Analysis of longitudinal *nef* sequence variation throughout HIV-2 infection

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Background

The HIV/AIDS infection is considered a public health problem and both types of virus, HIV-1 and HIV-2, are circulating in Portugal [1]. About 4% of the notified HIV/AIDS cases at the National Surveillance System were diagnosed as HIV-2 infections [2]. Although HIV-2 infection may cause a severe immunodeficiency, lower transmission and disease progression rates were described, compared with HIV-1 infection [3,4].

The *nef* gene encodes for a multifunctional protein. Nef sequence motifs were associated with several viral processes: down-regulation of CD4, CD3, CD28, MHC-I and MHC-II molecules, interference with intracellular signalling and apoptotic pathways, and increase of replication and virion infectivity [5,6]. The role of Nef protein during the development of AIDS has been clearly demonstrated in simian immunodeficiency virus model [7]. However, determinants which play a part in the pathogenesis of HIV-1 are relatively poorly understood, and even less is known about this protein and the HIV-2 infection.

Objectives

To analyze the *nef* gene sequence variation throughout HIV-2 infection, including the Nef functional motifs, from infected-individuals in different clinical stages of infection, in order to establish an association between the reconstruction of mutated sequences and/or motifs with the progression of infection.

Materials and Methods

HIV-2 Sample Collection: HIV-2 infected subjects were selected based on available biological samples, collected in several time points, between 1994 and 2009 and corresponding to different clinical stages of the infection.

Amplification and Sequencing of HIV-2 *nef* gene: HIV-2 proviral DNA was extracted from PBMC with QIAmp DNA Mini Kit (QIAGEN) according to manufacturer's instructions. Nested PCR amplification of the *nef* gene was performed using outer and inner primers and the reaction conditions described in Pádua *et al*,. 2003 [8]. The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN) and sequenced in both strands by using the inner primers, the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and an automated capillary sequencer (ABI PRISM 310, Applied Biosystems). Sequence consensus corresponding to each sample was generated using BioEdit 7.0.9.0 [9], Consensus Maker [10] and ClustalX 2.0.10 [11].

Sequence Analysis: MEGA computer package 4.0.2 [12] was used for phylogenetic analysis and distance calculations, aligned sequences were trimmed to remove gaps. The tree was constructed by Neighbor-joining method, supported by performing 1000 bootstrap replicates using SIVmm239 reference sequence as outgroup.

Hypermutation analysis was performed by Hypermut 2.0 [10] . The DS:DN ratio was calculated using SNAP program [10] in accordance with the method of Nei and Gojobori, 1986 [13]and incorporating a statistic developed by Ota and Nei, 1994 [14]. Positively selected sites were identified in *nef* sequences using Bayesian M8 model and likelihood ratio test for statistical analysis [15]. Both analyses were performed at Selecton server [16].

Statistical Evaluation: The SPSS 17.0 was used with a level of significance of 5%. Mann-Whitney test was performed to compare means and medians between unpaired variables and Fisher bilateral exact test to study the association between variables. The Pearson parametric correlation was used for association between two quantitative variables. Confidence intervals of percentages were also calculated.

Results

Characterization of the HIV-2 Population (Table 1)

❑ A total of 17 HIV-2 infected individuals, with a time of infection range from 2 to 34 years, were enrolled in the study: 6 (35%) were male and 11 (65%) were female. A minimum of 2 and a maximum of 9 blood samples were studied for each individual and collected with an interval time from 2 to 88 months.

❑ Only 2 patients (A and B) were at AIDS clinical stage and 15 were asymptomatic when the first sample was collected; while 12 cases remained asymptomatic during time, 3 patients (C, J, M) have progress to AIDS.

❑ The majority of subjects have born outside of Portugal (70.5%) and the HIV-2 infection was acquired in African countries (83%), namely Guinea Bissau, Cape Vert and Angola, and mainly by heterosexual contacts (88%).

❑ All HIV-2 symptomatic cases fulfill antiretroviral therapy. Only 6 asymptomatic individuals were naïve to antiretroviral drugs.

❑ The mean of CD4 counts was 602.3 332.7cells/mm3 for asymptomatic group and 173.8 83.6 cell/mm3 for AIDS cases (Mann-Whitney, p<0.001).

Classification and Analysis of HIV-2 Nef Sequences

❑ A total of 48 sequences, classified as HIV-2 *nef* subtype A, were obtained in the study (Figure 1). All sequences encode a Nef full-length protein without any deletions or truncations. The tree showed long branches for P2 and J3 sequences but only J3 was hypermutated (ρ=0.0001) and removed from further analysis (Figure 1).

❑ A high degree of amino acid conservation in Nef sequences was observed in AIDS patients [63%; C. I. of 95% (62 ;63)], comparatively to asymptomatic individuals [19%; C. I. of 95% (18 ;19)] (Table 2).

Table I – Epidemiological and clinical data of the HIV-2 infected studied population.

Individual	Gender	Age[1]	Birthplace	Country of Infection	Route of Infection	Year of Infection	Estimated time of Infection[2] (years)	Sequence	Time between samples (months)	Clinical Stage	ART	Viral load (copies/ml)	CD4+ Cells Count (cells/mm³)	Genbank accession numbers
A	M	8	Guinea Bissau	Guinea Bissau	Blood Transfusion	1987	12	A1	60	AIDS	YES	NA	199	AJ344370
								A2		AIDS	YES	12590	196	AJ344377
B	M	42	Guinea Bissau	Guinea Bissau	Heterosexual	Before 1988	>8	B1	10	AIDS	YES	NA	46	AJ344371
								B2		AIDS	YES	NA	53	AJ344373
C	F	44	Portugal	Portugal	Heterosexual	1993	16	C1	34	ASN	YES	NA	NA	AJ344383
								C2	47	ASN	YES	NA	NA	AJ344384
								C3	40	ASN	YES	NA	580	AY622984
								C4	3	AIDS	YES	NA	287	GQ890616
								C5	2	AIDS	YES	2700	206	GQ890617
								C6	10	AIDS	YES	5700	196	GQ890618
								C7	6	AIDS	YES	390	336	GQ890619
								C8	4	AIDS	YES	470	145	GQ890620
								C9		AIDS	YES	660	138	GQ890621
D	F	53	Guinea Bissau	Guinea Bissau	Heterosexual	1998	9	D1	3	ASN	YES	<50	480	GQ890622
								D2		ASN	YES	<100	429	GQ890623
E	F	29	Portugal	ND	Heterosexual	Before 2000	>5	E1	36	ASN	YES	NA	742	GQ890624
								E2		ASN	YES	<50	538	GQ890625
F	F	30	Angola	Portugal	Heterosexual	1988	16	F1	33	ASN	NO	<200	610	AJ344385
								F2	55	ASN	NO	NA	1550	AJ344386
								F3		ASN	NO	NA	NA	GQ890626
G	M	53	Cape Vert	Cape Vert	Heterosexual	Before 1992	>7	G1	6	ASN	NO	2259	407	AJ344390
								G2		ASN	NO	NA	400	AJ344411
H	F	21	Guinea Bissau	Guinea Bissau	Heterosexual	Before 2000	>4	H1		ASN	YES	<200	427	AJ344404
								H2	26	ASN	YES	NA	NA	AY622983
								H3	23	ASN	YES	NA	NA	GQ890627
I	F	23	Portugal	Portugal	Heterosexual	1999*	>2	I1	6	ASN	NO	<200	NA	AJ344392
								I2	17	ASN	NO	NA	NA	AJ344397
								I3		ASN	NO	NA	NA	AY622982
J	M	48	Guinea Bissau	Guinea Bissau	Heterosexual	Before1988	>20	J1	88	ASN	NO	<200	288	GQ890628
								J2	4	ASN	NO	310	623	GQ890629
								J3	8	ASN	NO	380	566	GQ890630
								J4	8	ASN	NO	<50	570	GQ890631
								J5		AIDS	YES	<50	NA	GQ890632
K	M	55	Portugal	Africa	Heterosexual	1965	38	K1	48	ASN	NO	NA	1050	DQ640016
								K2		ASN	NO	NA	1207	DQ640017
L	F	23	Cape Vert	NA	Heterosexual	2002*	>2	L1	29	ASN	NO	NA	NA	GQ890633
								L2		ASN	NO	NA	NA	GQ890634
M	M	57	Guinea Bissau	Guinea Bissau	Blood Transfusion	1974	34	M1	4	ASN	YES	<50	271	GQ890635
								M2	11	AIDS	YES	6900	148	GQ890636
								M3		AIDS	YES	6000	136	GQ890637
N	F	NA	African	NA	Heterosexual	2000*	>2	N1	22	ASN	YES	NA	NA	AY622980
								N2		ASN	YES	NA	NA	AY622981
O	F	25	Guinea Bissau	NA	Heterosexual	2003*	>2	O1	33	ASN	YES	NA	NA	GQ890638
								O2		ASN	YES	NA	269	GQ890639
P	F	27	Guinea Bissau	NA	Heterosexual	2001*	>5	P1	64	ASN	NO	NA	NA	GQ890640
								P2		ASN	NO	NA	NA	GQ890641
Q	F	32	NA	NA	Heterosexual	1999*	>2	Q1	18	ASN	YES	NA	NA	GQ890642
								Q2		ASN	YES	NA	NA	GQ890643

F – female; M – male; ASN – asymptomatic; ART – Antiretroviral therapy; NA – not available; 1 – age of patient at the first sample; 2 – When the year of infection was unknown, it was replaced by diagnostic's year * ; 3 – Time of Infection calculated by the difference between the estimated date of infection and the time of the last sample collection.

Evolution of HIV-2 Nef Sequences and Variation of Functional Motifs

❑ The residue M_{20} and the PxxP motif in Nef terminal region described in HIV-1 infection, were absent in HIV-2 sequences analyzed. However, the $MGxxxS_5$ motif is 100% conserved (Figure 3).

❑ While several motifs ($DDDD_{93}$, RR_{137} and DD_{205}) and also residues (G_{127}, I_{141} and L_{142}) remained fairly conserved, others sequences ($YSRF_{39}$, $LRAR_{21}$, $PxxP_{101}$, EE_{185}, LL_{195}) revealed intra and inter-individuals amino acid changes (Figure 3).

❑ The di-acidic motif described as conserved in HIV-1 sequences, was identified as EG_{185} configuration for the majority of HIV-2 studied cases. Nevertheless, the EE_{185} configuration was also observed and statistically associated with the symptomatic stage of infection (Fisher's bilateral test, ρ =0.002) (Figure 3).

❑ Disruption of the minimal core PxxPLR motif was observed in 11 sequences exclusively related to asymptomatic individuals (Fisher's bilateral test, ρ=0.021). The variation towards the complete configuration which is typically observed in HIV-1 sequences was identified in HIV-2 symptomatic patients. Reconstruction to a complete tetra-proline motif was observed in patient "A" after 12 years in AIDS clinical stage, and also observed in patient "J" during the switch of his clinical status to a symptomatic phase of infection. Nevertheless, this complete motif was also observed in only one asymptomatic individual but with 16 years of HIV-2 infection (individual F) (Figure 3).

❑ For patient C, with 9 samples studied during a follow up period of 146 months, it was observed a positive correlation among divergence and the time between samples collection (Pearson's correlation, r=0.4, ρ=0.016) (Figure 4).

❑ Data revealed the existence of a negative selective pressure in HIV-2 *nef* gene throughout infection. However, 5 individuals (C, F, H, I and M) exhibited codons under positive selective pressure in the *nef* sequences.

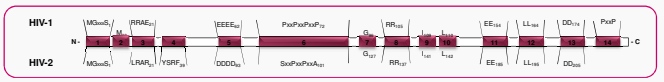

Figure 3 – Comparison of Nef functional motifs present in HIV-1 and studied HIV-2 Nef sequences.
1 – Myristoylation signal, 2 – Metionine residue 3 – α-helix , 4 – Tyr-based sorting signal, 5 – Acidic cluster, 6 – Tetra-proline motif, 7 – G residue, 8 – Pak association motif, 9 – Isoleucine residue, 10 – Leucine residue 11 – Di-acidic motif, 12 – Di-leucine motif, 13 – DD motif, 14 – Minimal proline motif

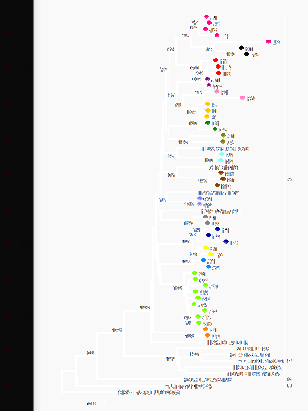

Figure 1 - Phylogenetic tree based on 48 HIV-2 *nef* sequences (761 nucleotides) derived from 17 infected-individuals. It was used reference sequences from subtype A, B G and U retrieved from HIV Los Alamos database. SIVmm239's *nef* sequence was used as outgroup and the nucleotide distances were estimated with Kimura's two-parameter model. Bootstrap values above 70% (of 1000 resamplings) are indicated. Each individual studied has a different color mark.

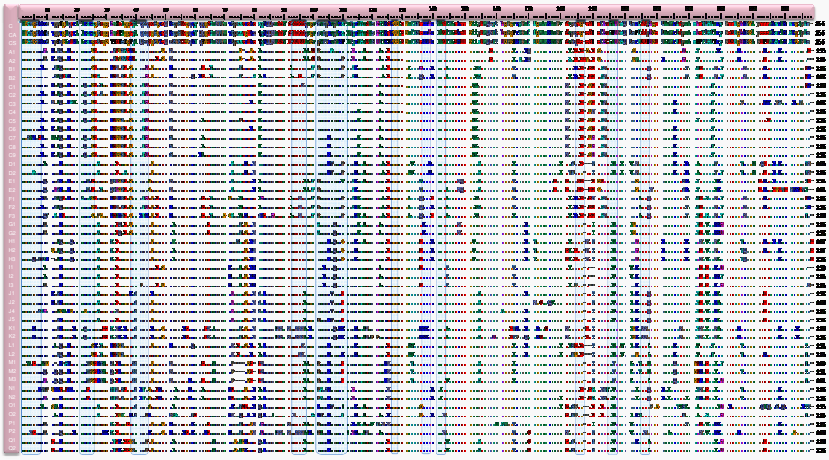

Figure 2 - Alignment of Nef sequences derived from 17 HIV-2 infected individuals. The multiple alignment sequences have been compared to the general consensus (C), the asymptomatic (CA) and the symptomatic consensus (CS). The capital letters corresponds to total conservation, lowercase letters represents partial conservation and ? indicate highly variable amino acid positions. Dots indicate amino acid identity, dashes indicate sequence gaps. Functional motifs are shaded in blue.

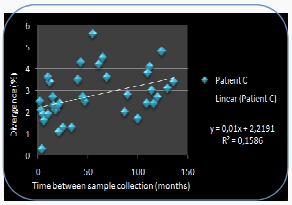

Figure 4 – Positive correlation among divergence and the time between samples collection observed in patient C (Pearson's correlation, r=0.4, ρ=0.016) (Figure 4).

References

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Conclusions

These data suggested that throughout HIV-2 infection, the sequences and particular functional motifs evolve to specific configurations that were usually observed in HIV-1 infection. The existence of motifs within the Nef protein with similar functions potentially allowed some disruption without a complete lost of effects in viral process. A greater proportion of conserved Nef sequences at the AIDS stage was observed in this study. Even though it seems necessary to maintain a functional Nef protein throughout infection, sequences altered in earlier stages of infection and potentially critical for the Nef function in vivo, may contribute at some level to a different pattern in viral pathogenesis and disease progression. To support this findings a continue follow up of the HIV-2 infected patients and further studies on Nef expression will be taken into account.