophages (MDM), perivascular macrophages and microglia. The *env* gene is subdivided into five variable (V1 – V5) and five constant regions (C1 – C5). The preference for coreceptor tropism of HIV *env* isolates is mainly dependent on the genetic composition within the variable region 3 (V3), but can also be influenced by the V1/V2 and V4 regions [16, 17].

In addition to its role in both cellular and coreceptor tropism, R5- and X4-tropic gp120s, exhibit different patterns of neuropathology [18, 19]. For instance, the X4-tropic gp120_{IIIB} specifically exhibits retrograde neuronal death within the rat brain [20]. In contrast, the R5-tropic gp120_{BaL} induces localized microglial activation [18]. Power et al 1998, demonstrated that recombinant viruses containing envelope regions from brain-derived HAND patients elicited greater neuronal death than *env* from patients with normal cognition [14]. *Env* isolates with macrophage tropism (M-tropic) often display neurotropic capabilities [21] and

are associated to HAD [22]. Herein, neurotropic M-tropic *env* sequences that the potential N-linked glycosylation site (PNLG) at position 308 efficiently replicated within cultured MDM. Also, R5X4 (dual-tropic) M-tropic isolates derived from brain exhibit potent neurotropic and a greater *in vitro* neurovirulent capability than isolates derived from peripheral tissues [19, 21, 23]. In study by Nieves *et al* 2007, co-receptor usage was analyzed from peripheral blood mononuclear cells (PBMC)-derived *env* isolates obtained from 21 women characterized for cognitive function [24]. PBMCs-derived *env* isolates from NCI HIV⁺ women showed both greater replication capacity and CXCR4 preference *in vitro* [24]. Whether genetic occurrences within the C2V4 region of isolates from NCI and non-NCI HIV⁺ Hispanic women are associated to HAND diagnosis remains obscure.

We previously reported the longitudinal genetic occurrences within the C2V4 of HIV *env* sequences in plasma and cerebrospinal fluid of a HIV⁺ subject with persistent ANI diagnosis [25]. Thus in the present study, we sought to characterize HIV *env* sequences obtained from PBMC co-cultures to determine the association between genetic occurrences within the C2V4 region and the severity of HAND diagnosis in 8 HIV⁺ Hispanic female patients from the SNRP cohort as previously described [24].

Materials and Methods

Ethics statement

This study was approved by the Institutional Review Board of the University of Puerto Rico, Medical Sciences Campus (Protocol 0720102). The samples were devoid of any personal identification.

Samples

Retrospective samples (n=8) of peripheral blood mononuclear cells (PBMC) supernatants collected from the Hispanic women cohort characterized for cognitive function from 2001 to 2008 were used for this study. These samples were collected as part of the Specialized Research Neuroscience Program in NeuroAIDS project entitled: “Monocyte Immunity and HIV dementia”. Inclusion criteria were: nadir CD4⁺ T-cell count ≥ 500 cells/mm³ or a viral load ≤ 1000 copies/ml. These parameters were evaluated at an AIDS Clinical Trial Group (ACTG) certified laboratory. Patients with a history of neuropsychiatric disorders, other infectious diseases such as hepatitis C, or with a positive toxicology report were excluded for this study. Evaluations of the patients were performed as described [24, 26]. Cognitive impairment was determined using a macro neurological exam and a battery of neuropsychological tests as described by the modified American Academy of Neurology criteria (m-AAN) for HIV-associated dementia [26]. According to m-AAN criteria patients were classified into normal cognition (NC), asymptomatic neurocognitive impairment (ANI), minor cognitive motor disorder (MC/MD), or HIV-1 associated dementia (HIV-D).

Viral isolation

Eight HIV-1 viral isolates (3 = normal cognition, 5 = cognitively impaired) that have been previously characterized for tropism, co-receptor usage, and replication capacity were used for this study [24]. Briefly, viral isolates were obtained after co-cultivation of peripheral

blood mononuclear cells (PBMCs) from HIV-seropositive patients with phytohemagglutinin (PHA)-activated PBMCs from normal donors. The PBMCs were obtained after Ficoll density gradient separation as previously described [24].

Sequencing

The eight HIV isolates were sequenced using *env*-specific primers. Sequences encompassing the HIV *env* V1-V5 hypervariable loop region were amplified from all eight isolates using nested PCR that recognizes flanking regions of the gp120 V1-V5 region. The first PCR was performed using the OneStep RT-PCR kit (Qiagen, Valencia, CA) with primers EnvA and EnvZ. The second PCR was performed with the GoTaq kit (Promega, Fitchburg Center, WI) and primers EnvB and EnvZ. The primer sequences for Env A, EnvB, and EnvZ were obtained from Dr. Maureen Goodenow [27]. The resulting fragment of approximately 1.4kb was obtained by gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and quantified. The purified PCR product was cloned into pCR2.1-TOPO or pDNA3.1 HIS/V5 vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Confirmation of cloning was made by EcoRI digestion. The resulting positive clones were used for further sequencing using the ABI Prism 310 DNA sequencing apparatus using T7, BGH and CV3R (5'TGATGGGAGGGGTATACATT3') primers.

Sequence alignment and phylogenetic analyses

All the sequence files were first manually verified and edited as necessary using the software Chromas Lite 2.0 (Technelysium Pty Ltd, Australia). Edited sequences were aligned using BioEdit version 7.0.5.2 [28] and Clustal W [29]. The Clustal W program (runs within BioEdit) was set to perform multiple sequence alignments using the default penalties. The sequences were then subjected to phylogenetic analysis, including tree construction, and divergence and diversity calculations using MEGA 6 [30]. Finally, sequences were translated to amino acids using BioEdit. Envelope gene sequences from Clade B HIV-1: HXB2, BaL, 89.6 and JR-CSF were used as reference sequences for CCR5-, CXCR4-, and dual-tropic strains, respectively. The evolutionary history was inferred using the neighbor-joining method [31], whereas the evolutionary distances were computed using the Jukes–Cantor method [32] and are in the units of the number of base substitutions per site.

Prediction of co-receptor usage and cellular tropism

We used validated tools for the prediction of co-receptor use and cellular tropism based on the V3 genotype [33] as previously described [25]. Briefly, the methods are based on the principle of the net V3 loop charge in combination with the presence of basic amino acid residues (i.e., lysine and aspartate) at position 11/25 in clade B subtypes. V3 sequences with a net charge +5 are associated with the X4 and R5/X4 phenotype, whereas a net charge of < +5 predicts an R5 phenotype [34, 35]. The genotypic algorithm position-specific scoring matrix (PSSM) (<http://indra.mullins.microbiol.washington.edu/webpssm/>) allows the prediction of co-receptor usage with a 97% specificity and 68% sensitivity [33, 36, 37]. We also supplemented co-receptor prediction analysis by using the geno2pheno tool (<http://coreceptor.bioinf.mpi-inf.mpg.de/>) [38].

Potential N-linked glycosylation sites (PNLGs)

The N-GlycoSite tool (<http://www.hiv.lanl.gov>) was used to highlight PNLGs from motifs with the amino acid context of N-X-S/T, in which an arginine (N) in the 1st position is followed by any amino acid (X, with the exception of proline) and a threonine or serine at the 3rd position [39].

Nucleotide sequences

Sequences in this report are available from GenBank (accession numbers: KJ882923-KJ882992; KT596903-KT596907).

Statistical analysis

Data were analyzed by One-way ANOVA followed by a post-hoc Tukey-Kramer all pairs comparisons test and correlation analyses with a 2-sided Spearman test. Values of $p < 0.05$ were considered to be statistically significant.

Results

Clinical findings and description of the study subjects

The SNRP cohort consists of Hispanic HIV⁺ female subjects who were infected by heterosexual contact. To determine neurocognitive impairment, the SNRP HIV-infected subjects undergo a comprehensive analysis of the neurological status by taking a battery of tests. Briefly, neurocognitive impairment was diagnosed based on the AAN HIV criteria (see materials and methods) to identify HAND status. Subjects with HAND were categorized based on the subsyndromic comorbidities: ANI, MCMD and HAD. Table 1 shows the clinical data for each isolate source, including CD4⁺ T cell counts, nadir CD4⁺ count, cART regimen, plasma and CSF viral load, and neurological status. The CSF viral load could not be determined for three of the *env* isolates: 3, 16 and 20. The CNS penetration effectiveness scores of the antiretroviral treatment was higher for subjects with normal cognition when compared to subjects with impaired cognition (3.33 ± 0.58 versus 1.20 ± 0.91 , $p = 0.012$). When analyzing the CPE scores based on the cognition status, there was a trend of lowest CPE scores in subjects with HAD (CPE scores: NC>ANI>MCMD>HAD; $p = 0.094$). In addition, comorbid depression was assessed using the Beck Depression Index (BDI) which scores depressive symptoms with the highest number being of greater severity. However, no significant differences were noted based on cognition status.

C2V4 sequence diversity correlates with the severity of neurocognitive impairment

Isolates evaluated in this study have been previously described for their coreceptor usage and replication capacity in both PBMCs and MDM [24]. Table 2 displays the phenotype cell tropism for each isolate being T-, M- or D-tropic [24]. We sought to determine the genetic relationship among C2V4 sequences of isolates from subjects with or without HAND. We performed phylogenetic analysis of the C2V4 region of isolates, using MEGA 6.0 with default penalties [25]. Figure 1 shows the resulting neighbor-joining phylogenetic tree of the 73 C2V4 *env* sequences from clinical isolates and 5 reference *env* sequences used as outgroup controls. None of the reference sequences formed monophyletic groups with the

clinical isolates (Figure 1). Four out of 5 reference sequences had a bootstrap value of 100% and the remaining sequence (*env* 89.6, open square) formed a single monophyletic taxonomic unit. *Env* C2V4 sequences from our clinical isolates rearranged as distinct monophyletic groups. Isolates from normal cognition (1, 3 and 7) formed unique monophyletic groups (100% bootstrap), without intermixing of isolates between other NC or NCI groups. Isolates from ANI (grey squares), showed a similar phylogenetic distribution pattern in which unique monophyletic groups were formed (100% bootstrap). We observed that 50% of sequences from isolate 15 (grey triangles) clustered within same MCMD monophyletic group as all sequences from isolate 16 (grey circles). In addition, we did not detect intermixing of C2V4 sequences derived from HAD isolates 20 (black squares) and 21 (black triangles). However, the monophyletic sequences from the HAD isolate 20 demonstrated a divergent pattern in branch length.

To determine whether the extent of *env* sequences diversity from PBMC co-cultures varied among HAND subsyndromes, we utilized the built-in tool in MEGA 6.0 to calculate the intragroup mean diversity for the *env* C2V4 region. Intra-group sequence diversity was higher in isolates obtained from HIV⁺ subjects with HAND (n= 5) compared to those with normal cognition (n=3). Specifically, NC isolates 1, 3 and 7 had C2V4 diversity of 0.001 (± 0.001), 0.002 (± 0.002), and 0.002 (± 0.001), respectively. The calculated diversity for ANI isolates was of 0.01 (± 0.005). Sequences from both MCMD isolates 15 and 16 respectively exhibited a diversity value of 0.059 (± 0.026) and 0.003 (± 0.002), being highest for isolate 15 (Figure 2A). Spearman correlation test show that C2V4 sequence diversity correlates with the severity of neurocognitive impairment ($p = 0.0286$; Figure 2B) and inversely correlates with CNS penetration effectiveness scores of the antiretroviral treatment (CPE; $p = 0.013$; Figure 2C).

The net charge of the V3 and V4 variable regions differed based on cognitive status

We analyzed the deduced amino acid sequence of each isolate by neurocognitive status. We performed sequence alignment of the variable regions V3 and V4 and compared them with clade B reference sequence. Table 2 lists the amino acids within the V3 region and the coreceptor tropism by using different inference methods. We inferred the coreceptor tropism *in silico* by using the bioinformatics tools geno2pheno (<http://coreceptor.bioinf.mpi-inf.mpg.de/>), and the position specific score matrix (PSSM) (<http://indra.mullins.microbiol.washington.edu/webpssm/>). We also report the coreceptor usage by calculating the V3 loop net charge and by applying the 11/25 rule. The V3 sequences from NC isolate 1 (n=10), and 7 (n=12) indicate a preference for using R5 as coreceptor of choice. The NC isolates 1 and 7 were characterized as being M-tropic phenotype *in vitro*. Among all sequences from NC isolate 1, only one sequence had lost the cysteine at position 330 of *env* (Table 2). All sequences from NC isolate 3 (n=8) exhibited an X4- or dual-tropic genotype that was in accordance with the D-tropic phenotype described by Nieves et al. 2007 [24]. The inferred genotype of all sequences from the ANI isolate 11 corresponded to that of an R5. All ANI isolates were previously demonstrated to have an M-tropic phenotype. The MCMD sequences from isolate 15 were classified as T-tropic, whereby three clones of these preferred either X4 or R5 as coreceptors of choice (Table 2). The PNLG at position 301 of V3 was lost in only two sequences from MCMD isolate 15 (X4/T-tropic).

Due to a lack of viral replication *in vitro*, the cell tropism could not be determined for isolates from subject 16. However, genotype inference analysis indicates that these isolates have a preference for using CCR5. In contrast, isolates from HAD subjects exhibited differences in both phenotype coreceptor usage and V3-inferred coreceptor tropism. The cellular tropism phenotype of HAD isolates 20 and 21 were respectively D- and M-tropic. Of the sequences from the HAD isolate 20 (n=7), three showed a preference for R5 and four for X4. In the case of HAD isolate 21, all sequences were inferred as being X4-tropic.

To further understand the genetic characteristic of HAND isolates, we calculated the net charge for both V3 and V4 loops in all sequences by subtracting the negative charge to that of the positive charges of amino acids residues. Group comparison of the net charge of the V3 region demonstrated a significant difference ($p = 0.018$). Analysis of pair comparisons revealed that the net charge of the V3 region of NC isolates did not significantly differed from any of HAND isolates (Figure 3A). However, a significant difference in V3 net charge was detected when comparing the isolates from ANI vs. HAD ($p = 0.033$) and when comparing MCMD vs. HAD ($p = 0.032$). The mean V3 net charge $\pm$ SD in the isolates of ANI, MCMD and HAD neurocognitive status were 6.0 ± 0.0 , 6.2 ± 0.4 , and 7.2 ± 1.3 , respectively. Figure 3B shows the V4 net charge values by HAND diagnosis. Statistical analyses of the V4 net charge between isolates demonstrated a significant difference in the V4 charge by status of neurocognitive impairment ($p < 0.0001$). When compared to sequences of the NC isolates, there was a significant difference in V4 net charge from sequences of ANI ($p = 0.005$) and MCMD ($p < 0.0001$) isolates. The V4 net charge was also significant between ANI isolates versus MCMD isolates ($p = 0.034$). For HAD isolates, the V4 net charges did not differed between those calculated for NC isolates (Figure 3B) but significantly differed between ANI ($p = 0.035$) and between MCMD ($p < 0.0001$).

We also analyzed the length of V4 region and found that it was distinct among all isolates by neurocognitive status. Within the V4 region, sequences from NC exhibited having the same length as clade B reference sequence (30 amino acids). Sequences from ANI isolates had 33 amino acids in length in V4. There were three out of six sequences from MCMD isolate 15 that had shorter V4 loops (28 amino acids) (Table 3). The remaining three sequences from MCMD isolate 15 and in eleven sequences of MCMD isolate 16 the V4 length was of thirty-two amino acids. Only one sequence from isolate 16 showed a V4 length of thirty-one amino acids. HAD isolates had a V4 length of thirty-two amino acids, with the exception of three out of seven sequences of HAD isolate 20, which had a length of thirty amino acid (Table 3).

The pattern of potential N-linked glycosylation sites differed based on neurocognitive status

We also determined the number of PNLG in sequences by using the N-glycosite tool (<http://www.hiv.lanl.gov>) within the V4 region (Table 3). Group comparison of the number of PNLGs in V4 sequences by neurocognitive showed a significant difference ($p < 0.0001$). Specifically, the mean PNLGs from MCMD isolates (2.3 ± 0.8) was significantly lower than the mean PNLGs of the other groups: NC (4.5 ± 0.9 , $p < 0.0001$); ANI (4.5 ± 0.5 , $p < 0.0001$); HAD (4.3 ± 0.8 , $p < 0.0001$). We identified that the PNLG at position 386 was absent in three sequences of isolate 15 and in all sequences from isolate 16 (Table 3). In

addition, loss of the PNLG at 386 was also evident for three sequences in HAD isolates from subject 20.

DISCUSSION

In the present study, we analyzed the HIV *env* C2V4 sequences of clinical isolates obtained from PBMCs of HIV⁺ women with varying degrees of HAND. Here we provided a genetic characterization of the C2V4 region from clinical HIV *env* isolates matched with well-defined HAND diagnoses according to the ANN criteria [24]. We complemented the corresponding *in vitro* cellular tropism for each clinical isolate [24] by examining the amino acid sequences of C2V4. Thus for all isolates, the tools for inferring coreceptor tropism provided additional clues about the predominant HIV *env* strains that may present during the different HAND subsyndromes.

Phylogenetic analyses showed that the majority of the *env* C2V4 sequences clustered within distinct monophyletic groups. The NJ-phylogenetic tree indicated that outgroup references showed no monophyletic clustering among our clinical isolates. Interestingly, subjects 15 and 16 were diagnosed with MCMD and share isolates with similar V3 *env* sequences. We observed that three sequences from isolate 15 (grey triangles) and all sequences from isolate 16 (grey circles) grouped within the same monophyletic group with a 100% bootstrap value. A bootstrap value provides strong support that there is an epidemiological link between isolate 15 and 16. This finding suggests that subjects 15 and 16 may have become infected from a singular HIV source.

Analysis of the mean sequence diversity among clinical isolates by neurocognitive impairment revealed that sequences from MCMD and HAD isolates exhibited a greater viral diversity than sequences from NC or ANI. In a classic study, *env* sequences from HAD brains displayed a greater genetic diversity within the C2V3 region [15]. In said study, neurocognitive impairment in HIV⁺ subjects was determined using the AAN Criteria by the DANA Consortium [40]. In our study, we extended our analysis to include the V4 region and categorized the clinical isolates based on the updated ANN criteria for HAND diagnosis, to include the asymptomatic category [1]. Van Marle et al 2002 demonstrated that HAD subjects had impaired viral neutralization responses compared to HIV⁺ subjects without dementia. Considering the observations by Van Marle et al 2002, a greater C2V4 diversity may present in isolates obtained from with severe neurocognitive impairment apparently resulted from altered immune function [15]

The C2V4 *env* diversity was greatest for MCMD sequences of isolate 15 (Figure 2A). The extent of the genetic diversity in sequences from the MCMD isolate 15 may have mostly resulted from the distinct lengths seen for the V4 region. For instance, 3 sequences had a length 28 amino acids whereas 3 sequences had length a 32 amino acid in V4. The C2V4 diversity in sequences from HAD subject 20 were greater than either normal or ANI. Considering that C2V4 *env* diversity was greater in MCMD and HAD isolates, we analyzed the degree of association between C2V4 diversity and cognitive impairment. The severity of neurocognitive impairment correlated with increased C2V4 diversity. These results suggest that sequences from NCI isolates are more heterogeneous within the C2V4 region than

isolates obtained from subject with NC or ANI. Interestingly, we also found that sequence diversity within the C2V4 region of *env* inversely correlated with greater CPE scores. The observation that HIV⁺ subjects on cART regimens with higher CPE scores and lower *env* sequence diversity suggests that effective cART penetrance is required to reduce *env* heterogeneity of residual viral isolates. Antiretroviral treatment regimens with CPE scores equal to or greater than 2 have been shown to correlate with improved neurocognitive function by 12 weeks after initiating therapy [41]. In our study, HIV-infected subjects with either normal cognition or ANI were receiving cART regimens with CPE scores >2, and their respective isolates exhibited lower C2V4 genetic diversity compared to MCMD or HAD receiving cART with CPE < 2. To our knowledge, this is the first study to demonstrate a correlation between CPE scores and viral *env* gene diversity. Although we cannot exclude the possibility that additional interplay between host factors can affect neurocognitive status, one tentative explanation in the context of viral factors is that a greater C2V4 *env* heterogeneity may result in the emergence of either neurotropic or neurovirulent isolates. Although M-tropism better predicts neurotropism than does the specificity of coreceptor usage [21], M-tropic isolates display a higher affinity towards CCR5 and a reduced dependence towards CD4 and CCR5 [42] by mechanisms involving distinct gp120-CD4 interactions [43]. Neurovirulence also may depend on coreceptor preference and enhanced capabilities of *env* to engage a preferred coreceptor. For instance, neurovirulent *env* strains trigger neuronal apoptosis and often exhibit R5 or R5/X4 phenotypes with greater capacity to engage CCR5 [19]. The *in vitro* phenotype determined for MCMD isolate 15 was that of an T-tropic with an X4 or R5 coreceptor preference (Table 2), whereas the determined phenotype of HAD isolates were either M-tropic or dual-tropic that preferentially uses CXCR4 (Table 2).

Besides studying the V3 region because its usefulness to infer coreceptor tropism, we extended our analysis to the V4 region. We observed that genetic occurrences and the extent of variability within the V4 region varied among all sequences. Dunfee et al 2006 demonstrated that an *env* isolate from a HAD subject lost the PNLG of position 386 of V4. Specifically, the absence of the PNLG 386 was greatly associated to the enhanced infection of MDM. In our study we found that three MCMD sequences and three HAD sequences had lost the PNLG 386. The associated phenotypes with these sequences were either T-tropic or M-tropic but demonstrated to prefer X4 as coreceptor.

To our interest, the mean number of PNLGs in MCMD isolates was significantly lower ($p<0.0001$) compared to all isolates from other neurocognitive status. Also, we found various lengths within the V4 sequences among isolates. A length of 33 amino acids was detected for only V4 loops of ANI isolates. Considering that the mean PNLG number in MCMD sequences was significantly lower, we found that three sequences with shorter V4 loops (28 amino acids) had conserved 4 PNLGs. In contrast, fourteen out of the eighteen of MCMD sequences had longer V4 loops and retained only 2 PNLGs. Thus the number of PNLGs did not correspond with V4 length.

In a longitudinal study using autologous sequences from an ANI patient, we found a significant difference in the V4 net charge when comparing clones from plasma and CSF [25]. As in our previous study using plasma derived-clones, clones derived from the PBMCs

of a subject with ANI also had an overall charge of -2 . Interestingly, the clones analyzed in the current study from the subjects with MCMD also had higher overall negative charges in V4 when compared to NC and HAD. Thus, the variability within the V4 region may be highly associated with the mild forms of neurocognitive impairment: ANI and MCMD.

This study had several limitations including the sample size within neurocognitive impairment classifications. In some cases, HIV *env* could not be amplified from PBMCs which may have been a function of the impact of subjects variable cART exposure and regimens. For this similar reason, we believe that CSF HIV isolates were unsuccessfully amplified despite various attempts. This hinders our analysis for brain or CSF isolates which can provide better clues about the C2V4 genotypes present *in vivo*. In addition, this study used stored samples from a cohort of Hispanic women that were evaluated for cognition status with limited previous studies of the viral genetic variance. Also, because our study is of cross-sectional design, comparisons of longitudinal changes in neurocognitive status and the occurrence of amino acid within C2V4 of *env* could not be obtained. Lastly, we had limitations in the analysis of the full-length of *env* due to incomplete regions in some sequences. Nonetheless, our study is insightful in that we provide detailed characteristics of the inherent C2V4 sequences from *env* isolates obtained from PBMCs, categorized by HAND diagnoses. One of the main findings of our study is that the mean PNLG number within V4 region was significantly lower in MCMD isolates compared to isolates from other groups. The next generation sequence studies should consider the inclusion of V4 as an informative site associated with the neurocognitive status of HAND diagnoses. Thus determining *env* sequence characteristics for the isolates grouped in milder forms of HAND can provide insightful information that may be of supporting prognostic value to assess neurocognitive status in HIV⁺ subjects, particularly during the era of highly prevalent milder forms of HAND.

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Figure 1. Molecular phylogenetic analysis of HIV-1 viral isolates from subjects with normal cognition (NC), asymptomatic neurocognitive impairment (ANI), minor cognitive motor disorder (MCMD) or HIV-1 associated dementia (HAD)

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [44]. The tree with the highest log likelihood (−3443.0425) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 78 nucleotide sequences. Codon positions included were 1st+2nd +3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 493 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [30].

Figure 2. Diversity within the C2V4 region correlates with the cognition status

Panel A: The mean sequence diversity analysis within the C2V4 region of each HIV-1 viral isolate was conducted using the Maximum Composite Likelihood model [45]. Evolutionary analyses were conducted in MEGA6 [30]. Error bars indicate the mean standard error and were obtained by a bootstrap procedure (500 replicates). Panel B: Correlation of the C2V4 sequence diversity with cognition status. Patient cognition was classified as normal cognition (0), asymptomatic neurocognitive impairment (1), minor cognitive motor disorder (2), and HIV-dementia (3). Panel C: Correlation of the C2V4 sequence diversity with CPE scores. Correlation was statistically analyzed using a 2-sided Spearman correlation test. The cut-off for significance was $p = 0.05$. CB: Confidence band; CPE: CNS penetration effectiveness.

Figure 3. The V3 and V4 net charges significantly differed between the cognition statuses
 The net charges of the gp120 regions V3 (Panel A) and V4 (Panel B) were calculated by subtracting the number of negatively charged amino acids from the number of positively charged amino acids. Black squares indicate the mean $\pm$ standard deviation. Statistical comparisons were done using single factor analysis of variance (ANOVA) followed by a Tukey-Kramer all pairs comparisons test. Values of $p < 0.05$ were considered significant.

Figure 4. HIV-1 viral isolates from MCMD have decreased PNLG sites within the gp120 V4 region

Sequences from gp120 V4 region were translated and aligned. N-linked glycosylation sites [N(X)T/S] were predicted using the N-glycosite software from Los Alamos Database. Black squares indicate the mean $\pm$ standard deviation. Statistical comparisons were done using single factor analysis of variance (ANOVA, *p* value indicated in the graph) followed by a Tukey-Kramer all pairs comparisons test. Values of $p < 0.05$ were considered significant (* $p < 0.0001$).

Table 1**Viral and immune parameters of study subjects**

Blood samples were collected and used to determine CD4⁺ T-cell counts and viral load, and the CSF samples were used for viral load. The neurological status of the subject was examined by a battery of neurological tests.

Viral Isolate	Age	CD4 ⁺ Nadir cells/mm ³	CD4 ⁺ T-cell count cells/mm ³	Antiretrovirals	Plasma viral load (Log ₁₀ copies/mL)	CSF viral load (Log ₁₀ copies/mL)	CPE Score	Clinical (Neurological)/BDI*
1	37	39	190	Amprenavir, AZT-3TC, AZT	4.67	2.53	3	NC / 25
3	37	185	100	d4T, 3TC, Delavirdine, Indinavir, Risperidol	4.99	ND	4	NC / 18
7	48	426	466	Kaletra, Efavirenz, AZT-3TC	< 1.7	< 1.7	3	NC / 0
11	29	22	22	d4T, 3TC, Kaletra	5.68	2.27	2	ANI / 1
15	37	296	296	d4T, ddI, Nelfinavir	3.14	1.83	0.5	MCMD / 7
16	34	225	210	Efavirenz, AZT-3TC	4.17	ND	2	MCMD / 12
20	30	337	204	Naïve	4.32	ND	0	HAD / 1
21	29	35	46	d4T, Indinavir, Ritonavir, Viread	4.83	1.97	1.5	HAD / 30

* Cognitive impairment was defined according to the American Academy of Neurology (AAN) HIV criteria modified to identify asymptomatic cognitive impairment as described in the Materials and Methods.

CPE: CNS penetration effectiveness. BDI= Beck's depression index. NC = normal cognition; ANI = asymptomatic neurocognitive impairment; MCMD = minor cognitive motor disorder; HAD = HIV-associated dementia.

Table 2
V3 amino acid sequences and co-receptor usage prediction of HIV-1 isolates obtained from the supernatants of PBMCs co-cultures

Co-receptor usage was determined using various computational methods. V3 loop sequences from PMBCs-derived isolates are presented. Changes in the amino acid sequence as compared to the V3 loop of the Clade B consensus sequence are in red. The PNLG site at position 301 is underlined.

Clones ^a	V3 region	b	V3 charge	c	11/25 Rule	d	Geno2pheno	e	PSSM	f	Cell tropism
Clade B	CTRPNNNTRKS-IHIGPGRAFYTTGEIIIGDIRQAH	5		R5		R5		R5		-	
Normal											
Isolate 1											
9	CTRP G NNNTRKS-IH M GPGR A F H AT G AIIIGDIR A HC	8		R5		X4		R5			
1	CTRP G NNNTRKS-IH M GPGR A F H AT G AIIIGDIR A HC	8		R5		X4		R5			M-tropic
Isolate 3											
6	CTRPNNNT GKR -ISIGPGRA FVAKRQ ITGDIRQAH	7		X4 / D ^g		X4		X4			
1	CTRPNSNT GKR -ISIGPGRA FVAKRQ ITGDIRQAH	7		X4 / D		X4		X4			Dual
1	CTRPNNNT GKR -IS M GPGR A F VAKRQ ITGDIRQAH	7		X4 / D		X4		X4			
Isolate 7											
11	CTRPNNNTRKS-I N IGPGRAFYTTG D IIIGNIRQAH	5		R5		R5		R5			
1	C P RPNNNTRKS-I N IGPGRAFYTTG D IIIGNIRQAH	5		R5		R5		R5			M-tropic
ANI											
Isolate 11											
3	C S RPNNNTRK G -IH V GPGR A IYTTG Q IVGDIRQAH	6		R5		X4		R5			
6	C S RPNNNTRK G -IHIGPGRA I YTTG Q IVGDIRQAH	6		R5		X4		R5			M-tropic
1	C S RPNNNTRK G -IH V GPGR K AVTTG Q IVGDIRQAH	6		R5		X4		R5			

Clones ^a	V3 region	b V3 charge	c 11/25 Rule	d Geno2pheno	e PSSM	f Cell tropism
Isolate 15						
3	CTRPNNNT TRKS -IHIGPGRAFYATGDIIGDIRAHC	6	R5	X4	R5	T-tropic
2	CTRP GYN IK RR IIHIGPGRAF HAT -G-GGDIRA SC	7	X4/D	X4	X4	
1	CTRP GYN IK RR IIHIGPGRAF HAT -G-GGDIRA FC	7	X4/D	X4	X4	
Isolate 16						
11	CTRPNNNT TRKS -IHIGPGRAFYATGDIIGDIRAHC	6	R5	X4	R5	ND ^h
1	CTRPNNNT TRKS -IHIGPGRAFYATGDIIGDTRAHC	6	R5	X4	R5	
<u>HAD</u>						
Isolate 20						
4	CTRPNNNT TRKR -IQIH PG AFYATGI-VGDIRKAHC	7	X4/D	X4	X4	Dual
2	CTRPNNNT TRKS -IHIGPGRAFYATGDIIGDIRQAHC	5	R5	R5	R5	
1	CARPNNNT TRKS -IHIGPGRAFYATGDIIGDIRQAHC	5	R5	R5	R5	
Isolate 21						
5	CTRPNNHKKRK-VTLGPGRVYYTTGEIVGDIRKAHC	8	X4/D	X4	X4	M-tropic
1	CTRPNNHKKRK-VTLGPGRVYYTTGEIVGDIRKARC	8	X4/D	X4	X4	
1	CTRPNNHKKRK-VTLGPGRVYYTTGEIVGDIRKAHC	8	X4/D	X4	X4	
1	CTRPNNHKKRK-VTLGPRRVYYTTGEIVGDIRKAHC	9	X4/D	X4	X4	

^aThe number indicates the total number of clones with the same *env* V3 sequence.

^bV3 charge was determined by subtracting the total number of negatively charged amino acids (D+E) from the total number of positively charged amino acids (H+K+R). Higher positive charge has been correlated with the likelihood of CXCR4 use.

^cSequences with positively charged amino acids at positions 11 and/or 25 within the V3 loop were classified as having an 11/25 genotype (believed as X4 or X4/R5 strain). Positions 11 and 25 are highlighted in grey.

^dGeno2pheno, *env* V3 sequence was aligned to the reference strain HXB2 and predicted as to whether the corresponding virus is capable of using CXCR4 as a coreceptor (R5/X4 or X4 variants) or not (R5 variants).

^ePSSM, Position-specific scoring matrix (PSSMX4R5 and PSSM SINSI)

^fCell tropism of viral isolates was previously determined by Toro-Nieves et al. 2007.

I_{ND} : not determined because of lack of viral replication in phytohemagglutinin (PHA)-stimulated PBMCs.

I_D : Dual tropic

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Table 3
V4 amino acid sequences of HIV-1 isolates obtained from the supernatants of PBMCs co-cultures

V4 loop sequences derived from plasma-derived of NCI patients are presented. The arrow marks the PNLG site at position 386 as described in Dunfee et al. 2007.

Clones ^a	V4 region	V4 charge ^b	PNGL ^c
Clade B	↓ C <u>N</u> STQ <u>L</u> F <u>N</u> STW <u>N</u> ---VTE-ES <u>N</u> NTV <u>E</u> NN <u>T</u> ITLPC	-4	5
Normal			
Isolate 1			
8	C <u>N</u> STPL <u>F</u> <u>N</u> STW <u>N</u> I <u>N</u> -STG <u>N</u> GT <u>D</u> --GSK- <u>N</u> ITL <u>Q</u> C	0	5
2	C <u>N</u> STPL <u>F</u> <u>N</u> STW <u>N</u> I <u>N</u> -GTG <u>N</u> GT <u>D</u> --GSK- <u>N</u> ITL <u>Q</u> C	0	5
Isolate 3			
8	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>H</u> <u>N</u> <u>N</u> <u>D</u> --TSP <u>N</u> A--GGDDK <u>I</u> L <u>P</u> C	-1	3
Isolate 7			
11	C <u>N</u> TTK <u>L</u> F <u>N</u> STW <u>S</u> <u>S</u> <u>N</u> NTW <u>N</u> GTEG <u>N</u> W <u>N</u> GTDP <u>I</u> L <u>P</u> C	-1	5
1	C <u>N</u> TTRL <u>F</u> <u>N</u> STW <u>S</u> <u>S</u> <u>N</u> NTW <u>N</u> GTEG <u>N</u> W <u>N</u> GTDP <u>I</u> L <u>P</u> C	-1	5
ANI			
Isolate 11			
3	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>N</u> GTER <u>Y</u> <u>N</u> YTVG-- <u>N</u> ESDP <u>I</u> L <u>P</u> C	-2	5
3	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>N</u> GNER <u>F</u> <u>N</u> YTVG-- <u>N</u> ESDP <u>I</u> L <u>P</u> C	-2	4
3	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>N</u> GTER <u>F</u> <u>N</u> YTVG-- <u>N</u> ESDP <u>I</u> L <u>P</u> C	-2	5
1	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>N</u> GNER <u>Y</u> <u>N</u> YTVG-- <u>N</u> ESDP <u>I</u> L <u>P</u> C	-2	4
MCMD			
Isolate 15			
3	C <u>N</u> TLK <u>L</u> F <u>N</u> STW <u>N</u> GTLDEHSTEE--SGDDT <u>I</u> L <u>P</u> C	-4	2
3	C <u>N</u> TTQ <u>L</u> F <u>N</u> STW <u>N</u> DTKGS <u>I</u> <u>N</u> ITG-----HFTL <u>P</u> C	1	4
Isolate 16			
11	C <u>N</u> TLK <u>L</u> F <u>N</u> STW <u>N</u> GTLDEHSTEE--SGDDT <u>I</u> L <u>P</u> C	-4	2
1	C <u>N</u> TLK <u>L</u> F <u>N</u> ST- <u>N</u> GTLDEHSTEE--SGDDT <u>I</u> L <u>P</u> C	-4	2
HAD			
Isolate 20			
1	C <u>N</u> TSK <u>L</u> F <u>N</u> STW <u>N</u> DT <u>S</u> ARTY <u>T</u> ED--P <u>N</u> STEIT <u>I</u> P <u>C</u>	-2	4
1	C <u>N</u> TSK <u>L</u> F <u>N</u> STW <u>N</u> DT <u>S</u> AWSY <u>N</u> KE-- <u>S</u> <u>N</u> STEITL <u>P</u> C	-1	4
2	CDTTK <u>L</u> F <u>N</u> STW <u>N</u> KTS <u>A</u> WKY <u>T</u> EG--TD <u>N</u> --FT <u>I</u> P <u>C</u>	0	3
1	CDTTK <u>L</u> F <u>N</u> STW <u>N</u> KTS <u>A</u> WKY <u>T</u> EG--TD <u>N</u> --LT <u>I</u> P <u>C</u>	0	3
1	C <u>N</u> TSK <u>L</u> F <u>N</u> STW <u>N</u> DT <u>S</u> AWNY <u>N</u> KE-- <u>S</u> <u>N</u> STEITL <u>P</u> C	-1	4
1	R <u>N</u> TSK <u>L</u> F <u>N</u> STW <u>N</u> DT <u>S</u> AWTY <u>T</u> ED--P <u>N</u> STEIT <u>I</u> P <u>C</u>	-2	4

Clones ^a	V4 region	V4 charge ^b	PNGL ^c
Isolate 21			
7	CNSTKLFNSTWHINNTWEGLN--NTEENITLPC	-1	5
1	CNSTKLFNSTWHINNTWEGLN--NTEKNITLPC	1	5

^aThe number indicates the total number of clones with the same *env* V4 sequence.

^bV4 net charge was determined by subtracting the total number of negatively charged amino acids (D+E) from the total number of positively charged amino acids (H+K+R) [23].

^cPNLG: Potential N-Linked Glycosylation Site; PNLG in position 386 (arrow). Loss of PNLG at this site is described to confer higher macrophage tropism and is associated with severe HIV-associated dementia as described by Dunfee et al. 2007.