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Selected amino acid changes in HIV-1 subtype-C gp41 are associated with specific gp120$_{V3}$ signatures in the regulation of co-receptor usage

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ABSTRACT

The majority of studies have characterized the tropism of HIV-1 subtype-B isolates, but little is known about the determinants of tropism in other subtypes. So, the goal of the present study was to genetically characterize the envelope of viral proteins in terms of co-receptor usage by analyzing 356 full-length *env* sequences derived from HIV-1 subtype-C infected individuals. The co-receptor usage of V3 sequences was inferred by using the Geno2Pheno and PSSM algorithms, and also analyzed to the "11/25 rule". All reported env sequences were also analyzed with regard to *N*-linked glycosylation sites, net charge and hydrophilicity, as well as the binomial correlation phi coefficient to assess covariation among gp120$_{V3}$ and gp41 signatures and the average linkage hierarchical agglomerative clustering were also performed.

Among *env* sequences present in Los Alamos Database, 255 and 101 sequences predicted as CCR5 and CXCR4 were selected, respectively. The classical V3 signatures at positions 11 and 25, and other specific V3 and gp41 amino acid changes were found statistically associated with different co-receptor usage. Furthermore, several statistically significant associations between V3 and gp41 signatures were also observed. The dendrogram topology showed a cluster associated with CCR5-usage composed by five gp41 mutated positions, A22V, R133M, E136G, N140L, and N166Q that clustered with T2V$_{V3}$ and G24T$_{V3}$ (bootstrap = 1). Conversely, a heterogeneous cluster with CXCR4-usage, involving S11GR$_{V3}$, 13–14insIG/LG$_{V3}$, P16RQ$_{V3}$, Q18KR$_{V3}$, F20ILV$_{V3}$, D25KRQ$_{V3}$, Q32KR$_{V3}$ along with A30T$_{gp41}$, S107N$_{gp41}$, D148E$_{gp41}$, A189S$_{gp41}$ was identified (bootstrap = 0.86).

Our results show that as observed for HIV-1 subtype-B, also in subtype-C specific and different gp41 and gp120V3 amino acid changes are associated individually or together with CXCR4 and/or CCR5 usage. These findings strengthen previous observations that determinants of tropism may also reside in the gp41 protein.

1. Introduction

Ninety percent of HIV-1-infected people worldwide harbors non-B-subtype variants, and consequently the vast majority of cases of infections are due to these viruses (Arien et al., 2007). Globally, the C-subtype is the most prevalent circulating viral clade and accounts for nearly half of infections, followed by A, B, G subtypes and the recombinant form CRF02-AG and CRF01-AE (Hemelaar et al., 2011).

The higher rate of non-synonymous mutations tends to occur in regions of the HIV-1 *env* gene and is submitted to strong selective pressure from the immune system (Choisy et al., 2004; Lemey et al., 2006; Mikhail et al., 2005; Zhang et al., 2005). A structure of particular importance in this process is the third variable loop (V3) of the surface glycoprotein gp120 which is essential for HIV-1 co-receptor usage (de Jong et al., 1992; Fouchier et al., 1992; Huang et al., 2005). In most European countries, HIV tropism is identified with tropism phenotype testing. New data support genotype analysis of the V3 for the identification of HIV-1 tropism (Vandekerckhove et al., 2011).

HIV-1 enters into the host cell by binding gp120 to CD4 receptor on a target cell, leading to conformational changes within gp120 that allows for the engagement of a second host cell receptor (co-receptor) (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Trkola et al., 1996; Wu et al., 1996). About 20 G-protein-coupled receptors

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(GPCRs) have been shown to act *in vitro* as co-receptors (Neil et al., 2005; Shimizu et al., 2009; Simmons et al., 2000), but only CCR5 and CXCR4 are considered essential and apparently relevant in HIV pathogenesis (Berger et al., 1998; Simmons et al., 2000; Zhang et al., 1998). Moreover, the interaction with co-receptor induces the arrest of the gp41 transitions at a pre-hairpin intermediate stage that leads to the insertion of the fusion peptide into the target cell membrane and ultimately to virus-cell fusion activity (Eckert and Kim, 2001; Wyatt and Sodroski, 1998).

The gp41 is a transmembrane glycoprotein that retains the gp120 on viral surface with non-covalent interactions (Helseth et al., 1991) and some studies indicate that several mutations in gp41 were involved to be significantly associated with co-receptor usage (Dimonte et al., 2011a; Huang et al., 2008; Stawiski et al., 2009; Thielen et al., 2009, 2010), beyond the primary classical determinants of gp120 including particularly positions 11 and 25 in V3-loop (de Jong et al., 1992; Fouchier et al., 1992; Resch et al., 2001), and secondly by other flanking domains (as V1, V2, C3, C4 and V5) (Carrillo and Ratner, 1996; Huang et al., 2008, 2011; Koito et al., 1995; Labrosse et al., 2001; Lin et al., 2011; Pastore et al., 2006; Svicher et al., 2011b; Suphaphiphat et al., 2007). Both CCR5 and CXCR4 co-receptors interact with the same region of the surface gp120 viral protein that encompasses not only the V3 loop but also specific regions from the V1/V2 and the C4 domains (Sierra et al., 2007).

Moreover, few amino acid substitutions and an increasing net charge of the V3-loop were sufficient to confer a change from CCR5 to CXCR4 in cellular tropism (de Jong et al., 1992; De Wolf et al., 1994). On the other hand, the previous studies have defined the loss of a Potential *N*-linked Glycosylation Site (PNGS) at V3 positions 6–8 (Pollakis et al., 2001), as a close association between the V3-loop *N*-linked glycosylation motifs (sequons) and CXCR4 usage (Clevestig et al., 2006).

Molecular mechanisms underlying the transition from CCR5 to CXCR4 usage of clade C virus remain poorly known. With the recent introduction of HIV-1 chemokine receptor antagonists on the market as components of antiretroviral therapy, it is increasingly important to properly screen co-receptor usage for all infected patients prior to therapy (Hunt and Romanelli, 2009; Sayana and Khanlou, 2009). Hence, simple and efficient processes for routinely characterizing and monitoring HIV-1 co-receptor usage are needed to replace slow and resource-intensive phenotypic assays. Existing methods do not consider the other gp120 regions, mainly for limited data available, although incorporating the V2-loop is known to improve prediction methods based on V3 sequence information (Prosperi et al., 2009), and key genetic-elements in V1, V2, and C4 domains tightly and differentially modulate HIV-1 dependency on CXCR4 or CCR5, irrespective of V3 genetic-background (Svicher et al., 2011a). Nevertheless, genotypic determinants of co-receptor usage located outside V3 could also explain some of the mispredictions (Raymond et al., 2010).

In this study, large datasets of HIV-1 $gp120_{V3}$ and gp41 C-subtype sequences were analyzed to genetically characterize them in terms of co-receptor usage. In addition, according to CCR5 and/or CXCR4 usage, the association between amino acid signatures, average hydrophilicity, net charge, and number of *N*-linked glycosylation sites were defined for the V3 and the gp41.

2. Materials and methods

2.1. Sequence analysis

The analysis included 312 HIV-1 C-subtype *env* full-length sequences and other 44 HIV-1 C-subtype V3 sequences, retrieved from the Los Alamos Database (overall from 356 infected individuals at all stages of infection, with one isolate *per* single patient) (http://www.hiv.lanl.gov) (Table S1). The treatment status for the individuals is not available in the Los Alamos Database. The multiple sequence alignments of V3 and gp41 segments were performed by using ClustalX (Thompson et al., 1997) and manually edited with the Bioedit software (Hall, 1999). Published *env* consensus sequences of pure HIV-1 subtypes (A, B, C, D, F1, F2, G, H, J, and K) were used, and multi-aligned sequences were subjected to phylogenetic inference through the Neighbor-Joining method and Kimura two-parameter model implemented in the MEGA 4 package (Tamura et al., 2007). One thousand bootstrap replicates were used to assess the phylogenetic robustness of the clusters.

2.2. Tropism prediction

Within all 356 env-sequences, the V3 region was extrapolated and submitted for tropism prediction to Geno2Pheno algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de) and to the Position Specific Scoring Matrices (PSSM) algorithm (http://fortinbras.us/cgi-bin/fssm/fssm.pl) (Vandekerckhove et al., 2011).

Geno2Pheno was preferred because it features an adjustable cutoff. Beyond tropism prediction, it assigns to each V3 sequence a score, called False Positive Rate (FPR), ranging from 0% to 100%, which represents the probability for a sequence to belong to a CCR5-virus. According to FPR values, arbitrarily we selected sequences with FPR $\leq$5% (indicating a strong CXCR4 prediction) and sequences with FPR $\leq$80% (indicating a strong CCR5 prediction) for CXCR4- and CCR5-tropic viruses, respectively. These sequences, together with the related gp41 sequences, were then used for all at the rest of the study.

For Fortinbras PSSM, an easy and rapid bioinformatic method for viral tropism estimation written by the original WebPSSM developer, the subtype-C specific matrix (that recently was provided) was used (Jensen et al., 2003).

2.3. N-linked glycosylation motifs prediction

We assessed the *N*-linked glycosylation motifs (sequons) in all 356 V3 HIV-1 C-subtype sequences using the LANL N-glycosite program (http://www.hiv.lanl.gov) (Table S1). The sequons were governed by the amino acid order asparagine-X-threonine/serine-Y (N-X-S/T-Y) (Marshall, 1972), where X can be any amino acid except proline (P) in the threonine (T) context (Gavel and von Heijne, 1990; Kasturi et al., 1997; Mellquist et al., 1998) and also not tryptophan (W), aspartic acid (D) or glutamine (E) in a serine (S) context (Kasturi et al., 1997). These parameters provided us with a high probability of oligosaccharide addition (Gavel and von Heijne, 1990; Kasturi et al., 1997; Mellquist et al., 1998; Shakin-Eshleman et al., 1996) and the criteria for evaluating each sequon as a possible *N*-linked glycosylation site. Sequences exhibiting ambiguities in a site were included from this calculation.

2.4. V3-loop amino acid physical and chemical properties

Determinations of the net charge and the average of hydrophilicity of the V3-loop for each sequence at pH 7.0 were determined using a desktop-based bioinformatics system Peptide Property Calculator from Innovagen (http://www.innovagen.se). All possible permutations were assessed when amino acid mixtures were found at some codons of V3. To compare the values between C- and B-subtype, the V3 B-subtype sequences (one *per* patient) with available phenotypic determination of HIV-1 tropism (114 CXCR4- and 582 CCR5-tropic viruses, respectively; Los Alamos Database) were used (Table S2). Using the Jameson–Wolf methodology, the

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values of hydrophobicity and surface probability of gp120 V3-loop region were calculated.

2.5. Verification of tropism prediction

To further support the correlation of V3 and gp41 mutations with different co-receptor usage and the correlation among these Env amino acid signatures, all sequences available from Los Alamos Database with pure phenotype and/or co-receptor determinations have been considered (for V3: 423 CCR5- and 48 CXCR4-using viruses, respectively; for gp41: 106 CCR5- and 19 CXCR4-using viruses, respectively) (Table S1).

2.6. Statistical analysis

To analyze gp41 and V3 mutations, we calculated the frequency of all mutations in the 353 gp41 amino acids and 35 V3 amino acids, using the *env* selected sequences. Fisher exact tests were used to determine whether the differences in frequency between the 2 groups of patients were statistically significant (isolates with strong CCR5 and CXCR4 prediction, respectively).

The Benjamini–Hochberg method has been used to identify results that were statistically significant in the presence of multiple-hypothesis testing (Benjamini and Hochberg, 1995). A false discovery rate of 0.05 was used to determine statistical significance. To identify significant patterns of pairwise associations between V3 and gp41 mutations, we calculated the φ coefficient and its statistical significance for each pair of mutations. A positive and statistically significant correlation between mutations at two specific positions ($0 < \varphi < 1$; $P < 0.05$) indicates that the latter mutate in a correlated manner in order to confer an advantage in terms of co-receptor selection and that the co-occurrence of these mutations is not due to chance. Moreover, to analyze the covariation structure of mutations in more detail, we performed average linkage hierarchical agglomerative clustering (Dimonte et al., 2011b; Svicher et al., 2009). Mann–Whitney *U* tests have been used to assess statistically significant differences among all the pairwise mutations associated. Statistical tests have been corrected for multiple-hypothesis testing by using Benjamini–Hochberg method at a false discovery rate of 0.05 (Benjamini and Hochberg, 1995). Using again the nonparametric Mann–Whitney *U* tests, we compared the mean changes in the mean net charge and in the mean hydrophilicity respectively, in 255 CCR5- and 101 CXCR4-using viruses V3 amino acid sequences.

3. Results and discussion

The genotypic algorithms built from B-subtype virus data are questioned whether they correctly predict the tropism of non-B viruses (Garrido et al., 2008), despite recent observations suggesting that they performed well for predicting the tropism of HIV-1 clade C virus (Raymond et al., 2010). Moreover, a study comparing the predictive performance of Geno2Pheno, PSSM and other methods against the first-generation Trofile® assay (validated for HIV-1 tropism determination), concluded that the concordance being as high as 91% (Raymond et al., 2008). Similarly, another work described that HIV-1 tropism determination via plasma viral V3 RNA genotyping coupled with Geno2Pheno interpretation may represent a valid alternative to enhanced sensitivity Trofile® assay (Prosperi et al., 2009).

In HIV-1 B-subtype, gp120 mutations in the V3 and V1/V2 domains are required for co-receptor switching, but in C-subtype there is a much stronger genetic barrier to co-receptor switching that involves the requirement for more extensive changes outside the V3 region (Coetzer et al., 2011). Hence, the contribution of the other gp120 regions in directing co-receptor usage was excluded in this study.

3.1. Physical and chemical V3 properties and prevalence of V3 mutations

356 HIV-1 C-subtype V3-containing env-sequences were collected from the Los Alamos HIV Sequence Database. Among them, 312 contained also gp41 genome region. Geno2Pheno algorithm was used to infer HIV-1 co-receptor usage for all the 356 V3-containing env-sequences. Among them, 255 were CCR5-using (with FPR ≥80%), and 101 CXCR4-using (with FPR ≤5%). The prediction of co-receptor usage was fully confirmed using both Fortinbras PSSM algorithm, and the "net charge" and "11/25" rules (Table 1) (Vandekerckhove et al., 2011). Thus, these 3 interpretation methods for tropism-prediction provide superimposable results.

Previous studies have shown that CXCR4-using viruses were infrequently found in HIV-1 C-subtype infection compared to B-subtype (Cecilia et al., 2000; Ndung'u et al., 2006; Pollakis et al., 2004; Zhang et al., 2006): thus, this can explain the low number of CXCR4-related env sequences retrieved and employed for the entire study.

By evaluating the V3-loop sequences, we have identified 11 amino acids at specific V3 positions whose prevalence was significantly higher in CCR5-using than in CXCR4-using viruses (*P* values from 1.40E−30 to 1.66E−2) (Fig. 1). All of them (D25D and S11S, and T2V, N5N, N6N, N7N, K10ET, P16P, G24T, D29N and Q32E) had a prevalence ≥10% (ranging from 12.2% to 100%) in CCR5-using viruses. We also identified 46 amino acids at specific V3 positions whose prevalence was significantly higher in CXCR4-using than in CCR5-using viruses, suggesting their association with CXCR4-usage (*P* values from 2.34E−38 to 4.49E−2). Among them, 18 (S11R and D25KRQ, and N5G, T8KR, K10R, S11G, 13–14insIL/IG/VG, P16RQ, Q18KR, T19AV, F20ILV, A22TV, T23A, T23HK, G24DE, G24KR, I26V, and Q32KR) had a prevalence ≥10% (ranging from 10.9% to 91.1%) in CXCR4-using viruses, suggesting (and mimicking the trend observed in B-subtype) that within the V3 region, much more mutations are associated with CXCR4 usage (Fig. 1). In fact, in a study enlarged to flanking V3 regions that used samples with experimentally determined phenotype, mutations at 23 positions within V3 were significantly associated with HIV-1 B-subtype X4 viruses, as well as for 13 positions in V2 and 2 in C4, respectively (Thielen et al., 2009).

A detailed analysis of the classical V3 positions 11 and 25 showed that the wild-type amino acid at positions 11 and 25 (S11S and D25D) were significantly associated with CCR5-usage ($P = 6.77E{-}10$; $\varphi = 0.41$), respectively, while S11GR and D25KRQ mutations were significantly associated with CXCR4 usage ($P = 3.36E{-}4$; $\varphi = 0.31$) (Fig. 1). Among the other mutations found at V3 position 25 of HIV-1 C-subtype, the prevalence of E (wild-type for B-subtype) was higher in CCR5-using than CXCR4-using viruses (15.7% and 6.9%, respectively, $P = 0.071$). Conversely, the mutations K, N, P, Q, R, T and V at position 25 were mainly found in CXCR4-using viruses (1.2% in CCR5 versus 56.4% in CXCR4). Only the mutations AGS at position 25 had a similar prevalence in CCR5- and CXCR4-using viruses.

The analysis of position 11 showed the complete absence of the Lysine at this position in HIV-1 C-subtype (while S11K is common in HIV-1 B-subtype CXCR4-using viruses) and the presence of glycine. This glycine is completely absent in all V3 sequences from CCR5-using viruses, while it was observed in 12.8% of CXCR4-using viruses ($P = 4.74E{-}8$) (Fig. 1). When the position 11 was mutated (47.5%) the corresponding virus was always CXCR4-using.

We also analyzed the V3 region encompassing the amino acids 5–8 including the *N*-linked glycosylation site (N_6XT_8). This region has been shown to be critical for CCR5-usage. In particular,

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Table 1
V3 and gp41 chemico-physical properties of CCR5- and CXCR4-tropic viruses.

	CCR5-using viruses, $N = 255$	CXCR4-using viruses, $N = 101$	P-value[b]
Mean average hydrophilicity[a]	0.06	0.12	<0.001
Mean net charge at pH 7.0[a]	2.85	5.32	<0.001
Number of V3 sequences without *N*-linked glycosylation sites	4 (1.6%)	21 (20.8%)	<0.001
	CCR5-using viruses, $N = 255$	CXCR4-using viruses, $N = 57$	P-value[b]
Number of gp41 *N*-linked glycosylation sites (=3)	7 (2.7%)	4 (7.0%)	>0.05
Number of gp41 *N*-linked glycosylation sites (=4)	180 (70.6%)	36 (63.1%)	>0.05
Number of gp41 *N*-linked glycosylation sites (≥5)	68 (26.7%)	17 (29.8%)	>0.05

[a] The mean hydrophilicity and the mean net charge were calculated by using Innovagen's Peptide Property Calculator (http://www.innovagen.se).
[b] P values were calculated by using Mann–Whitney *U* test (for continuous variables) and χ^2 test (for categorical variables).

mutations at position 7 have been shown to abrogate the binding with CCR5 co-receptor (Huang et al., 2007), while the loss of the glycosylated site has been associated with CXCR4-usage in both B- and C-subtypes (Back et al., 1994; Li et al., 2001; Losman et al., 1999; Malenbaum et al., 2000; McCaffrey et al., 2004). In our dataset, N7K mutation was found only in CXCR4-using viruses (prevalence 7.9% in CXCR4-using versus 0% in CCR5-using viruses; $P = 5.48E{-}6$) (Fig. 1). This suggests that N7K can be a CXCR4 related marker also in C-subtype. In addition, the loss of the *N*-linked glycosylation site was observed in 1.6% of CCR5- and 20.8% of CXCR4-using viruses ($P < 0.001$) (Table 1) (Nabatov et al., 2004; Polzer et al., 2002).

Considering the physical and chemical properties of CCR5- versus CXCR4-using viruses (Table 1), the net charge of CCR5-using viruses (mean 2.85, median 3.00, IQR 2.00–3.00) was significantly lower than that observed in CXCR4-using viruses (mean 5.32, median 5.10, IQR 4.00–6.09) ($P < 0.001$, Mann–Whitney *U* tests), as expected and already known for the group M subtypes (Clevestig et al., 2006). This was due to the presence of increased numbers of K and R residues that were scattered throughout the V3 region of CXCR4-using viruses, including positions 11 and 25. Moreover, we observed an increase in the V3 hydrophilicity in CXCR4-using viruses compared to CCR5-using viruses in both C- (median 0.07 for the V3 sequences from CCR5-using viruses, and median 0.13 for V3 sequences from CXCR4-using viruses [$P < 0.001$, Mann–Whitney *U* tests]) and B-subtype (median 0.03 for the V3 sequences from CCR5-using viruses, and median 0.13 for V3 sequences from CXCR4-using viruses [$P < 0.001$, Mann–Whitney *U* tests]). The increased hydrophilicity of V3 sequences from CXCR4-using viruses (for both B- and C-subtypes) can be one of the potential factors affecting the drift from CCR5 to CXCR4 tropism in HIV-1 C-subtype (Choge et al., 2006; Cilliers et al., 2003; McCormack et al., 2002; Ndung'u et al., 2006). This could (at least in part) explain tropism changes observed in HIV-1 C-subtype infected patients who had progression to AIDS during the pre-highly active antiretroviral therapy (HAART) era (Connor et al., 1997). All these results are consistent with previously published papers showing correlations between an increased hydrophilicity and net charge with syncytium inducing ability and CXCR4 usage (Fouchier et al., 1992; Wang et al., 1998). These two parameters can be markers of tropism changes acting on secondary structure of the V3-loop.

Another V3 region critical in modulation HIV-1 subtype co-receptor usage is the GPGQ crown (at positions 15–18) (Coetzer et al., 2006; Lin et al., 2011). This motif forms a proteic β-turn and specific amino acid changes have been shown to be critical determinants of co-receptor usage (Cormier and Dragic, 2002; Hartley et al., 2005; Hu et al., 2000; Pollakis et al., 2004; Shimizu et al.,

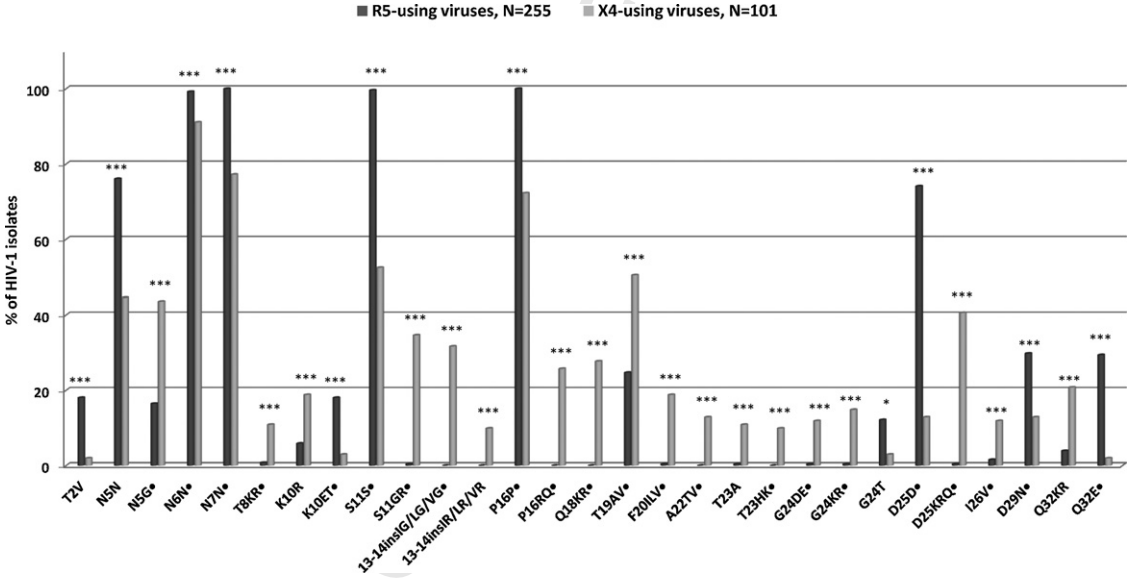

Fig. 1. Frequencies of HIV-1 $gp120_{V3}$ amino acid changes. Frequencies of V3 signatures in HIV-1 CCR5-tropic isolates with FPR ≥80% by Geno2Pheno-algorithm prediction (dark gray) and HIV-1 CXCR4-tropic isolates with FPR ≤5% by Geno2Pheno-algorithm prediction (light gray). The analysis was performed in sequences derived from 356 patients, 255 reported as CCR5-tropic and 101 reported as CXCR4-tropic at genotypic test. The co-receptor usage of the sequences was confirmed using Fortinbras PSSM algorithm and the combination of criteria from the net charge and "11/25" rules. Statistically significant differences were assessed by chi-square tests of independence. *P* values were significant at a false-discovery rate of 0.05 following correction for multiple tests. $^{*}P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. The codons with a black dot (22/29) were significant and confirmed also using a dataset of V3 sequences with phenotypic tropism determination (423 CCR5- and 48 CXCR4-using viruses, respectively).

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1999; Suphaphiphat et al., 2003). In this region, the wild-type amino acid at V3 position 18 is an R in B-subtype and a Q in C-subtype. In B-subtype, the position 18 was found never mutated in 114 (90.4%) of CXCR4-using viruses (one sequence *per* patient), suggesting and sustaining a functional role for the wild type arginine (Resch et al., 2001), possibly predisposing B-subtype viruses to use CXCR4 co-receptor. In C-subtype, Q18R was a frequent mutation in CXCR4-using viruses (24.7%), followed by Q18H (6.9%). Accordingly, the switch to CXCR4 usage may require the acquisition of Q18RH in order to increase the V3 net charge and/or to alter the V3 conformation (Hartley et al., 2005).

In addition, the V3 position 18 (along with position 20) resides in a domain shown to be involved in the binding with two specific glycosphingolipids (GSLs): galactosylceramide and sphingomyelin. This binding has been shown to mediate the attachment of HIV-1 to plasma membrane microdomains (rafts) (Fantini et al., 2002; Rawat et al., 2005; Hammache et al., 1998). Several works suggest that GSLs are involved in the entry of a broad range of HIV-1 isolates into cell lines expressing CD4, CCR5 and/or CXCR4, and that a GSL depletion blocked subsequent viral fusion and infection (Hug et al., 2000; Puri et al., 1998). Hence, mutations at V3 positions 18 and 20 may have an impact on HIV-1 ability to recognize these membrane microdomains.

Additionally, 29.7% of HIV-1 C-subtype CXCR4-using viruses had an insertion of 2 amino acids between V3 positions 13 and 14 (Fig. 1). This signature has been observed in other analysis on C-subtype CXCR4-tropic viruses (Cilliers et al., 2003; Coetzer et al., 2006; Raymond et al., 2010; Singh et al., 2009). Recently, Zhang et al. (2010) have shown that removal of this insertion abolished CXCR4 utilization by dual-tropic viruses, indicating its critical role in modulating the binding to CXCR4 co-receptor. Differently, this insertion was never found in CCR5-using viruses (Fig. 1).

The high variability of the V3-loop is not surprising, since positive selection has been implicated in the maintenance of such diversity (at individual- as well as at population-level) It is likely that the principal driving force in the evolution of HIV-1 gp120-V3 region is the cell receptor usage, the escape from host immune response, or a combination of the two (Leal et al., 2007; Lemey et al., 2007; Ross and Rodrigo, 2002; Shankarappa et al., 1999; Williamson, 2003; Yang et al., 2003).

Additionally, we selected from Los Alamos Database a new set of 471 HIV-1 C-subtype V3-containing sequences (one sequence *per* patient), with a phenotypic characterization of HIV-1 tropism (423 CCR5- and 48 CXCR4-using viruses, respectively) in order to confirm the correlation of V3 mutations with different co-receptor usage. By using this "phenotypic" dataset, despite the low number of CXCR4-sequences available, the majority (22/29; 76%) of V3 signatures identified using genotypic tropism prediction were confirmed (Fig. 1). Of note, in order to assess the reliability of genotypic tropism testing in HIV-1 C-subtype, we applied Geno2Pheno and PSSM algorithms to predict the co-receptor usage of the 471 V3 sequences with phenotypically determined viral tropism. Geno2Pheno and PSSM algorithms were 96.2% and 87.5% concordant with the phenotypic determination of viral tropism and showed a sensitivity of 87.5% and 87.7%, and a specificity of 95.2% and 93.8%, respectively. These results support that genotypic tropism testing can be a valuable tool to predict co-receptor usage in HIV-1 C-subtype and is in line also with other studies (Raymond et al., 2010).

3.2. Prevalence of gp41 amino acid changes

Among the 312 *env* sequences containing the V3 and gp41 encoding regions, both Geno2Pheno and PSSM algorithms predicted 57 CXCR4-using and 255 CCR5-using viruses. By analyzing these C-subtype gp41 sequences, we found 63 out of 353 gp41 positions significantly associated with different co-receptor usage (P value from 7.56E−9 to 4.86E−2) (Fig. 2). In particular, 17 mutations, whose prevalence was significantly higher in CCR5-using than in CXCR4-using viruses, were identified: 16 of them had a prevalence ≥10% (ranging from 12.2% to 34.5%) in CCR5-predicted viruses (A14ILV, A22V, R133M, E136G, N140L, S154K, K156Q, N166Q, N212I, R221E, F263L, A270V, G293KR, S294G, D312N, and I339LV). Conversely, we identified 51 gp41 mutations whose prevalence was significantly higher in CXCR4-using than in CCR5-using viruses, suggesting their association with the CXCR4-usage. Among them, 19 mutations had a prevalence ≥10% (ranging from 10.5% to 71.9%) in CXCR4-using viruses (F8IL, F11ILV, T67A, I84LM, A96N, S107N, Q108L, S125N, N140T, K147Q, D148E, T165S, I187TV, F188LMV, A189G, N195QR, G220E, Q297L, and I332AF) (Fig. 2). The majority of statistically significant gp41 minor variants associated with different co-receptor usage reside within the Heptad Repeat 1 and 2 (HR1 and HR2) (A22, S23, A30, L34, I37, T67, A71, K77, D78, I124 S125, R133, E136, N140, K147, and D148), within the cluster I epitope (transiently exposed during fusion) (I84, L91, A96, S101, S107, and Q108), and within the tryptophan-rich membrane-proximal external region (MPER) (S154, K156, D163, T165, and N166). All these positions are localized in gp41 ectodomains known to be immunodominant and to induce high-titer antibodies in the majority of HIV-1-infected individuals (Cheung et al., 2005; Cleveland et al., 2003; Hollier and Dimmock, 2005; Hrin et al., 2008; Montero et al., 2008; Prabakaran et al., 2007; Xu et al., 1991). The fact that all these polymorphisms are localized in the extracellular domains is consistent with the idea that the gp41 may act as a scaffold in order to maintain the stability of the gp120/gp41 complex, and may influence (directly or indirectly) the viral tropism and plausibly other functions such as the envelope conformation or the interaction with other cell-surface molecules. Intriguingly, the localization of some specific residues within recognized epitopes may support the idea of potential role in the modulation of antibody recognition in this viral glycoprotein (Blish et al., 2008; Ringe and Bhattacharya, 2012).

To further support the correlation of gp41 mutations with different co-receptor usage, we collected from Los Alamos Database 125 HIV-1 C-subtype gp41-containing *env*-sequences with a phenotypic determination of viral tropism (106 CCR5- and 19 CXCR4-using viruses, respectively). Despite the very low number of available phenotypic CXCR4-tropic viruses, the trend of correlation with different co-receptor using was confirmed for the majority of gp41 mutations identified using genotypic tropism testing (44/68; 65%). For 27/68 (40%) mutations the statistical significance was also confirmed ($P < 0.05$). Probably, the low number of CXCR4-using sequences is a limiting factor that objectively may produce a mutational pattern that only partially describes the signatures of the CXCR4-using viruses.

3.3. Association among V3 and gp41 signatures

By analyzing the associations among mutations, statistically significant correlations between V3 and gp41 signatures in HIV-1 clade C were found (for the first time in the literature). Some of these correlations involved the classical V3 positions 11 and 25 (Table 2). Specifically, the $D25D_{V3}$ showed positive correlations with several gp41 substitutions (A22V, R133M, E136G, N140L, and N166Q) (Table 2). All these mutations correlated with CCR5-usage and localized in gp41 ectodomain (Cheung et al., 2005; Cleveland et al., 2003; Hollier and Dimmock, 2005; Hrin et al., 2008; Montero et al., 2008; Prabakaran et al., 2007; Xu et al., 1991). Conversely, $S11S_{V3}$ established a positive correlation with only one gp41 mutation (S154K) localized in HR2 domain (Table 2). Among positive correlations between V3 and gp41 signatures associated with CXCR4-usage, a correlation was observed for $A30T_{gp41}$ with

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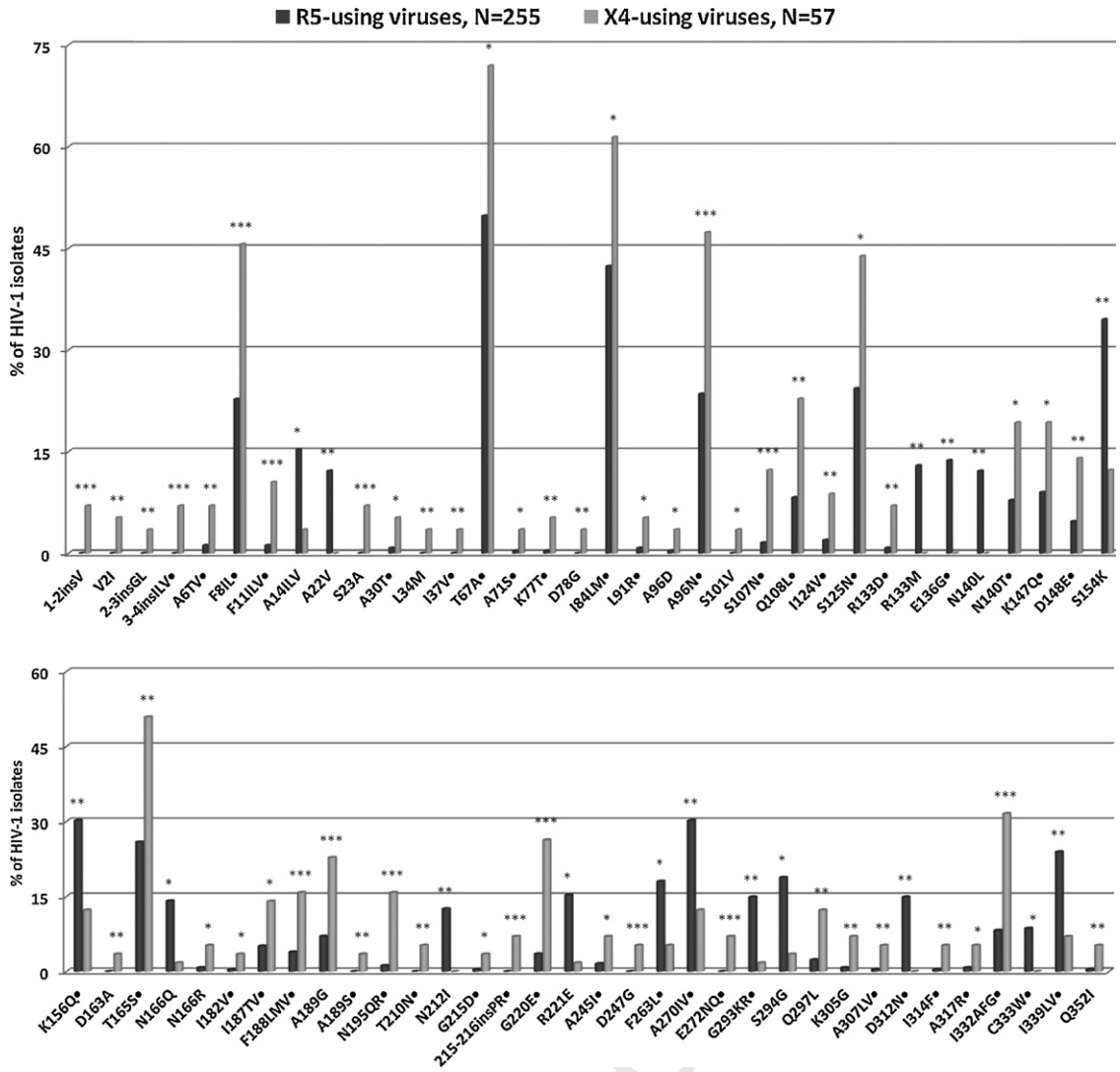

Fig. 2. Frequencies of HIV-1 gp41 amino acid changes. Frequencies of gp41 signatures in HIV-1 CCR5-tropic isolates with FPR ≥80% by Geno2Pheno algorithm prediction (dark gray) and HIV-1 CXCR4-tropic isolates with FPR ≤5% by Geno2Pheno-algorithm prediction (light gray). Among the 312 *env* sequences containing the V3 and gp41 encoding regions, both Geno2Pheno and PSSM algorithms, and the combination of criteria from the net charge and "11/25" rules, predicted 255 CCR5-using and 57 CXCR4-using viruses, respectively. Statistically significant differences were assessed by chi-square tests of independence. *P* values were significant at a false-discovery rate of 0.05 following correction for multiple tests. $^{*}P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. The codons with a black dot were validated also using a dataset of gp41 sequences with phenotypic tropism determination (106 CCR5- and 19 CXCR4-using viruses, respectively): in 44/68 mutations identified using genotypic tropism prediction the trend of correlation with different co-receptor using was confirmed.

F20ILV$_{V3}$ ($P = 0.023$; $\varphi = 0.28$) (Table 2). Of note, F20ILV$_{V3}$ were found in 40% of patients with A30T$_{gp41}$, thus further supporting that these variants are enough correlated with each other (Table 2). Moreover, it has been recently shown that F20L$_{V3}$ could determine a moderate increase of V3-loop antigenic index in HIV-1 B-subtype (Svicher et al., 2011b) that is probably resulting in a more protruding V3-crown exposure. Using the same approach (Jameson–Wolf methodology), the mutation F20L$_{V3}$ in HIV-1 C-subtype correlates with a decreased hydrophobicity and surface probability (−0.11 and −0.05, respectively). In *in vitro* studies the association of A30T$_{gp41}$ signature with CXCR4 usage in HIV-1 B-subtype was recently found (Stawiski et al., 2009; Thielen et al., 2009, 2010, 2011). The position 30 of gp41 is located in a specific region of HR1 involved in a direct interaction with gp120 (Park and Quinnan Jr, 1999). In addition, the presence of A30T$_{gp41}$ was observed in HIV-1 B-subtype CXCR4-using isolates characterized by a high infectivity and/or replication capacity in CXCR4-expressing cells, thus supporting their involvement in the mechanism underlying CXCR4 usage (Stawiski et al., 2009; Leavitt et al., 2003; Rodrigo, 1997). Overall, this supports the role of these two substitutions in the stabilization of non-covalently complex gp120/gp41, and/or in viral receptor attachment and membrane fusion. The F20ILV$_{V3}$ also established a positive correlation with the gp41 polymorphism (A189S) which localized in the transmembrane domain ($P = 0.003$; $\varphi = 0.47$) (Table 2).

To further confirm the correlation among mutations V3 and gp41, we selected from Los Alamos Database a new set of 125 *env* sequences containing HIV-1 C-subtype V3 and gp41 (106 CCR5-using and 19 CXCR4-using viruses, respectively), all with a phenotypic tropic determination of viral tropism available. In this set of sequences the correlation between the classical D25D$_{V3}$ and S11S$_{V3}$ ($P = 7.71E{-}7$; $\varphi = 0.41$), and between D25KRQ$_{V3}$ and S11GR$_{V3}$ ($P = 6.67E{-}12$; $\varphi = 0.78$) was confirmed. Moreover, other associations, involved F20ILV$_{V3}$ and A189S$_{gp41}$ ($P = 8.00E{-}3$; $\varphi = 1$), A30T$_{gp41}$ and D148E$_{gp41}$ ($P = 4.54E{-}3$; $\varphi = 0.48$), N140IL$_{gp41}$ and N166Q$_{gp41}$ ($P = 2.84E{-}2$; $\varphi = 0.29$), N140IL$_{gp41}$ and N136Q$_{gp41}$ ($P = 2.84E{-}2$; $\varphi = 0.29$), S11S$_{V3}$ and D148E$_{gp41}$ ($P = 3.16E{-}4$; $\varphi = -0.44$), and P16RQ$_{V3}$ and D25KRQ$_{V3}$ ($P = 3.54E{-}11$; $\varphi = 0.85$)

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Table 2
Novel V3 and gp41 amino acid changes with each other significantly associates.

Env mutations	Frequency no. (%) of isolates[a]	Frequency % in X4-tropic viruses[b]	Correlated mutations	Frequency no. (%) of isolates[a]	Covariation frequency no. (%) of isolates[c]	φ[d]	P[e]
$S154K_{gp41}$	95 (30.4)	12.3	$T2V_{V3}$	47 (15.1)	40 (42.1)	0.50	9.71E−17
			$S11S_{V3}$	284 (91.0)	91 (95.8)	0.14	3.51E−02
			$G24T_{V3}$	32 (10.2)	31 (32.6)	0.49	1.95E−16
			$A22V_{gp41}$	31 (9.9)	31 (32.6)	0.50	8.78E−18
			$R133M_{gp41}$	33 (10.6)	31 (32.6)	0.48	2.34E−15
			$E136G_{gp41}$	35 (11.2)	33 (34.7)	0.49	1.46E−16
			$N140L_{gp41}$	31 (9.9)	31 (32.6)	0.50	8.78E−18
			$N166Q_{gp41}$	37 (11.9)	34 (35.8)	0.49	3.05E−16
			$T2V_{V3}$	47 (15.1)	39 (19.6)	0.18	6.83E−03
			$S11GR_{V3}$	14 (4.5)	1 (0.5)	−0.29	2.08E−06
$D25D_{V3}$	199 (63.8)	17.5	$S11S_{V3}$	284 (91.0)	197 (99.0)	0.41	9.70E−13
			$13–14insLG/IG_{V3}$	10 (3.2)	1 (0.5)	−0.19	3.49E−03
			$P16RQ_{V3}$	8 (2.6)	0	−0.21	1.57E−03
			$Q18KR_{V3}$	14 (4.5)	1 (0.5)	−0.24	9.89E−05
			$F20ILV_{V3}$	9 (2.9)	1 (0.5)	−0.18	7.89E−03
			$A22TV_{V3}$	13 (4.2)	1 (0.5)	−0.23	2.49E−04
			$G24T_{V3}$	32 (10.2)	31 (15.6)	0.24	1.90E−05
			$Q32KR_{V3}$	27 (8.7)	7 (3.5)	−0.21	1.70E−03
			$A22V_{gp41}$	31 (9.9)	31 (15.6)	0.25	1.31E−06
			$R133M_{gp41}$	33 (10.6)	33 (16.6)	0.26	7.49E−07
			$E136G_{gp41}$	35 (11.2)	33 (16.6)	0.23	5.39E−05
			$N140L_{gp41}$	31 (9.9)	31 (15.6)	0.25	1.31E−06
			$N166Q_{gp41}$	37 (11.9)	34 (17.1)	0.22	2.12E−04
A30T gp41	5 (1.6)	8.8	$F20ILV_{V3}$	9 (2.9)	2 (40.0)	0.28	2.35E−02
			$D148E_{gp41}$	20 (6.4)	3 (60.0)	0.28	7.57E−03
D25KRQ V3	22 (7.1)	36.8	$S11GR_{V3}$	18 (5.8)	3 (13.6)	0.31	2.67E−04
			$S11S_{V3}$	284 (91.0)	11 (50.0)	−0.34	1.00E−05
			$13–14insLG/IG_{V3}$	10 (3.2)	7 (31.8)	0.45	1.78E−06
			$P16RQ_{V3}$	8 (2.6)	7 (31.8)	0.51	1.49E−07
			$Q18KR_{V3}$	14 (4.5)	4 (18.2)	0.18	3.52E−02
			$A22TV_{V3}$	13 (4.2)	4 (18.2)	0.19	2.76E−02
			$Q32KR_{V3}$	27 (8.7)	7 (31.8)	0.20	1.44E−02
			$S107N_{gp41}$	11 (3.5)	4 (18.2)	0.22	1.46E−02
F20ILV V3	9 (2.9)	14.0	$S11GR_{V3}$	18 (5.8)	4 (44.4)	0.29	3.21E−03
			$S11S_{V3}$	284 (91.0)	4 (44.4)	−0.24	4.49E−03
			$Q32KR_{V3}$	27 (8.7)	5 (55.6)	0.23	1.05E−02
			$A189S_{gp41}$	2 (0.6)	2 (22.2)	0.47	3.05E−03
S107N gp41	11 (3.5)	12.3	$S11GR_{V3}$	14 (4.5)	5 (45.5)	0.25	7.27E−03
			$Q18KR_{V3}$	18 (5.8)	3 (27.3)	0.21	3.04E−02
			$Q32KR_{V3}$	27 (8.7)	5 (45.5)	0.26	2.91E−03

[a] Frequency was determined in 312 env-isolates from HIV-1 infected patients having FPR ≤5% and FPR ≥80%, using the Geno2Pheno algorithm. The co-receptor usage of the sequences was confirmed using Fortinbras PSSM algorithm and the combination of criteria from the net charge and "11/25" rules.
[b] Frequency was determined in 57 HIV-1 isolates reported as CXCR4-using by Geno2Pheno algorithm (FPR ≤5%) and by Fortinbras PSSM.
[c] Percentages were calculated in patients with each specific env-mutation listed in the first column of the table.
[d] Positive and negative correlations with $\varphi > 0.10$ and $\varphi < -0.10$, respectively are shown.
[e] P values significant ($P < 0.05$) after correction for multiple hypothesis testing (Benjamini and Hochberg, 1995).

were also identified. Overall, these data strengthen the association between amino acid signatures in V3 and gp41 viral regions, likely implying the contribution of gp41 in the machinery of viral tropism. These results support that the genetic determinants of viral tropism are also inside the gp41, which becomes increasingly important to better understand the viral infection.

3.4. Clusters of correlated amino acid changes

The correlation of the V3 and gp41 signatures was also confirmed by hierarchical clustering analysis. In particular, the topology of the dendrogram suggests the existence of a cluster associated with CCR5-usage composed by five gp41 mutations, A22V, R133M, E136G, N140L, and N166Q (among them four localized close to each other in gp41 structure) that are clustered with the $T2V_{V3}$ and $G24T_{V3}$ (bootstrap = 1). This cluster was linked to $S11S_{V3}$ and $D25D_{V3}$ (bootstrap = 1) (Fig. 3). Conversely, a large cluster was found associated with CXCR4-usage. This involves the V3 signatures S11GR, 13–14insIG/LG, P16RQ, Q18KR, F20ILV, D25KRQ, and Q32KR, along with the gp41 mutated positions A30T, S107N, D148E, and A189S (bootstrap = 0.86) (Fig. 3). Overall, our results suggest that specific additional gp41 signatures could be taken to implement the genotypic prediction.

The algorithms are currently in common use, as already demonstrated by Thielen et al. (2010, 2011), who observed an improvement (albeit marginal) of CXCR4 co-receptor usage prediction in HIV-1 B-subtype. Moreover, the same group has identified 48 amino acid changes in gp41 (including A30T and A189S) correlated with different co-receptor usage (Stawiski et al., 2009). The authors affirmed that the gp41 region could theoretically be used to predict co-receptor usage alone or in combination with the V3 region. In particular, the $A30T_{gp41}$ has been postulated to be a critical determinant for co-receptor usage. In our study, the A30T minor variant (1.6% in 312 one sequence/patient) was 100% associated to CXCR4-tropic viruses (Table 2) which is reinforcing the role of this amino acid substitution in CXCR4-usage. In addition, Huang et al. (2008) showed that mutations in the fusion peptide and cytoplasmic tail of HIV-1 B-subtype gp41 can contribute to CXCR4 usage by a dual-tropic clone, while the addition of G515V mutation in a CCR5-using strains determines the switch from CCR5 to dual tropism. Possibly, the associations among V3 and gp41 amino acid changes may also have an impact on the HIV-1 pathogenesis and it is known

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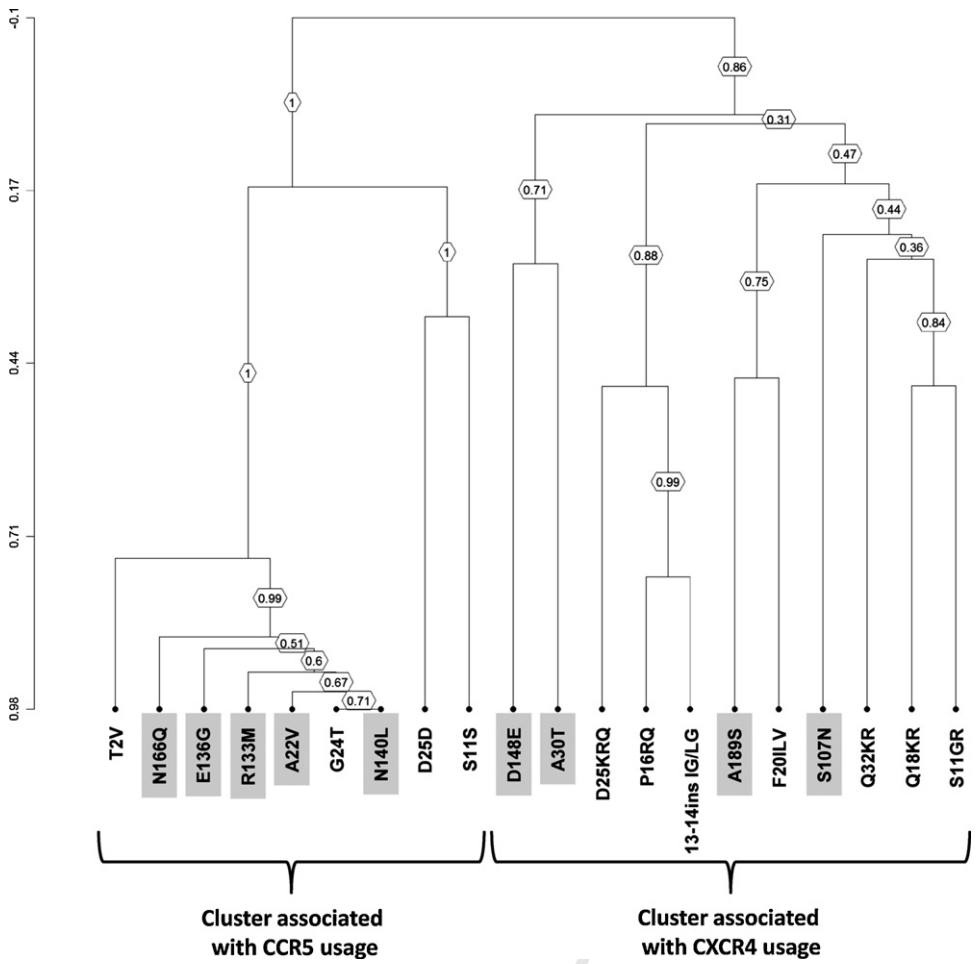

Fig. 3. Clusters of correlated amino acid changes. Dendrogram obtained from average linkage hierarchical agglomerative clustering showing significant clusters involving gp41 (shown in the gray box) and V3 amino acid changes. The length of branches reflects distances between signatures in the original distance matrix. Boostrap values, indicating the significance of clusters, are reported in the boxes. The analysis was performed in sequences derived from 312 patients, 255 reported as CCR5-tropic and 57 reported as CXCR4-tropic at genotypic test.

that CXCR4 phenotype has been associated with progression and increased severity of HIV-1 disease, and several gp41 mutations are associated with viral fitness and cytopathic effects.

3.5. Localization of the gp41 amino acid changes in the available proteic structures

Analyzing the crystal structures of HIV-1 gp41 so far available (Chan et al., 1997; Shi et al., 2010; Tan et al., 1997; Weissenhorn et al., 1997), the positions A22V, A30T, S107N, R133M, E136G, N140L, D148E, S154K and N166Q are all exposed on the surface of the glycoprotein (in HR1, cluster I epitope transiently exposed, HR2 or MPER domains). Differently, the position N195 is located near the classical single membrane spanning domain (172–198 amino acids) that recently has been proposed to shuttle between two different conformations during the viral-cell fusion process (Gangupomu and Abrams, 2010). Based on another work, the same residue is a part of an external loop of gp41 in an alternative membrane-spanning model, suggesting its alternating intra- and extra-membrane localization (Hollier and Dimmock, 2005). However, Steckbeck and his co-workers indicated an apparent distinct C-terminal tail topologies of gp41 for virion- and cell-associated Env species, as reconsideration of a topology which is more complex than the currently envisioned (Steckbeck et al., 2010). Consequently, we could speculate that gp41 A22V, A30T, S107N, R133M, E136G, N140L, D148E, S154K, N166Q and A189S signatures may act together (directly or indirectly) with specific V3 amino acid positions, via allosteric effects on the gp120/gp41 complex. This may allow the best conformational structural plasticity of gp41 and gp120 for their appropriate binding to the cellular receptors and co-receptors. In fact, the X-ray crystal structures of CD4-bound HIV-1 gp120 have revealed that the gp120 "core" consists of a gp41-interactive inner domain, a surface-exposed and heavily glycosylated outer domain and a conformationally flexible bridging sheet (Xiang et al., 2010). Furthermore, studying the quaternary density movement of the gp140 trimer upon CD4 binding in samples of C-subtype indicated an outward domain shift of the three gp120 subunits (diminishing gp120/gp41 interactions), and a "flat open" concave trimer apex related to the gp120 tilting away from threefold axis, juxtaposing the fusion peptide with the host membrane (Moscoso et al., 2011).

Anastassopoulou and his colleagues have shown that viruses resistant to the small molecule CCR5 inhibitor, Vicriviroc, can be caused by conservative changes in the fusion peptide of HIV-1 gp41 (Anastassopoulou et al., 2009) and a downstream residue (Anastassopoulou et al., 2011). Similarly, Pfaff *et al.* found the involvement of gp120 and gp41 mutations in modulating the magnitude of drug resistance to another small CCR5 antagonist, Aplaviroc (Pfaff et al., 2010). Overall, these studies, which focus on changes toward drug resistance without assessing the issue of tropism-switch, are complementary to our results.

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4. Conclusions

Genotypic methods are mainly based on sequencing of the gp120 V3-loop as it is commonly regarded as the main determinant of HIV-1 co-receptor usage. In this study, we found that in HIV-1 subtype-C specific gp41 polymorphisms are significantly associated with different co-receptor usage and with specific V3 mutations, thus providing new information that could be taken into account for improving co-receptor usage prediction. These findings strengthen previous observations that determinants of tropism may reside also outside the V3-loop, even in the gp41 protein. Additional studies are needed to confirm which of these gp41 amino acid changes contribute directly to co-receptor use and to establish the specific and precise utility of this information.

Q3 Uncited reference

Sing et al. (2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2012.06.019.

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