HIV-1 SUBTYPE B TAT GENE ACTIVITIES AND DISEASE PROGRESSION IN HIV-1 CRF01_AE INFECTION

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Abstract. HIV-1 tat gene function and immunogenicity of HIV-1 Tat protein from 3 low (PS01, PS40, PS58) and 3 high (PS19, PS65, LP22) viral load infected, untreated and asymptomatic individuals from Thailand were compared. Levels of Tat-dependent chloramphenicol acetyltransferase (CAT) induced in HL3T1 cells with tat¹ gene from HIV-1 isolates of high viral load group was significantly higher than those from low viral load group. HIV-1 subtype determination using env (C2-V4) gene demonstrated that 2/3 (PS01 and PS40) and 1/3 (PS58) from low viral load group were CRF01_AE and subtype B, while all 3 HIV-1 isolates from high viral load group were CRF01_AE. However, all 3 HIV-1 tat nucleotide sequences from low viral load group, which contained env CRF01_AE sequence, belonged to subtype B whereas all those from high viral load group contained CRF01_AE sequence. HIV Tat recombinant proteins from these groups were tested for immunogenicity in mice. All recombinant Tat proteins (except from PS58) were immunogenic in a dose-dependent manner, but with significantly differences of the immunogenicity levels between high and low viral load groups. These results indicated that HIV-1 subtype B tat gene activities might be associated with reduced disease progression of HIV-1 CRF01_AE infected individuals.

INTRODUCTION

Human immunodeficiency virus (HIV), the etiologic agent of AIDS, is a complex retrovirus, which contains several structural and accessory genes. Among viral regulatory genes, tat gene encodes proteins playing a central role in HIV-1 gene expression and viral replication. Tat is an early regulatory protein encoded by two exons of the HIV genome, the first exon located within the central region of the viral genome and encoding the initial 72 amino acids, whereas the second exon overlaps the envelope gene and encodes the remaining 14 to 32 amino acid residues (Jeang et al, 1999). Tat is implicated in HIV-1 pathogenesis not only by its indispensability for virus replication but also by its capacity to prime quiescent T cells for productive HIV-1 infection and to induce apoptosis in uninfected T cells. It could directly or indirectly affect multiple steps in the virus life cycle to facilitate HIV-1 infection, considering its pleiotropic biological properties, such as regulation of both viral and cellular gene expression and modula-
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Following infection with HIV-1, the rate of clinical disease progression varies between individuals. Factors such as host susceptibility, genetics and immune function, health care and co-infections as well as viral genetic variability, may affect the rate of progression to AIDS. Three clinical outcomes of HIV infection can be distinguished: a typical progression over 8 to 10 years, a rapid progression with 2 to 5 years, and a long-term survival or non-progression in which an individual remains healthy for more than 10 years (Levy, 2007). Several studies of the predictive power of viral load for outcome of HIV infection have been published and consistently show a strong correlation between baseline viral load and disease progression, regardless of the group studied (Mellors et al., 1995; Mofenson et al., 1997; Shearer et al., 1997).

Some data have provided in vivo evidence that increasing HIV-1 replication efficiency may be related to a concomitant increase in HIV-1 diversity, which in turn may be a determining factor in disease progression (Troyer et al., 2005). We hypothesized that efficient replication of HIV-1, which is a prerequisite for progressing infection, depends on the Tat protein functions. So, Tat protein from HIV-1 infected individuals with slow and typical progression of disease were analyzed.

MATERIALS AND METHODS

Samples

Peripheral blood mononuclear cells (PBMCs) from HIV-1 untreated infected patients (3 high and 3 low viral load individuals) were obtained from the National HIV Repository and Bioinformatic Center (NHRBC), Thailand. The PS subjects were women who visited the antenatal clinic at Siriraj Hospital, Bangkok, Thailand from February 1999 through November 2000 and who were confirmed to be HIV-1 seropositive were counseled regarding their HIV-1 infection status, the antiretroviral regimens available for prevention of mother-to-child transmission of HIV-1, and appropriate infant feeding. The LP subject was a patient from Lampang Province in 1998. All the patients in this study were in heterosexual transmission risk group. PBMCs were isolated from the asymptomatic HIV-1 infected patients without antiretroviral therapy and the viral loads were stable for at least 6 months. The patients were classified by using viral load value. The patients with viral load more than 500,000 copies/ml were designated as high viral load group and those with viral load less than 5,000 copies/ml were designated as low viral load group. Details of the subjects are shown in Table 1.

Amplification of HIV-1 tat gene

HIV-1 tat1 and tat2 genes were amplified by nested PCR using 4 primer sets including 1) tat1 outer primers: 5'-GGC AGG AAT GGA GCT GGT AG-3' and 5'-GGA ATT CTT TCC TCC AGG TCT GAA-3'; 2) tat1 inner primers: 5' GGT ACC AGG GAG AAG AGG CAG GAA TGG A 3' and 5' GGA CCA CAC AAC TAT TGC TAT TAT T 3'; 3) tat2 outer primers: 5' GAG TTA GGC AGG GAT ACT CAC 3' and 5' GGA CCA CAC AAC TAT TGC TAT TAT T 3'; and 4) tat2 inner primer: 5' GAG TTA GGC AGG GAT ACT CAC 3' and 5' GGT AGC TGA AGA GGC ACA GG 3'.
round PCR cycles were consisted 35 cycles of 94°C denaturation step for 1 minute, 50°C annealing step for 1 minute, and 72°C extension step for 1 minute and a final extension at 72°C for 10 minutes. For the second round PCR, the cycles consisted of 35 cycles of 94°C denaturation step for 1 minute, 55°C annealing step for 1 minute, and 72°C extension step for 1 minute and a final extension at 72°C for 10 minutes.

Gene splicing overlap (SOE) method
To combine tat exon 1 and tat exon 2 (whole tat), the tat1 and tat2 PCR products were used in SOE reaction by mixing with 20 pmol of primers of tat1 forward primer (5’ GGC AGG AAT GGA GCT GGT AG 3’) and tat1 reverse primer (5’ CTG CTC TGG TAT AGG ATA TTG 3’) and tat2 forward primer (5’ CAA TAT CCT ATA CCA GAG CAG CCC CTA CCC ATC 3’) and tat2 reverse primer (5’ GGT AGC TGA AGA GGC ACA GG 3’). The cycling parameters were 30 cycles of 94°C for 2 minutes, 50°C for 2 minutes, and 72°C for 3 minutes.

Plasmid construction
The specific PCR amplified products containing 3’A-overhangs of tat1 and whole tat were cloned into pDrive, PCR cloning vector containing 3’T overhangs (QIAGEN, Germany), resulting in pDrive/tat1 and pDrive/whole tat respectively. The KpnI/EcoRI tat1 and KpnI/EcoRI whole tat fragment from pDrive/tat1 and pDrive/whole tat, respectively, was then cloned into expression vector, pBAD/HisA (Invitrogen, Germany), resulting in pBAD/tat1 and pBAD/whole tat, respectively. For Tat functional analysis, the KpnI/BamHI tat1 fragment from pDrive/tat1 was then cloned into eukaryotic vector, pcDNA3.1(+) (Invitrogen, Germany), resulting in pcDNA/tat1.

Sequencing and phylogenetic analysis
HIV-1 tat nucleotide sequence was analyzed by cycle sequencing reaction of tat PCR product. Chromatogram of HIV-1 nucleotide sequences were edited using Chromas version 1.45. Lasergene Software version 5.0 (DNASTAR, WI) was used to edit and translate HIV-1 nucleotide sequences. The edited nucleotide sequences were aligned using ClustalX software. Phylogenetic analysis, statistical robustness of the neighbor-joining tree with F84 evolutionary model and bootstrap test, were performed with PHYLIP 3.63 software package for Window to determine subtype of HIV-1.PHYLIP (Phylogenetic inference package, Felsenstein 1989-1996) available from Dr J Felsenstein at http://evolution.genetics.washington.edu/phylip.html (Felsenstein, 1993). Maximum likelihood tree with HKY evolutionary model and genetic distance of HIV-1 was computed by TREE PUZZLE 5.2 software package to study phylogenetic relationship and estimate the most recent common ancestor (MRCA) of HIV-1 circulating in Thailand. TREE PUZZLE is a maximum likelihood analysis for nucleotide, amino acid data, available from http://www.treepuzzle.de/ (Schmidt et al, 2002). Phylogenetic tree constructed from these software was viewed by TREEVIEW software. The accession number of env (C2-V4) nucleotide sequence of PS01, PS40, PS58, PS19, PS65, and LP22 was AY692104, AY692108, AY692128, AF449932, AF449929, and AF449835, respectively; and of tat sequence was FJ517637, FJ517639, FJ517640, FJ517638, FJ517641, and FJ517636, respectively.

HIV-1 recombinant breakpoint analysis
Bootscanning analysis contained in SimPlot version 3.2 was used to identify and to determine the significance of recombination breakpoints. A neighbor-joining algorithm with 500 replicates, using sliding windows of 200 nucleotides with and overlap of 50 nucleotides, was applied to the analysis.
The background sequences included CRF01_AE (CM240), subtype B (MN), and subtype C (96BW05), all of which are reference strains available at the HIV Sequence Database website (http://hiv-web.lanl.gov). Breakpoint were determined by a significant ($p < 0.001$) difference in the ratio of informative sites of each HIV-1 subtype on either side of the breakpoint, as assessed by Maximum chi-square (Martin et al., 2005).

**HIV- Tat recombinant protein expression and purification**

pBAD/\textit{tat}1 and pBAD/\textit{whole tat} were transformed into competent \textit{E. coli} LMG194. Arabinose with a final concentration of 0.2% was used to induce the production of polyhistidine tagged recombinant protein. The recombinant protein purification was performed with nickel affinity resin (ProBond, USA) according to the manufacturer’s instructions. Protein concentration was measured using BCA assay (Bio-Rad, USA).

**Tat-induced transactivation assay**

Transactivation was assayed by inducing Tat-dependent chloramphenicol acetyltransferase (CAT) production in HeLa HL3T1 cells harboring an integrated HIV-1 LTR-CAT gene construct. Plasmid pcDNA/\textit{tat}1 were transfected into HL3T1 cells seeded in a 24-well plate (40,000 cells/well) with Polyfect (QIAGEN, Germany). The transfected cells were washed after 24 hours, and then were lysed within 24 hours to assay CAT activity. CAT production induced by Tat-dependent LTR-transactivation was assayed using a CAT ELISA kit (Roche Diagnostics, France) according to the manufacturer’s instructions.

**Mice immunization with recombinant Tat protein**

Animal use was according to national guidelines and institutional policies. Mice were purchased from the National Laboratory Animal Center, Thailand. Six to 8-week-old female BALB/c mice were immunized intramuscularly (IM) with recombinant whole Tat proteins at various doses (10, 20, 50 and 100 \(\mu\)g) in 200 \(\mu\)l of an emulsion prepared with Alum adjuvant and phosphate-buffered saline (PBS). Control mice were injected with pBAD/HisA vector protein as control. Boosts were given at 2 and 3 weeks after the first immunization with the same concentration of whole Tat mixed with Alum adjuvant. One week after the last boosting, mice were sacrificed and whole blood was collected for immunogenicity analysis. Serum was prepared by centrifugation of clotted blood at 1,800g for 5 minutes, stored at -80°C and utilized for the anti-Tat serology and neutralization assay.

**Detection of Tat-antibody from immunized mice**

The presence of serum anti-Tat specific immunoglobulins was determined by an ELISA assay. Briefly, 96-well microplates were coated overnight at 4°C with 1 \(\mu\)g per well of purified Tat protein in 0.05 M sodium carbonate-bicarbonate buffer pH 9.6. Plates were washed 5 times with PBS containing 0.05% Tween 20. Sera were diluted in blocking solution (PBS/Tween 20 containing 1% BSA, 200 \(\mu\)l per well), and plates were washed 5 times with PBS/Tween 20 prior to the addition of 100 \(\mu\)l per well of horseradish peroxidase-labeled goat anti-mouse IgG(M+G) diluted 1:1000 in blocking solution and incubated for 60 minutes at 37°C. Plates were then washed 5 times with PBS/Tween 20, and 100 \(\mu\)l of ABTS substrate were added for 30 minutes at room temperature. Absorbance was measured at 405 nm with a microplate reader.

**Western blot analysis**

HIV- Tat recombinant proteins (1 \(\mu\)g) were subjected to SDS-PAGE (15%) under sulphydryl reducing conditions. Proteins were then electro-transferred to nitrocellulose
membrane. Immunoblotting was performed on strips. Strips were incubated for 1 hour with 5% skim milk and 1 hour with specific antibody. The secondary antibody was peroxidase-labeled anti-mouse Ig (G+M+A) (Sigma, USA) diluted 1:1000 and bands were revealed with H$_2$O$_2$/0.1%, diaminobenzidine tetrahydrochloride (Sigma, USA) in PBS buffer as substrate.

RESULTS

Recombinant Tat protein production

The 14 kDa Tat1 and 17 kDa whole Tat recombinant proteins from 6 HIV-1 isolates were detected by Western blot hybridization using anti-Xpress antibody and anti-Tat monoclonal antibody (Centralized Facility for AIDS reagents) (Fig 1 shows results for Tat 1). Purified proteins 8-10 mg of almost homogeneity were obtained from 1 liter of Escherichia coli culture. There were no differences in the amounts of protein production among HIV-1 isolates in high and low viral load groups. The purified recombinant proteins were lyophilized and kept at -20ºC. These recombinant Tat proteins then were used for immunogenicity tests in BALB/c mice.

Comparison of Tat function

It has been shown that only the portion of Tat protein encoded by the first exon (tat1), which consists of 4 different short domains, is required for transactivation (Jeang et al, 1999). To determine and compare the transactivation activity tat1 from 6 isolates were cloned into pcDNA resulting in pcDNA/tat1, which were then transfected into HL3T1 cells harboring an integrated HIV-1 LTR-CAT gene construct. Transactivation was assayed by measuring Tat-dependent chloramphenicol acetyltransferase (CAT) production in the cells by CAT ELISA. The average amount of CAT production induced by Tat1 from pcDNA/tat1 of PS01, PS40 and PS58 from low viral load group was 0.178, 0.243 and 0.124 ng, respectively. The average amount of CAT in high viral load group PS19, PS65 and LP22 was 0.381, 0.408 and 0.307 ng, respectively. The basal level of CAT in negative control was only 0.008 ng. To compare the transactivation activity of Tat1 from patients in these two groups, the amount of CAT production induced by Tat1 was converted to relative CAT activity, determined as the ratio of CAT activity between pcDNA/tat1 transfected and pcDNA/Zero transfected cells. The mean (range) fold activation of CAT in low and high viral load group was 27.23 (19.05-35.95) and 53.38.
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There is a significant difference in the amount of CAT production induced by Tat proteins between low and high viral load groups ($p < 0.001$).

Immunogenicity of whole Tat protein

Immunogenicity of whole Tat protein from slow progression group (PS01, PS40 and PS58) and normal progression group (PS19, PS65 and LP22) was studied in mice. Dose responsive curve of each isolate was plotted as concentration of protein against ELISA titer (Fig 3). All whole Tat proteins were immunogenic, inducing immunogenicity in mice in a dose-dependent manner. Tat protein from PS19 was the best immunogen when compared to other isolates while Tat protein from PS58 was the worst immunogen. At doses of 10, 20 and 50 µg, there was a difference in the immunogenicity of HIV-1 Tat protein from low and high viral load groups, while there was not difference between 2 groups at dose of 100 µg (Fig 3).

HIV-1 tat nucleotide sequences

HIV-1 tat1 genes from low viral load group (PS01, PS40 and PS58) and high viral load group (PS19, PS65 and LP22) were sequenced and analyzed. Of the HIV-1 isolates from low viral load group, HIV-1 subtype determination using C2-V4 region of env gene demonstrated that PS01 (AY692104) and PS40 (AY692128) were CRF01_AE while PS58 (AF449932) was subtype B. However, subtype determination of these 3 isolates (PS01: FJ517637; PS40: FJ517639; PS58: FJ517640) using tat1 gene showed them to be subtype B (Table 1). The C2-V4 and tat1 nucleotide sequences of 3 isolates from high viral load group (LP22: AF449835 & FJ517636; PS19: AY692108 & FJ517638; and PS65: AF449929 & FJ517641) were subtype CRF01_AE.

From deduced Tat1 amino acid alignment, the amino acid at position 23 of those from high viral load group was serine (PS19 and PS65) and asparagine (LP22) while that from low viral load group was threonine (PS01, PS40, PS58). The other amino acid positions were different between these two
There are two B cell immunodominant epitopes of Tat protein, one epitope at N-terminus amino acids 1-10 (MEPVDPRLREP) and the other one at amino acids 62-73 (SQTHQVSLSKQP). From deduced amino acid alignment, amino acids at position 1-10 of HIV-1 isolates in high viral load group: N\textsuperscript{7}, W\textsuperscript{32}, L\textsuperscript{36}, K\textsuperscript{40}, K53H\textsuperscript{54}, P\textsuperscript{59}, K63D\textsuperscript{64}, Y67P68I69P70E\textsuperscript{71}); high viral load group: N\textsuperscript{7}, W\textsuperscript{32}, L\textsuperscript{36}, K\textsuperscript{40}, K53H\textsuperscript{54}, P\textsuperscript{59}, K63D\textsuperscript{64}, Y67P68I69P70E\textsuperscript{71}).

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Table 1
Demographic data of samples used in the study and subtype identification of HIV-1 isolates by env (C2-V4) and tat1 gene.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Subtype</th>
<th>C2-V4 env (accession no.)</th>
<th>tat (accession no.)</th>
<th>Sex</th>
<th>Age</th>
<th>Viral load (copies/ml)</th>
<th>CD4+ (cells/mm\textsuperscript{3})</th>
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<tr>
<td>PS01</td>
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<td>720</td>
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<td>AY692128 (AY692128)</td>
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<td>F</td>
<td>28</td>
<td>&lt;400</td>
<td>885</td>
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<tr>
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<td>AF499932 (AF499932)</td>
<td>B</td>
<td>F</td>
<td>22</td>
<td>&lt;400</td>
<td>864</td>
</tr>
<tr>
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<td>M</td>
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DISCUSSION
During acute HIV-1 infection, the virus is replicating extensively in the absence of any detectable adaptive immune response, reaching levels of up to 100 million copies HIV-1 RNA/ml (Mellors et al., 1995). It is during this initial cycle of viral replication that important pathogenic processes are thought to occur. These include the seeding of virus to a range of tissue reservoirs and the destruction of HIV-1-specific CD4+ T lymphocytes. The very high levels of HIV-1 viremia are normally short-lived, indicating that the host is able to generate an immune response that controls viral replication. Over the following weeks, viremia declines by several orders of magnitude before reaching a viral set point, a term used to describe the quasi-
steady viral load during the asymptomatic period of HIV-1 infection, with high set points correlated with rapid disease progression (rapid progressor), high infectiousness, and poorer responses to treatment (Mellors et al, 1995, Morgan et al, 2002). The viral set point is established within 3 to 12 months after primary infection. In this study, the subjects were asymptomatic without antiretroviral therapy. Based on the viral load, the asymptomatic untreated HIV-1 infected subjects in this study were categorized into 2 group: 1) low viral group (viral load less than 5,000 copies/ml) and 2) high viral load group (viral load more than 500,000 copies/ml).

HIV-1 tat gene from patients in low and high viral load groups have been analyzed. It has previously been suggested that defective tat gene may limit the spread of HIV-1 in vivo, and a single individual with a high frequency of about 50% tat-defective proviruses has been described (Sabino et al, 1993). Others found defective tat1 gene with a frequency of 10 to 15% in sequential samples taken over a 4-year period from a progressing HIV-1 infected individual (Meyerhans et al, 1989). No selection for more active tat1 alleles during disease progression was found in this individual (Delasus et al, 1992). The defects in the promoter/TAR region or tat1 gene of HIV-1 from different rates of disease progression were also reported (Kirchhoff et al, 1997). In our study all 6 tat genes from high and low viral load groups had intact reading frames. However, we found that all tat nucleotide sequences from high viral load group clustered together as subtype CRF01_AE strain while those from low viral load group clustered with subtype B. Moreover, subtype determination using env (C2-V4) gene demonstrated that two isolates from low viral load group (PS01 and PS40) and all three isolates of high viral load group were CRF01_AE. This indicated that these HIV-1 isolates (PS01 and PS40) were recombination HIV-1 and Tat subtype B might responsible for low viral load when compared to HIV-1 CRF01_AE infection. These results are consistent with a recent report that Tat proteins derived from HIV-1 clades C and E contained stronger transactivator activity when compared with Tat derived from HIV-1 clade B (Desfosses et al, 2005).

To determine the function of Tat protein from HIV-1 isolates of low and high viral load groups, eukaryotic plasmid vectors that carried tat1 genes of these HIV-1 isolates were transfected into HL3T-1 cell line and CAT production was examined. This transient assay showed differences in the levels of CAT transactivation of tat1 gene isolated from high and low viral load groups. These results suggest that tat1 gene activity may be associated with severity of disease progression in HIV-1 infected individuals.

HIV-1 is one of the most complex retroviruses with regard to its genetic organization. At least 6 gene products are devoted to the regulatory pathway of the virus expression. One of these proteins, Tat, is a powerful transactivator which is highly conserved among various HIV-1 isolates. HIV-1 CRF01_AE Tat protein is 101 amino acids in length and is encoded by two exons. It has been shown that only the portion of Tat encoded by the first exon, which consists of 4 different short domains, is required for transactivation (Dayton et al, 1986; Fisher et al, 1986; Malim et al, 1988; Kuppuswamy et al, 1989; Jones et al, 1994; Jeang et al, 1999). Thus, many studies dealing with Tat have been conducted with this 72 amino acid long portion of the Tat molecule and sometimes with even smaller fragments. Only few studies on the gene expressed or chemically synthesized full length Tat protein have been reported (Dillon et al, 1991; Zauli et al, 1995). Purified recombinant whole Tat proteins from HIV-1 isolates of
low and high viral load groups were used to immunize in BALB/c female mice by IM route with different doses. All whole Tat proteins were immunogenic in a dose-dependent manner. These results are consistent with many studies using Tat protein as an immunogen in animal models, such as mouse, rabbit and monkey (Ensoli and Cafaro, 2002). HIV-1 Tat protein is an attractive vaccine candidate based on: 1) early expression of Tat and its critical role in the virus life cycle, 2) correlation of anti-Tat immune response with non-progression in infected individuals, 3) unique property of Tat to be efficiently taken up by antigen presenting cells and be presented in the context of the MHC class I, 4) conservation among geographically distinct isolates, and 5) safety, immunogenicity, and efficacy in macaques (Dayton et al, 1985; Matsui et al, 1996; Harrich et al, 1997; Ensoli and Cafaro, 2002; Silvera et al, 2002). The differences in the levels of immunogenicity of whole Tat protein from low and high viral load groups might be associated with the B cell immunodominant epitopes (amino acid positions 1-10 and 62-73) of HIV-1 Tat. Further analysis of the epitopes of these proteins and Tat-neutralizing properties of these sera are essential for understanding of these effects. Recent data have shown that only sera with antibody specificity to both N-terminal and basic functional domains are able to inhibit extracellular Tat-dependent transactivation significantly in vitro (Re et al, 1995, 2001). These results demonstrated that immunization with Tat protein elicits the production of Tat-neutralizing antibodies and suggested that Tat vaccination could be used to block in vivo extracellular Tat transactivation of HIV-1 replication (Caputo et al, 2004; Ruckwardt et al, 2004).

In summary, we have shown differences in the functional and immunogenic potentials of recombinant Tat protein from high and low viral load HIV-1 infected subjects. Genetic differences of tat gene might have an effect on these Tat properties. Further studies of factors involved HIV-1 disease progression are needed.

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