Full Length Research Paper

Rapid progression to human immunodeficiency virus infection / acquired immunodeficiency syndrome (HIV/AIDS) correlates with variation in viral '*tat'* sequences

Mary Bridget Nanteza[2], David Yirrell[1,5], Benon Biryahwaho[2], Natasha Larke[3], Emily Webb[3], Frances Gotch[4] and Pontiano Kaleebu[1,2]

[1]MRC/UVRI Uganda Research Unit on AIDS, c/o Uganda Virus Research Institute, Plot 51-59 Nakiwogo Road, P. O. Box 49, Entebbe, Uganda.
[2]Uganda Virus Research Institute, Plot 51-59 Nakiwogo Road, P. O. Box 49, Entebbe, Uganda.
[3]MRC Tropical Epidemiology group, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom.
[4]Department of Immunology, Imperial College, Chelsea and Westminster Hospital 369 Fulham Rd, London SW10 9NH, United Kingdom.
[5]Department of Medical Microbiology, Ninewells Hospital, Dundee, DD1 9SY United Kingdom.

Gene sequence diversity plays an important function in determining survival of micro-organisms. Pathogenicity of HIV is correlated to host as well as viral factors. We aimed to identify sequence variations in *tat, nef* and the membrane-proximal *gp41.* These genes regulate important viral functions: *tat* for trans-activation, *nef* for enhancing infectivity and the membrane-proximal gp41 for fusion which could correlate with HIV disease progression. We studied HIV sequences from ART naïve adult Ugandans. Sequence diversity was analysed for 19 rapid progressors and 22 long-term survivors, Rapid progressors were individuals who progressed to a CD4 count of <200 cells/μl in a median time of 3.7 (range 1.3 to 4.9) years. The median time is calculated as being from mid-way between the last HIV sero-negative result and the index HIV sero-positive result, to the time of obtaining the study blood sample. Long-term survivors were individuals who had a CD4 count of >500 cells/μl after a median time of 8.8 (range 7.5 to 9.3) years, measured from the time of the index HIV sero-positive result to the time of obtaining the study blood sample. Amplification of DNA by polymerase chain reaction (PCR) and subsequent sequencing of *tat, nef* and membrane-proximal gp41 was performed starting from viral DNA directly obtained from frozen uncultured peripheral blood mononuclear cells. A 'long' *tat* protein was only observed in rapid progressors (RPs). The 'long' *tat* appears to predict rapid disease progression and could be relevant for designing an HIV-1 prognostic assay.

Key words: HIV-1, progression, *tat*, *nef*, gp41.

INTRODUCTION

Disease progression among people living with human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) seems to be strongly associated with multiplicity of factors such as host HLA alleles and HIV-1 subtype. Genetic variation in individual viral sequence such as an insertion of two amino acids in the C-terminus of exon 2 of *tat* (Tzitzivacos et al., 2009) and deletions in *nef* were in some studies shown to correlate with slow disease progression. In gp41, low levels of anti-bodies to the epitope '*ELDKWA*' have been associated with advanced HIV-1 disease (Srisurapanon et al., 2005).

The *tat* protein trans-activates transcription by attaching to the trans-activating *responsive (TAR)* element of the 5' long terminal repeat (*LTR*). Studies have been performed to delineate the functional mechanisms of *tat* (Kuppuswamy et al., 1989, LeGuern et al., 1993). Peloponese et al.

(1999) showed that HIV strains circulating in Africa were more virulent than strains in Europe and America and was attributed to variations in the *tat* protein. Furthermore, Niyasom et al. (2009) also showed that subtype B *tat* activity was associated with reduced disease progression. Humoral and cytotoxic T-cell responses to *tat* have also shown inverse correlation with slow and non- progressive HIV-1 disease (van Baalen et al., 1997, Zagury et al., 1998, Gupta and Mitra, 2007). However this association was not replicated in a study conducted among Ugandans (Senkaali et al., 2008). The trans-activating and immuno-responsive functions of *tat* have been attributed to the cysteine-rich region (region III) and the basic region (region IV) of *tat* exon 1. *Tat* has also been shown to enhance HIV-1 replication.

With regard to *nef,* several studies have demonstrated that *nef* deleted mutants of HIV-1 and simian immuno-deficiency virus (SIV) were associated with diminished viral virulence and attenuated infection however some other work did not confirm this observation (Hofmann-Lehmann et al., 2003, Chakrabarti et al., 2003). The *nef* gene has been shown to exert its effect through acceleration of HIV-1 activation from latency and enhancement of viral replication. There are indications that *nef* is implicated in the downregulation of CD4 and MHC class I molecules (Jin et al., 2008), and Geriach et al., 2010) thus disabling the humoral and cytotoxic responses.

Apobec3 cytidine deaminases are antiviral proteins that inhibit the replication of HIV-1. The *'YXXL'* motif in the membrane-proximal cytoplasmic gp41 has been identified to mediate the binding to the human Apobec3 (Pery et al., 2009). It has been hypothesised that sequence variations in the *'YXXL'* motif could interfere in the binding of Apobec3 and result in up regulation of viral replication. Such events could subsequently result in rapid disease progression. We report on DNA sequences of *tat, nef and membrane-proximal gp41* and attempts to correlate the variation observed to HIV-1 disease progression.

METHODOLOGY

Study subjects

This was a retrospective cross sectional study conducted among HIV-infected adults from a natural history population-based cohort maintained by the MRC/UVRI Uganda Research Unit on AIDS in Uganda (Morgan et al., 1997). Rapid progressors were individuals who progressed to a CD4 count of <200 cells/µl (median 173) in a

*Corresponding author. E-mail: marybridgetnanteza@yahoo.com. Tel: +256 772 525 704. Fax: +256 0414 320483.

Abbreviations: RP, Rapid progressors, **LTS,** long term survivors.

median time of 3.7 (range 1.3 to 4.9) years. This median time was calculted from mid-way between the last HIV sero-negative result and the index HIV sero-positive result, to the time of obtaining the study blood sample. Long-term- survivors were individuals who had a CD4 count of >500 cells/µl (median 689) in a median time of 8.8 (range 7.5 to 9.3) years. The median time was measured from the time of the index HIV sero-positive result to the time of obtaining the study blood sample. 64% of the long-term survivors were prevalent cases with no prior documen*tati*on of a negative result. The remainder were incident cases where the true length of infection could be documented (data not shown). The blood samples were obtained before anti-retroviral therapy (ART) was widely implemented in Uganda and participants were selected on the basis of having no previous exposure to anti-retroviral drugs. The Uganda Virus Research Institute Scientific and Ethical Committee approved the study.

CD4/CD8 count estimation

CD4/CD8 lymphocytes were quantified from 50 ∝l of fresh ethylene diaminetetraacetic acid (EDTA) blood using flowcytometry on a fluorescence activated cell sorting (FACS) count according to the manufacturer's instructions (Becton Dickinson International, Belgium).

DNA extraction

DNA was extracted from 300 µl frozen uncultured PBMC using the Puregene kit (Gentra Systems Inc., North Carolina, USA) according to the manufacturer's protocol.

DNA amplification

We designed *tat* and *gp41* primers and the *nef* primers were adapted from (Jubier- Maurin et al., 1999) to suit HIV-1 subtype A and D that were dominantly present in Uganda. All the *tat*, *gp41* and *nef* primers were synthesized by Oswel DNA, Southampton, UK. For the first round PCR, 5 ∝l of the DNA extract (~1.0 ∝g DNA) was added to a 15 ∝l reaction containing x1 PCR buffer; 200 ∝M-of dCTP, dATP, dTTP and dGTP [Sigma, USA]; 0.2 pmoles outer primer pairs (*tat-1* and *tat-2*) or (*nef-1* and *nef-2*) or (*env-7* and *env-8*); 1.5 mM $MgCl_2$ for (*tat-1* and *tat-2*) and (*nef-1* and *nef-2*); and 1.4 mM $MgCl_2$ for (*env-7* and *env-8*). Finally, 0.05 U of Taq DNA polymerase was added. DNA samples were cycled: (i) 94°C (1 min), 55°C (1 min) and 72°C (1 min); for three cycles; (ii) 94°C (30 s), 55°C (45 s), 72°C (1min); for 30 cycles; and (iii) 72°C (5 min). Two microlitres of the first round PCR product was transferred to an 18 ∝l reaction mixture containing x1 PCR buffer; 1.4 mM $MgCl_2$; 200 ∝Mof dCTP, dATP, dTTP and dGTP; 0.2 pmoles of the inner primer pairs (*tat-3* and *tat-4*) or (*nef-3* and *nef-4*) or (*env-5* and *env-6*) or (*env-5* and *env-4*); and 0.05U of Taq DNA polymerase. Amplification was performed using the cycling conditions stated above. The details of the primers used are shown in Table 1.

Sequencing

The template for sequencing was generated from a 120 ∝l secondary PCR reaction containing 3 ∝l of the corresponding primary PCR product. The generated product was cleaned using the QIAquick PCR Purification kit [QIAGEN, UK]. A sequencing PCR reaction was carried out in a volume of 10 ∝l which consisted of 1 ∝l of 3.2 pmole/∝l single secondary primer; 4 ∝l dRhodamine deoxy terminator mix [Applied Biosystems, Warrington, UK]; and 5 ∝l of cleaned PCR product. The mixture was subjected to thermal cycling

Table 1. Details of primers used.

Primer name	Primer location on HXB2	Primer sequence
*Tat*1	5711-5730 outer	5'GGATACYTGGGMAGGAGTTG 3'
*Tat*2	6227 -6207 ,,	5'CATTGCCACTGTCTTCTGCTC 3'
*Tat*3	5775-5795 inner	5'CAGAATTGGGTGYCWACATAG 3'
*Tat*4	6137-6116 ,,	5'C*TAT*RGTCCACACAAC*TAT*TGC 3'
EnvVII	7932-7952outer	5'GTCTGGGGCATTAAACAGCTC 3'
EnvVIII	8782-8761 ,,	5'CTTTCTAAGCCCTGTCTGATTC 3'
EnvV	8004-8032 inner	5'GGAATTTGGGGCTGCTCTGG 3'
EnvVI	8707-8686 ,,	5'C*TAT*CTRTCCMCYCAGCTACTG 3'
EnvIV	8537-8516 ,,	5'CAAKYGGTGGTAGCTGAAGAGG 3'
*Nef*1	8513-8533 outer	5'GTGCCTCTTCAGCTACCACCG 3'
*Nef*2	9508-9488 ,,	5'AGCATCTGAGGGGYTAGCCACT 3'
*Nef*3	8696-8717 inner	5'GKGGAYAGATAGGGY*TAT*AGAA 3'
*Nef*4	9467-9448 ,,	5'CRCCTCCCCTGGAAAGTCCCC 3'

The second exon of *tat* from a different reading frame in gp41 was added to the first exon.

at 90°C (30 s), 50°C (15 s), and 60°C (4 min) for 25 cycles. The product was precipitated with ethanol and sequenced on an ABI 373A automated sequencer according to the manufacturer's instructions.

Sequence analysis

Sequences from each region were separately aligned with homologous regions from consensus HIV-1 strains D and A obtained from the Los Alamos database [http://hiv-web.lanl.gov] using version 2.2 of the Genetic Data Environment [GDE] package (Smith et al., 1994). Neighbour joining phylogenetic trees for each region were generated using the PHYLIP set of computer programs (Felsenstein et al., 2003) implemented through the Treecon package (Van de Peer et al., 1994) employing a Kimura distance matrix (Kimura, 1980). The nucleotide sequences were submitted to the Genbank; *tat* exon 1 AF425936 – AF425974, *nef* AF425870 – AF425900 and gp41 AF425901 – AF425935].

S*tat*istical analysis

The frequencies of residues at each amino acid position were compared between RPs and long term survivors (LTSs) in an alignment with the corresponding consensus subtypes D and A using a Chi squared test. The crude association between presence/ absence of the long *tat* and clinical outcome (RP vs. LTS) was evaluated using a Chi square test. To allow for the large number of statistical tests and correlations between positions, p-values adjust-ted for multiple testing were also calculated using an empirical permu*tat*ion procedure with 100,000 iterations (Churchill and Doerge, 1994). To investigate the possibility that predictor residues could be specific for subtype, frequencies of residues at each position were examined separately by subtype; however formal statistical analyses were not undertaken because this was among a small sample size. Nevertheless several crude p values were found and evidence of an association at p<0.05 was provided, many of the adjusted p values were considerably larger, this was as a conse-quence of adjusting for the multiple s*tat*istical tests conducted in a small sample size. All analyses were carried out in Stata 10.

RESULTS

We found major and minor sequence variations affecting the three target HIV-1 genes.

The 'long' *tat* was only found in RPs;

Tat sequence variations were observed in *tat* exons 1 and 2. A major variation was found in exon 2; the 'long' *tat* (115+ amino acids) was only found in RPs (seven out of 18 RPs, 36.8% compared to 0 out of 14LTSs, 0% crude p = 0.002, adjusted p=0.008 chi square test (Figure 1). Furthermore, there was some suggestion that positions 75, 79, and 86 of *tat* exon 2 seem to be associated with rapid disease progression (crude p= 0.032, adjusted p = 0.301) (Figure 2A). Position 82 also seems to be associated with rapid disease progression (crude p = 0.036, adjusted p=0.356). Whilst these adjusted p values do not provide strong evidence (p > 0.05), this was among a small sample size. Sequence variations were observed among "long" *tat* subtype A sequences as 28% sequences had proline (P) at position 75, glutamine (Q) at position 79 and glutamic acid (E) at position 86. Position 79 constitutes part of the cellular adhesion region of *tat* and variations at this position could play a role in HIV pathogenesis. In *tat* exon 1, lysine (K) at position 63 appears to be associated with rapid disease progression (crude p=0.011, adjusted p = 0.073) and was also associated with the "long" *tat* in subtype A sequences. Position 8 in *tat* exon 1 was conserved in LTSs; leucine

(L) was exclusively found in viruses isolated from LTSs whereas both leucine (79%) and isoleucine (I) (21%) were found in RPs (crude p = 0.034 adjusted p = 0.351)
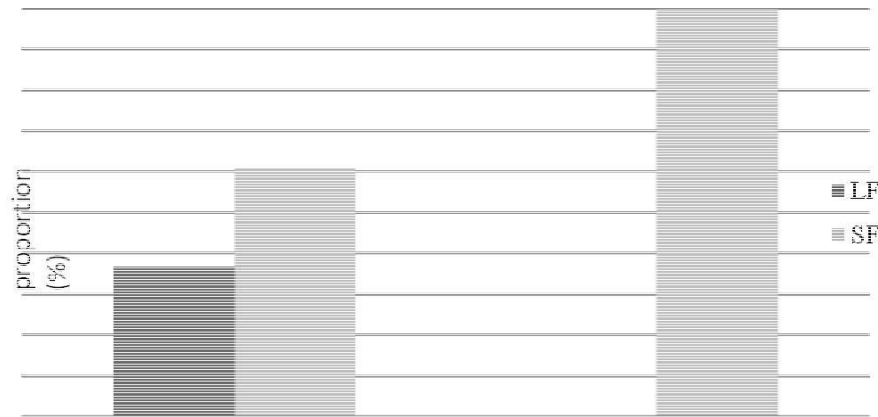
Figure 1. *Tat* sequence length variation and disease progression. LF, Long form; SF, short form; The figure shows the proportion of participants with 'long' *tat* and 'short' *tat* expressed as a percentage of total RPs and LTSs.

(Figures 2A and B). Among the "short" forms of *tat,* serine (S) at position 75, arginine (R) at position 79, and lysine (K) at position 86 were perfectly conserved in all LTSs and the amino acid residue at these positions was the same as the consensus subtype D sequence (Figure 2B). A neighbour joining phylogenetic tree for the *tat* exon 1 sequences is shown in Figure 3. Eight of the study isolates clustered with reference strains subtype A. Six were RPs and two were LTSs. Twenty six (26) clustered with reference strains subtype D. Nine were RPs and 17 were LTSs. Five study isolates did not identify with specific reference strains. These were 19RP, 04RP, 12RP, 14RP and 15LTS.

Nef sequence variation

The *nef* region exhibited a minor sequence variation at the protein kinase C binding site (data not shown). The alanine residue in the protein kinase C binding site of *nef 102'PMTYKAA'108* was more common in RPs (56%) than LTSs (15%) whereas glycine (G) at the same site was more common in LTSs (85%) than RPs (44%) (crude p=0.023, adjusted p=0.381). A similar pattern was shown by Walker et al. (2007).

Gp41 sequence variation;

The epitope '*55NWFSI'---'LW64'* of the membrane-proximal external region *(MPER)* for gp41 neutralizing antibody '4E10' showed a minor sequence variation (data not shown). The serine residue within the epitope was more common in LTSs (76.5%) than RPs (27.8%) whereas aspartic acid (D) was more common in RPs (38.9%) than LTSs (17.6%) (crude p = 0.038, adjusted p = 0.502). The '*96YXXL99*' of the membrane-proximal cytoplasmic gp41 was conserved in the RPs and LTSs.

DISCUSSION

Campbell et al. (2004) performed a functional study on two subtype D sequences from our study population 05RP and 11LTS. A short alpha helix was observed in *tat* 05RP. *Tat* from 05RP was more efficient than *tat* from 11LTS in the trans-activation function. The differences between 05RP and 11LTS were the minor sequence variations at positions 8 and 63 of *tat* exon 1. Position 8 in the acidic region of *tat* exon 1 was quite conserved among the subtype A and D isolates however it exhibited sequence variation with the isoleucine residue among the rapid progressors. The acidic region is a domain for the neutralization antibody epitope of *tat* (Sneham et al., 2012). Sequence variation at position 8 might represent neutralization escape mutants for the subtype A isolates. Position 63 is located in the glutamine rich region. The glutamic rich region is a vital domain and plays a role in *tat* mediated apoptosis of the T-cells. Sequence variation in this domain may affect the rate of disease progression. In this study, the proline residue at position 63 was found among the RPs. This might play a role in enhancing *tat* mediated apoptosis of the T-cells and thus disease progression.

Position 79 of *tat* exon 2 is part of the '79*RGD*80' motif of *tat* exon 2 that is involved in cellular adhesion, uptake of extracellular *tat*, apoptosis and enhancing HIV replication. The 'RGD' motif enhances the adhesion of the extracellular *tat* via the $\alpha_V\beta$ and $\alpha5T\beta$ integrins. Extracellular *tat* stimulates the HIV LTR and results in the up regulation of the transcriptional process. Sequence variations at this site might therefore have various effects. Variations could down regulate or up regulate the transcription process resulting in slow or rapid disease progression. The subtype A isolates exhibited sequence variation at position 79 *tat* exon 2. Position 79 contained the glutamine residue '79*Q/R*' among the RPs. The variation could not

Tat Amino acid sequence for subtype A study isolates in RPs and LTSs

A

B

Tat Amino acid sequence for subtype D study isolates in RPs and LTSs

Figure 2. The amino acid alignments of *tat* sequences from RPs and LTSs of HIV-1 infection The three published consensus sequences A1, A2 and D were obtained from the Los Alamos National Laboratory. The (.), denotes same identity with the respective consensus sequence; (-), deletion; (x), sequence residue was not clear; the letters underlined represent residue positions of potential importance to HIV-1 disease progression; letters in bold within the consensus represent the amino acid sequences of the cell adhesion signal motif of *tat* and the 'long' *tat*; (?), sequence that could not be classified into a subtype and (*), is a stop codon.
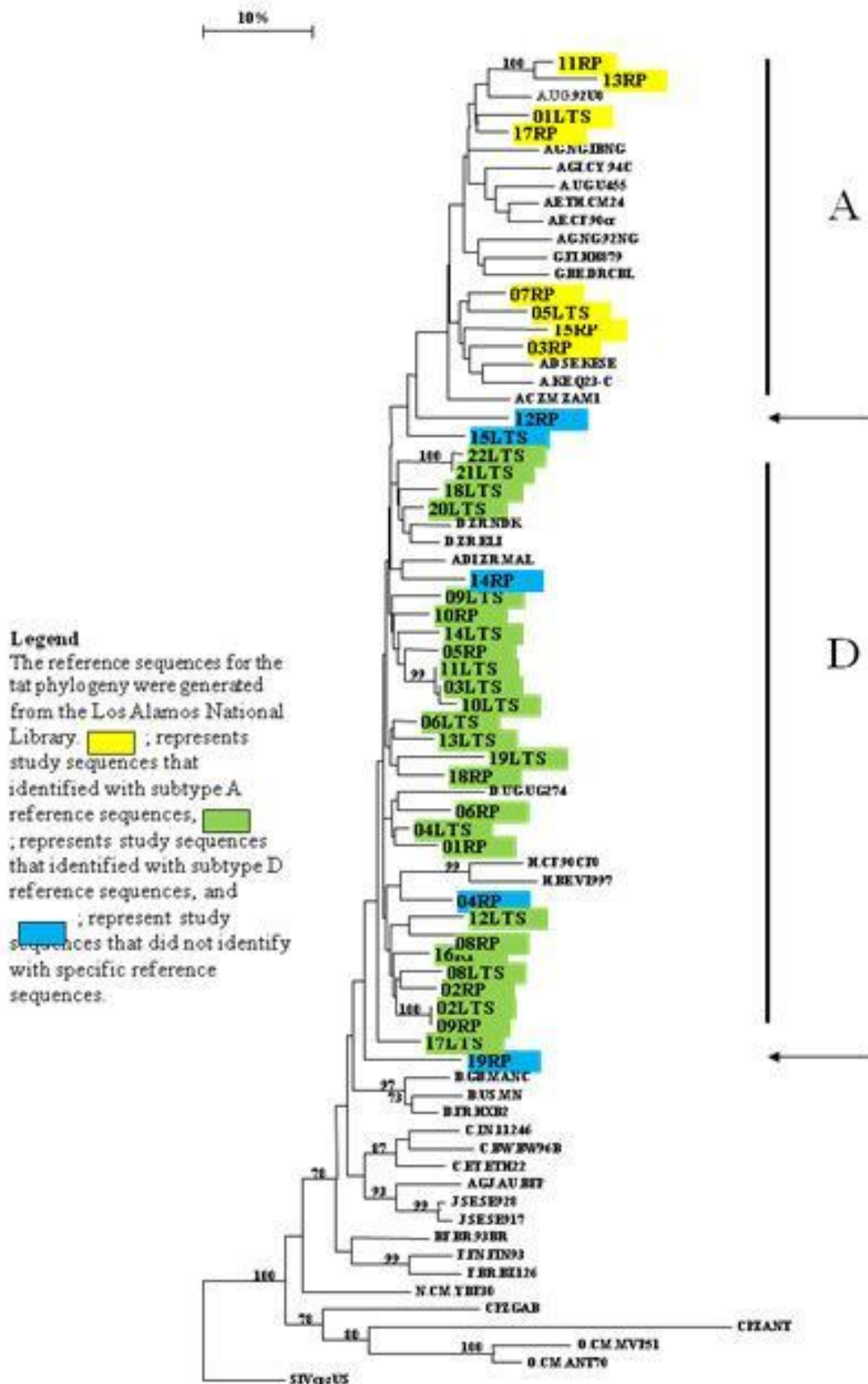
Figure 3. The phylogenetic tree of the first exon of TAT.

be studied in LTS because of the small sample size and short sequences. The 'long' tat was exclusively found in RPs in subtype A sequences. There was some suggestion that those residues with higher frequency in the sequences of RPs were likely to coexist with the 'long' tat in subtype A although this could not be formally evaluated due to the small study numbers. Tat exon 2 has been shown to improve the trans-activation process and induce HIV-1 pathogenic events (Lopez-Huertas et al., 2010). Thus variations within tat exon 2 could improve trans-activation function and contribute to disease progression. Although the cysteine-rich and basic regions have been reported to be critical for tat function data presented here in shows that tat exon 2 could also be critical for tat function. Functional studies of the 'long' and 'short' tat are required to understand better the role of the length of tat in disease progression. Future comparative studies using samples from European and American subjects could also give more insight on tat length and disease progression although the wide spread use of antiretroviral therapy makes this difficult.

CONCLUSION

In this study, the 'long' tat was associated with rapid HIV-1 disease progression. The 'long' tat could be a template for developing a prognostic screening assay as well as a therapeutic target for HIV disease control.

ACKNOWLEDGEMENTS

CONFLICT OF INTERESTS: The authors have declared that no conflict of interest exists.

REFERENCES

Campbell GR, Pasquier E, Watkins J, Bourgarel-Rey V, Peyrot V, Esquieu D, Barbier P, de Mareuil J, Braguer D, Kaleebu P, Yirrell DL, Loret EP (2004). The glutamine-rich region of the HIV-1 Tat protein is involved in T-cell apoptosis. J. Biol. Chem. 279:48197-48204.

Chakrabarti LA, Metzner KJ, Ivanovic T, Cheng H, Louis-Virelizier J, Connor RI Cheng-Mayer C(2003). A truncated form of nef selected during pathogenic reversion of Simian Immunodeficiency Virus SIVmac239Δnef increases viral replication. J. Virol. 77:1245-1256.

Churchill GA, Doerge RW (1994). Empirical threshold values for quantitative trait mapping. Genetics.138:963-971.

Felsenstein J (1993). Phylip Manual Version 3.52c. Berkeley University Herbarium, University of Calfornia Berkeley.

Geriach H, Laumann V, Martens S, Becker CF, Goody RS, Geyer M (2010). HIV-1 Nef membrane association depends on charge, curvature, composition and sequence. Nat. Chem. Biol. 6:46-53.

Gupta S, Mitra D (2007). Human immunodeficiency virus-1 Tat protein: immunological facets of a transcriptional activator. Indian J. Biochem. Biophys. 44:269-275.

Hofmann-Lehmann R, Vlasak J, Williams AL, Chenine AL, McCure HM, Anderson DC, O'Neil S, Ruprecht RM (2003). Live attenuated, nef deleted SIV is pathogenic in most adult macaques after prolonged observation. AIDS 17:157-166.

Jin YJ, Cai CY, Zhang X, Burakoff SJ(2008). Lysine 144, a ubiquitin attachment site in HIV-1 Nef, is required for Nef-mediated CD4 down-regulation.J. Immunol.180:7878-7886.

Jubier-Maurin V, Saragosti S, Perret JL, Mpoudi E, Esu-Williams E, Mulanga C, Liegeois F, Ekwalanga M, Delaporte E, Peeters M(1999). Genetic characterization of the nef gene for human immuno-deficiency virus type 1: Group M strains representing genetic subtypes A, B, C, E, F, G, and H. AIDS Res. Hum. Retroviruses 15:23-32.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol.16:111-120.

Kuppuswamy M, Subramanian T, Srinivasan A, Chinnadurai G (1989). Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. Nucleic Acids Res. 17:3551-3561.

LeGuern M, Shioda T, Levy JA, Cheng-Mayer C (1993). Single amino acid change in Tat determines the different rates of replication of two sequential HIV-1 isolates. Virology 195:441-447.

Lopez-Huertas MR, Callejas S, Abia D, Mateos E, Dopazo A, Alcami J, Coiras M (2010). Modifications in host cell cytoskeleton structure and function mediated by intracellular HIV-1 Tat protein are greatly dependent on the second coding exon. Nucleic Acids Res. 38:3287-3307.

Morgan D, Maude GH, Malamba SS, Okongo MJ, Wagner HU, Mulder DW, Whitworth JA (1997). HIV-1 disease progression and AIDS-defining disorders in rural Uganda. Lancet 350:245-250

Niyasom C, Horthongkham N, Sreephiang A, Kantakamafakul W, Louisirirofchanakuf S, Chuenchire T, Sufhent R (2009). HIV-1 subtype B Tat gene activities and disease progression in HIV-1 CRF01_AE infection. Southeast Asian J. Trop. Med. Public Health. 40:748-758.

Peloponese JM Jr, Collette Y, Gregoire C, Bailly C, Campese D, Meurs EF, Olive D, Loret EP(1999). Full peptide synthesis, purification, and characterization of six Tat variants. Differences observed between HIV-1 isolates from Africa and other continents. J. Biol. Chem. 274:11473-11478.

Pery E, Rajendran KS, Brazier AJ, Gabuzda D (2009). Regulation of APOBEC3 proteins by a novel YXXL motif in human immunodeficiency virus type 1 Vif and simian immunodeficiency virus SIVagm Vif. J. Virol. 83:2374-2381.

Senkaali D, Kebba A, Shafer LA, Campell GR, Loret EP, Van Der Paal L, Grosskurth H, Yirrell D, Kaleebu P (2008). Tat-specific binding IgG and disease progression in HIV type 1-infected Ugandans. AIDS Res. Hum. Retroviruses 24:587-594.

Smith SM, Pentlicky S, Klase Z, Singh M, Neuveut C, Lu CY, Reitz MSJr, Yarchoan R, Marx PA, Jeang KT (2003). An in vivo replication-important function in the second coding exon of Tat is constrained against mutation despite cytotoxic T lymphocyte selection. J. Biol. Chem. 278:44816-44825.

Sneham T, Madhavan PN Nair, Shailendra KS (2012). Rendezvous with Tat: Transactivator of Transcription during Human Immunodeficiency Virus Pathogenesis. American Journal of Infectious Diseases 8(2):79-91.

Srisurapanon S, Louisirirotchanakul S, Sumransurp K, Ratanasrithong M, Chuenchirtra T, Jintakatkom S, Wasi C (2005). Binding antibody to neutralizing epitope gp41 in HIV-1 subtype CRF 01_AE infection related to stage of disease. Southeast Asian J. Trop. Med. Public Health 36:221-227

Tzitzivacos DB, Tiemessen CT, Stevens WS, Papathanasopoulos MA (2009). Viral genetic determinants of nonprogressive HIV type 1 subtype C infection in antiretroviral drug-naive children. AIDS Res. Hum. Retroviruses 25:1141-1148

van Baalen CA, Pontesilli O, Huisman RC, Garetti AM, Klein MR, Miedema F, Gruterset RA, Ostahaus AD(1997). Human immunodefi-ciency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte

frequencies inversely correlate with rapid progression to AIDS. J. Gen. Virol. 78:1913-1918.

Van de Peer Y, De Wachter R(1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci.10:569-570.

Walker PR, Ketunuti M, Choge IA, Meyers T, Gray G, Holmes EC, Morris L (2007). Polymorphism in *nef* associated with different clinical outcomes in HIV type 1 subtype C-infected children. AIDS Res. Hum. Retroviruses 23:204-215.

Zagury JF, Sill A, Blattner W, Lachgar A, Le Buanec H, Richardson M, Rappaport J, Hendel H, Bizzini B, Gringeri A, Carcagno M, Criscuolo M, Burny A, Gallo RC, Zagury D(1998). Antibodies to the HIV-1 *Tat* protein correlated with nonprogression to AIDS: a rationale for the use of *Tat* toxoid as an HIV-1 vaccine. J. Hum. Virol. 1:282-292.