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Specific synonymous mutations tightly correlate with HIV-1 co-receptor usage and differentially affect the secondary structure of HIV-1 Env RNA

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Summary. – Human immunodeficiency virus (HIV) is a pathogen that infects blood cells, using CD4 molecule and two cell receptors CCR5 and CXCR4. The other major actor is gp120/gp41 viral protein complex, which interacts with receptors. Here, the presence of synonymous mutations associated with HIV-1 tropism and the related RNA secondary-structure in HIV-1 infected patients was evaluated. The analysis includes gp120-sequences from 340 HIV-1 subtype-B infected patients, all retrieved from Los Alamos database and with phenotypic HIV tropism determination based on recombinant-virus entry-assay. Frequencies of all nucleotide substitutions were calculated. Mfold and RNAfold algorithms were used to predict RNA secondary-structure of HIV-1. Nineteen codons in V2/C2, V3 and C3 domains were found to be closely related to CCR5 and CXCR4. Additionally, in X4-sequences, gp120 gc<u>a</u>303gc<u>u</u> and gu<u>a</u>222gu<u>c</u> synonymous mutations are positively related to the gp120 S11R and T8A/I codons in V3 protein domain. Furthermore, gu<u>a</u>222gu<u>c</u> increases stability of the viral RNA secondary-structure. Probably, it would not be surprising if a novel escape viral strategy therapy will be related to the gp120 synonymous mutations. Moreover, in relation to the pivotal role played by gp120 in polyvalent vaccine approaches, the impact of gp120 synonymous mutations may play an important role in HIV entry into the cell.

Keywords: gp120; tropism; v3; s11r; evolution; vaccine

Introduction

HIV-1 entry into the host cell is mediated by the Env glycoproteins, gp120 and gp41. The initial binding of gp120 to the domain of T cell surface glycoprotein CD4 activates spatial structural modifications in gp120 that promotes its interaction with one of the chemokine co-receptors, usually CCR5 or CXCR4. HIV-1 strains can be phenotypically classified according to their ability to use the CCR5 and/or CXCR4 co-receptors. This binding is based upon the presence of selected amino acids in gp120 (specifically within the V3-loop, but also in other viral protein domains, as V1/V2, C3, C4, V5), providing greater affinity to CCR5 or CXCR4 and therefore the viral tropism. The receptor binding induces dramatic structural rearrangements in Env (Wilen *et al*., 2008; Ma *et al*., 2018).

Especially, the study of HIV-1 tropism has also contributed to the understanding of viral pathogenesis (Shepherd *et al*., 2008; Parra *et al*., 2011; Scutari *et al*., 2018; Leda *et al*., 2020). Its determinations are mandatory before using CCR5 antagonists in clinical practice, and the maraviroc - the only FDA approved drug of this class - has been listed as part of anti-retroviral combination therapies. Some previous studies support that gp120 V3 domain alone is not sufficient for a comprehensive determination of co-receptor usage (Svicher *et al*., 2011; Monno *et al*., 2011). In a more specific detail, several different Env RNA region sequences encode genetic markers of HIV-1 tropism (Svicher *et al*., 2011; Dimonte *et al*., 2011a, 2012) and have a

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Abbreviations: gp120 = viral glycoprotein; Env = envelope; HIV = human immunodeficiency virus; R5 = viruses using CCR5 receptor; X4 = viruses using CXCR4 receptor

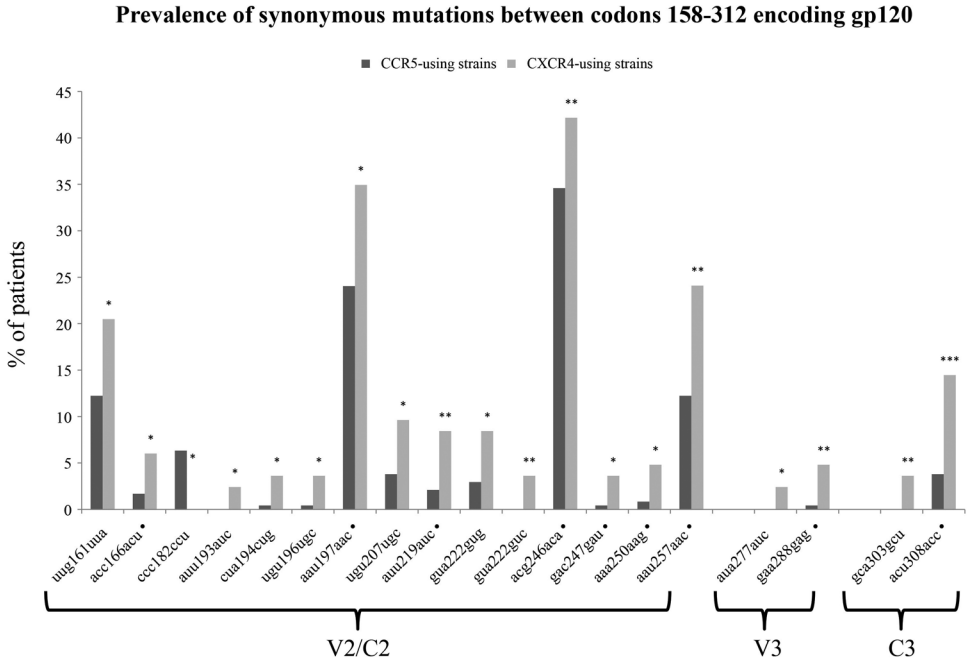

Fig. 1

Frequencies of HIV-1 gp120 synonymous mutations

The frequency of mutations was determined in 237 CCR5-using viruses and 83 CXCR4-using viruses Env sequences encompassing 158–312 encoding region with phenotypically-defined co-receptor usage. A total of 251 synonymous codon mutations were analyzed. The graph reports only synonymous mutations significantly correlated with different co-receptor usage. Statistically significant differences were assessed by Fisher exact tests (*, P <0.05; **, P <0.01; ***, P <0.001). The codons with a black dot were significant also after correction for multiple correction at a false-discovery rate of 0.05.

complex secondary structure (Watts *et al*., 2009; Jayaraman and Kenyon, 2018).

Very few studies have investigated the correlation of synonymous mutations at RNA level with co-receptor usage and their effect on RNA viral secondary structure. The aim of this study is to increase the understanding of HIV entry and, thus, the comprehension of HIV tropism and pathogenesis.

Materials and Methods

Sequences. The analysis includes gp120-sequences from 340 HIV-1 subtype-B infected patients, all retrieved from the Los Alamos database at all stages of infection (one isolate *per* single patient) (http://www.hiv.lanl.gov) and with phenotypic HIV tropism determination based on recombinant-virus entry-assay on U87 $CD4^+CCR5^+$/$CXCR4^+$-expressing cells (237 R5 viruses, 83 X4 viruses). The sequences were encompassing the encoding region between the gp120 amino acid positions 158–312. A total of 251 synonymous codon mutations were analyzed.

Frequency of nucleotide substitutions. To analyze Env codons, the frequency of all nucleotide substitutions was calculated. Fisher exact tests were used to determine the differences in frequency between the 2 groups of patients (infected with R5 and X4 viruses). 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.

Pairwise association. To identify significant patterns of pairwise associations between gp120 synonymous and non-synonymous mutations, the phi coefficient (φ) and its statistical significance for each pair of mutations was calculated. 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, average linkage hierarchical agglomerative clustering was performed (Svicher *et al*., 2009; Dimonte *et al*., 2011b). 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 tested by using Benjamini-Hochberg method at a false discovery rate of 0.05.

Secondary structure prediction. A HIV-1 consensus-B (corresponding with gp120 amino acid region 158-312) was used for RNA secondary structures prediction by using the Mfold program at 37°C, available at the UNAFold server (http://www.unafold.org/). This algorithm, based on thermodynamics of RNA structure motifs, including base-paired intramolecular stems and unpaired loops, provides the identification of putative optimal minimum free energy structures.

Results and Discussion

Innovatively compared to the previous observations, for the first time in literature specific synonymous mutations strongly correlated with phenotypically defined CXCR4- or CCR5-using viruses (Fig. 1). By evaluating the 340 HIV-1 subtype-B gp120 sequences analyzed, 19 codons at specific positions within V2/C2, V3 and C3 domains were identified: only one, the ccc182ccu, had a prevalence significantly higher in CCR5-using [6.3% (15 of 237)] than in CXCR4-using viruses [0% (0 of 83)] ($P = 0.023$) (Fig. 1). This is in agreement with previous observations in which the appearance of X4 HIV-1 populations and the viral adaptation in the $gp120_{C2\text{-}V3\text{-}C3}$ region of the *Env* gene was found related to the disease progression (Carvajal-Rodríguez *et al*., 2008), as well as the prediction between synonymous substitution rates of the *Env* gene and the disease progression in natural HIV-1 infection (Lemey *et al*., 2007).

From the remaining 18 synonymous mutated codons we here, observed significantly higher prevalence in CXCR4- than in CCR5-using viruses, where two are within the $gp120_{V3}$ domain [aua277auc (2.4% vs 0%) ($P = 0.016$) and gaa288gag (4.8% vs 0.4%) ($P = 0.009$ after multiple comparison correction)] and two in $gp120_{C3}$ domain [gca303gcu (3.6% vs 0%) ($P = 0.003$) and acu308acc (14.5% vs 3.8%) ($P = 6.88e^{-4}$ after multiple comparison correction)]. The residual 14 mutations are within the $gp120_{V2/C2}$ area: seven of them, were with statistical significance higher in CXCR4- than in CCR5-using viruses after multiple comparison correction ($P < 0.05$) (acc166acu; aau197aac; auu219auc; acg246aca; gac247gau; aaa250aag; aau257aac) (Fig. 1).

Consistent with its CXCR4 co-receptor usage, the synonymous mutation gua222guc (within the $gp120_{C2}$ domain) positively correlated with two V3 non-synonymous mutations, the agu274agg ($P = 0.019$; $\varphi = 0.313$) corresponding with S11R, and the aca271aua ($P = 3.81e^{-4}$; $\varphi = 0.638$) with T8I mutation. Both these signatures appeared with 0% in R5 viruses, while occurring in 27.7% and 8.4% in X4 viruses, respectively (Table 1). These missense amino acid substitutions are crucial for CXCR4 usage. S11R is the classical and well known V3 mutation of X4 viruses and was related to a decreased binding affinity for CCR5 N-terminus compared to the HIV-1 subtype-B wild-type gp120, while T8I appeared in viruses that easily switch to the CXCR4 co-receptor for the loss of the internal V3 *N*-linked glycosylation site (Svicher *et al*., 2011; Dimonte *et al*., 2011a; Polzer *et al*., 2002).

Similarly, the synonymous mutation gca303gcu correlates with the non-synonymous substitutions agu-274cgu ($P = 0.013$), aca271gca ($P = 8.82e^{-4}$), and aau270aag ($P = 0.005$), which all appeared with 0% in R5 variants, and with 7.2%, 2.4%, and 4.8% occurred in X4-using viruses, respectively (Table 1). Sequence agu274cgu is another substitution encoding the same $S11R_{V3}$, while aca271gca and aau270aag encode $T8A_{V3}$ and $N7K_{V3}$ again crucial for CXCR4 usage. Specifically, the V3 region encompasses the amino acids 5-8 including the *N*-linked glycosylation site (N_6XT_8). In particular, 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 (Dimonte *et al*., 2011a, 2012). Instead, T8A has been observed not to affect the gp120 binding affinity to the CCR5 N-terminus. It is conceivable that this mutation can mediate the interaction with the CXR4 co-receptor *via* the loss of the *N*-linked glycosylation site N_6XT_8 (Svicher *et al*., 2011).

Interestingly, innate immune responses have been appreciated to play an important role in both control and pathogenesis of HIV infection, as well as the HIV genomic RNA and the HIV-derived secondary structured RNA localized to peroxisomes, that has been shown to induce innate immune responses with low induction of type I IFN and higher induction of IFN-stimulated genes (Berg *et al*., 2012). Hence, the impact of above listed nucleotide substitutions in the predicted RNA secondary structure of singly spliced mRNAs – encoding Vif, Vpr, Vpu, and Env products – crucial for the HIV-1 life cycle was examined. By using the available Mfold algorithm, and zooming in the predicted RNA secondary structure of HIV-1 consensus-B model (corresponding with gp120 amino acidic region 158–312), gp120 codons 271 and 274 were localized in front of each other ($\Delta G = -84.5$ Kcal/mol) (Fig. 2a). The presence of gua222guc with agu274agg + aca271aua remarkably increases the stability of local RNA structure ($\Delta G = -88.8$ Kcal/mol) (Fig. 2a) compared to agu274agg + aca271aua (–86.9 Kcal/mol), agu274agg (–87.5 Kcal/mol), and aca271aua (–84.0 Kcal/mol) (figures of secondary structure not shown: similar folding to the "panel b"). These RNA model predictions were confirmed also by RNAfold algorithm (http://rna.tbi.univie.ac.at) (data not shown).

The correlation of signatures in gp120 synonymous and non-synonymous mutations consistent with HIV-1 CXCR4 co-receptor usage was also confirmed by hierar-

Table 1. Novel HIV_{gp120} synonymous signatures significantly associated with specific V2/C2, V3 and C3 domains codon changes

gp120 mutations (codon and aa mutations) found only in X4 viruses		Frequency No. (%) of isolates[a]	Correlated mutations (codon and aa mutations)		Frequency No. (%) of isolates[a]	Covariation frequency No. (%) of isolates[c]	φ[d]	P[e]
auu193auc	I193I	2 (2.41)	cau276aau	$H13N_{V3}$	1 (1.20)	1 (50.00)	0.703	0.024
			gaa288gag	$E25E_{V3}$	4 (4.82)	2 (100)	0.698	0.002
cua194cug	L194L	3 (3.61)	aau270acu	$N7T_{V3}$	1 (1.20)	1 (33.33)	0.570	0.036
			agu274ggc	$S11G_{V3}$	2 (2.41)	2 (66.67)	0.811	0.001
			aua277gua	$I14V_{V3}$	4 (4.82)	2 (66.67)	0.559	0.005
			aga281aaa	$R18K_{V3}$	9 (10.84)	2 (66.67)	0.348	0.030
			uuu283uua	$F20L_{V3}$	5 (6.02)	2 (66.67)	0.494	0.009
			aca285cca	$T22P_{V3}$	1 (1.20)	1 (33.33)	0.570	0.036
			gaa288gac	$E25D_{V3}$	10 (12.05)	3 (100)	0.523	0.001
			caa295gaa	$Q32E_{V3}$	1 (1.20)	1 (33.33)	0.570	0.036
auu219auc	I219I	7 (8.43)	aguU274ggu	$S11G_{V3}$	16 (19.28)	4 (57.14)	0.291	0.023
gua222guc	V222V	3 (0.94)	aau257aac	N257N	20 (24.10)	3 (100)	0.344	0.012
			aca271aua	$T8I_{V3}$	7 (8.43)	3 (100)	0.638	$3.81e^{-4}$
			agu274agg	$S11R_{V3}$	23 (27.71)	3 (100)	0.313	0.019
			gaa288caa	$E25Q_{V3}$	20 (24.10)	3 (100)	0.344	0.012
			gau292aau	$D29N_{V3}$	15 (18.07)	3 (100)	0.412	0.005
aaa250aag	K250K	4 (4.82)	aca265uca	$T2S_{V3}$	1 (1.20)	1 (25.00)	0.491	0.048
			aca286aau	$T23N_{V3}$	1 (1.20)	1 (25.00)	0.491	0.048
			caa295cau	$Q32H_{V3}$	1 (1.20)	1 (25.00)	0.491	0.048
aau257aac	N257N	20 (24.10)	aaa273aga	$K10R_{V3}$	23 (27.71)	2 (10.00)	-0.223	0.048
			agu274agg	$S11R_{V3}$	23 (27.71)	12 (60.00)	0.406	$4.82e^{-4}$
aua277auc	$I14I_{V3}$	2 (2.41)	cau276cgu	$H13R_{V3}$	12 (14.46)	2 (100)	0.382	0.019
			uau284guu	$Y21V_{V3}$	7 (8.43)	2 (100)	0.518	0.006
			aca286aua	$T23I_{V3}$	2 (2.41)	2 (100)	1	$2.94e^{-4}$
			gaa288aaa	$E25K_{V3}$	14 (16.87)	2 (100)	0.349	0.027
			gau292aau	$D29N_{V3}$	15 (18.07)	2 (100)	0.335	0.031
			aua293aug	$I30M_{V3}$	2 (2.41)	2 (100)	1	$2.94e^{-4}$
gaa288gag	$E25E_{V3}$	4 (4.82)	cau276aau	$H13N_{V3}$	1 (1.20)	1 (100)	0.491	0.048
			gga278gca	$G15A_{V3}$	1 (1.20)	1 (100)	0.491	0.048
gca303gcu	A303A	3 (3.61)	aau270aag	$N7K_{V3}$	4 (4.82)	2 (66.67)	0.559	0.005
			aca271gca	$T8A_{V3}$	2 (2.41)	2 (66.67)	0.811	0.001
			agu274cgu	$S11R_{V3}$	6 (7.23)	2 (66.67)	0.444	0.013
			cau276aau	$H13N_{V3}$	1 (1.20)	1 (33.33)	0.570	0.036
			gca282aca	$A19T_{V3}$	5 (6.02)	2 (66.67)	0.494	0.009
			aua290gua	$I27V_{V3}$	9 (10.84)	2 (66.67)	0.348	0.030
acu308acc	T308T	12 (14.46)	aga272aua	$R9I_{V3}$	2 (2.41)	2 (16.67)	0.382	0.019
			aua277aug	$I14M_{V3}$	14 (16.87)	5 (41.67)	0.272	0.026
			cca279aua	$P16I_{V3}$	3 (3.61)	2 (16.67)	0.471	0.002
			ggg280agg	$G17R_{V3}$	3 (3.61)	2 (16.67)	0.471	0.002

[a]Frequency was determined in 340 isolates from HIV-1 infected patients (237 R5 viruses, 83 X4 viruses). [b]Frequency was determined in 83 HIV-1 isolates reported as X4-tropic (phenotypic determination). [c]Percentages were calculated in patients with each specific gp120 mutation. [d]Positive and negative correlations with $\varphi > 0.2$ and $\varphi < -0.2$, respectively, are shown. [e]P values significant ($P < 0.05$) after correction for multiple hypothesis testing.

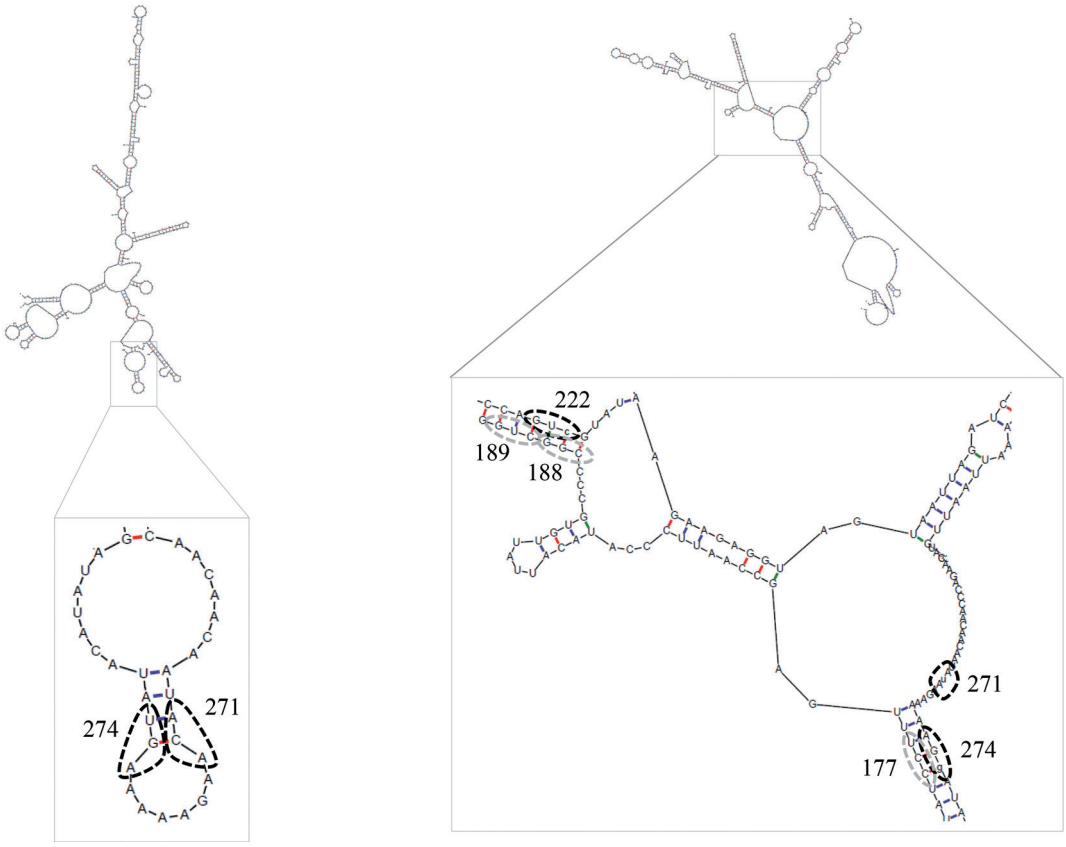

Fig. 2

Putative RNA secondary structure of HIV-1 gp120 region

(a) Predicted secondary structure consensus-B corresponding to gp120 amino acidic region 158–312. The wild-type codon of 271 and 274 positions are shown in black dashed areas. **(b)** Predicted putative secondary structure of the HIV-1 gp120 with nucleotide substitutions at position 222 (gua222guc), at position 271 (aca271aua) and at position 274 (agu274agg). The mutated codons are shown within black dashed areas, while in gray dashed areas the wild-type codons in base-pairing are shown.

chical clustering analysis. The existence of strong cluster involving the S11R$_{V3}$ and T8I$_{V3}$ together with the synonymous mutation gua222guc (bootstrap = 0.89) was observed (Fig. 3). Similarly, two sub-clusters involving the S11R$_{V3}$, T8A$_{V3}$ and N7K$_{V3}$ together with the synonymous mutation gca303gcu (bootstrap = 0.83), and the H13R$_{V3}$ and E25K$_{V3}$ together with the synonymous mutation aua277auc (I14I$_{V3}$) (bootstrap = 0.74) were revealed (Fig. 3) (Table 1).

The reason of the correlation among V3 amino acid changes and gp120 synonymous mutations could be related to different aspects. Firstly, the unique structure of mRNAs and the relative nearness between gp120$_{V3}$ mutations and the synonymous signatures associated, suggests that the viral tropism could determine the folding of the Env RNA and/or *vice versa*. Nevertheless, up to date, few data were reported in literature. Mens *et al.* described that during an *in vivo* virologically failing antiretroviral regimen, synonymous mutation rates of the *Env* gene have been observed with significant negative association between CD4 + T-cell count slopes (Mens *et al.*, 2009). Moreover, the significance of synonymous mutations had an impact probably mostly in the association between the "silent" evolutionary rate of HIV and the rate of disease progression in infected individuals (Lemey *et al.*, 2007). Thus, the highlight of the relation with tropism of HIV strengthens the role of synonymous assortment in the selection of more useful viral strain.

In an analysis of the Pol gene – including the PR and the RT regions – it was shown that the covariation between two amino acid mutations can be affected by selective interactions between amino acids, whereas covariation within amino acid and synonymous mutations pairs or synonymous and synonymous mutations pairs cannot. Specifically, synonymous mutation pairs covariation curves had a low but detectable level of background linkage disequilibrium (that indicates a specific interpretation of co-inheritance from a common ancestor) in HIV (Wang and Lee, 2007).

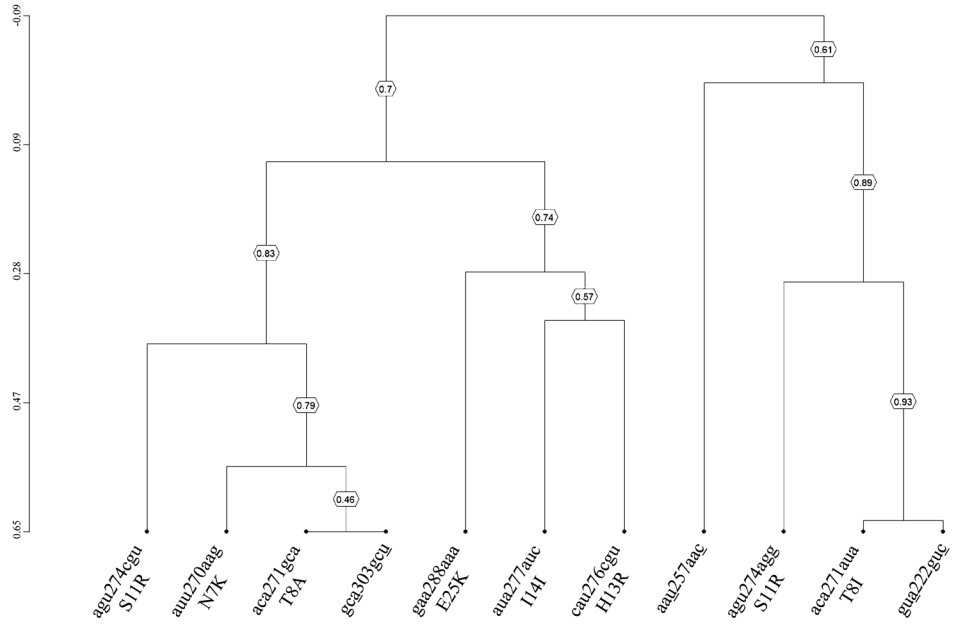

Fig. 3

Clusters of correlated gp120 synonymous and non-synonymous mutations in X4 viral variant
Dendrogram obtained from average linkage hierarchical agglomerative clustering, showing significant clusters involving synonymous and non-synonymous mutations. The length of branches reflects distances between signatures in the original distance matrix. Boostrap values, indicating the significance of clusters, are shown in the boxes.

Another study had shown extensive indication for positive epistatic interactions at both synonymous and non-synonymous nucleotide sites and in both recombinant and clonal RNA viruses, with the preponderance of these contacts covering small sequences. In RNA viruses, epistasis may therefore be central to epidemiologically important phenomena as host switching, immune escape, and the development of antiviral resistance (Shapiro *et al*., 2006). In fact, epistasis occurs when the phenotypic effect of a mutation is conditional to the presence of other mutation in the genome. Despite their structural simplicity and reduced size, the genomes of RNA viruses show complex patterns of epistatic interactions between and within genes. The existence of such complex patterns has profound implications in the evolutionary dynamics of these pathogens.

Thus, these ways of interpretation of synonymous mutations, could be a methodology applied in future insights and may be a possible specific answer to the interplay between the host selection and phylogeny in Env viral region.

Differently, with the ability of mutations to alter the effectiveness of retroviral drugs in treatment, just after specific drug exposure, the resistant viral variants selected with a synonymous mutation – within gp41 encoding region – in the Rev response element (RRE), was a classical and well-known drug pressure consequence in HIV-1 infected cells (Shuck-Lee *et al*., 2011).

During the last years, the introduction of new protease inhibitors (PIs) with broad activities against PI-resistant viral strains, the CCR5 antagonist maraviroc, the integrase inhibitor raltegravir and dolutegravir, and the second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) as etravirine and rilpivirine, has incredibly increased the efficacy of antiretroviral treatment. It would not be surprising if a novel escape viral strategy therapy will be related to the gp120 synonymous mutations: consequently, enhancement of the nuclear export and/or stability of the mRNA in the cell cytosol may determine a foremost viral power.

Conclusions

Here, 19 codons in gp120 V2/C2, V3 and C3 domains were found to be closely related in a statistically significant manner to CCR5 and CXCR4 receptor usage. Additionally, gp120 gu<u>a</u>222gu<u>c</u> synonymous mutation with ag<u>u</u>274ag<u>g</u>

(S11R) + aca271aua (T8I) had remarkable impact on the viral RNA secondary structure, increasing the energetic stability of local RNA structure.

Already other studies have underlined and confirmed the theoretical conjectures about the complexity of biological fitness and the importance of the high dimensionality of the genetic space in which adaptation takes place. The complexity and the high dimensionality need to be taken into account to describe adaptive processes in real biological systems. Nevertheless, the collaboration between biologists, medics and computation analysis is necessary because there are many advantages that bioinformatics can bring to the field of virology (Ibrahim *et al.*, 2018). HIV and the human immune system remain one of the best qualitatively and quantitatively characterized co-evolutionary systems. Hence, also in relation to the pivotal role played by gp120 in polyvalent vaccine approaches, the protein impact's evaluation of gp120 synonymous mutations could be a very important topic.

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