Original Article

Effect of HIV-1 Tat on Secretion of TNF-α and IL-1β by U87 Cells in AIDS Patients with or without AIDS Dementia Complex

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Abstract

Objective  To explore the role of HIV-1 tat gene variations in AIDS dementia complex (ADC) pathogenesis.

Methods  HIV-1 tat genes derived from peripheral spleen and central basal ganglia of an AIDS patient with ADC and an AIDS patient without ADC were cloned for sequence analysis. HIV-1 tat gene sequence alignment was performed by using CLUSTAL W and the phylogenetic analysis was conducted by using Neighbor-joining with MEGA4 software. All tat genes were used to construct recombinant retroviral expressing vector MSCV-IRES-GFP/tat. The MSCV-IRES-GFP/tat was cotransfected into 293T cells with pCMV-VSV-G and pUMVC vectors to assemble the recombinant retrovirus. After infection of gliomas U87 cells with equal amount of the recombinant retrovirus, TNF-α, and IL-1β concentrations in the supernatant of U87 cells were determined with ELISA.

Results  HIV-1 tat genes derived from peripheral spleen and central basal ganglia of the AIDS patient with ADC and the other one without ADC exhibited genetic variations. Tat variations and amino acid mutation sites existed mainly at Tat protein core functional area (38-47aa). All Tat proteins could induce U87 cells to produce TNF-α and IL-1β, but the level of IL-1β production was different among Tat proteins derived from the ADC patient’s spleen, basal ganglia, and the non-ADC patient’s spleen. The level of Tat proteins derived from the ADC patient’s spleen, basal ganglia, and the non-ADC patient’s spleen were obviously higher than that from the non-ADC patient’s basal ganglia.

Conclusion  Tat protein core functional area (38-47aa) may serve as the key area of enhancing the secretion of IL-1β. This may be related with the neurotoxicity of HIV-1 Tat.

Key words: HIV-1 tat gene; AIDS dementia complex; Cytokines; TNF-α; IL-1β; Neurotoxicity

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INTRODUCTION

HIV-1 can not only damage the peripheral immune system, but also cross blood-brain barrier into the brain to cause neurological damage, nerve system degeneration, and dysfunction\cite{1}. AIDS dementia complex (ADC) is one of the most common and serious complications of the nervous system caused by HIV-1 infection, which occurs in the late course of the disease in about 20% of the AIDS patients\cite{2}. The major clinical symptoms of ADC are cognitive impairment, emotional disturbance and motor disorder\cite{3}. In recent years highly active anti-retroviral therapy (HAART) has reduced ADC case fatality and prolonged the patient’s survival dramatically, but it fails to effectively prevent the occurring of ADC. Because of the improvement of life quality and survival of AIDS patients, ADC becomes a major cause of high AIDS related morbidity rate\cite{4,5,6}.

The pathogenesis of ADC is not completely understood. At present, neurons damage is viewed as an indirect mechanism. HIV-1 in the brain can only infect macrophages and glial cells, and the viral protein and nerve toxin released by the HIV-1 infected cells play an important role in the process of neurons damage\cite{7,8,9}.

HIV-1 Tat not only activates the gene transcription to enhance the virus replication, but also induces the activated macrophages and glial cells to secrete cytokines\cite{8}, such as TNF-α and IL-1β, which are two most important cytokines in the course of ADC. TNF-α and IL-1β can improve the permeability of blood brain barrier for HIV-1 infected mononuclear-macrophages to enter the brain\cite{10,11,12}, and meanwhile they can enhance the virus replication\cite{12,13}, and stimulate inflammatory reaction working together with chemotatic factors\cite{14}. In addition, TNF-α can boost the injury sensitivity of glial cells, and cause neurons apoptosis through the glutamic acid, calcium disorders and oxidative stress mechanism\cite{5}. IL-1β can affect cell migration, stimulate nerve cell proliferation, and mediate the virus neurotoxic action and neurodegeneration\cite{8}.

Studies have shown that the ADC pathological changes in the brain are not always related with the level of HIV-1 viral load, but may be also related with gene mutations and biological activity change of HIV-1 itself\cite{15,16}. Khanna and others have proved that different HIV-1 variants have different capacities of inducing peripheral blood mononuclear cells to secrete TNF-α\cite{17}. HIV-1 Tat protein can enhance the secretion of TNF-α and IL-1β, but whether the genetic diversity of tat can influence the secretion of TNF-α and IL-1β by neuroglial cells is unknown. In order to determine whether tat variations affect the secretion of TNF-α and IL-1β in basal ganglia tissue of the two patients, we analyzed 4 different tat genes from peripheral spleen and central basal ganglia of one AIDS patient with ADC and the other AIDS patient without ADC.

MATERIALS AND METHODS

Genomic DNA Extraction from the Organs of AIDS Patients with ADC or without ADC

The AIDS patient with ADC was aged 57 years, whose pathological examination showed an extensive atherosclerosis in the brain and a complication with cardiovascular disease, and who had received HAART before his death, with CD4<50/μL.

The AIDS patient without ADC was aged 31 years, whose pathological examination showed obvious multinucleated giant cells and vacuolization in the cerebral white matter, basal ganglia and cortex, with non-Hodgkin’s lymphoma, and who had received HAART for six years before his death, CD4<50/μL.

Genomic DNA was extracted from spleen and basal ganglia tissue of the two patients by using QIaamp DNA Mini kit (QIAGEN company, Valencia, CA) and quantified by absorbance using NanoDrop ND 1000 uv-vis spectrophotometer (NanoDrop Technologies, Wilmington, Delaware).

Amplification of HIV-1 tat Genes from Different Tissues of Two AIDS Patients

Spleen and basal ganglia of the two patients were used as template to amplify tat. The tat exon 1 gene (230 bp) was amplified with primary primers NP7 (CAGTAGAAGACATCAGCTGAAC) and ENV1 (TTCCACACAGGTACCCCA) and nested primer pair TATf (GCTCGAGATGGAGCCAGTAGATCTTAG) and TATr (GGATTCTGGATTTGATAWAGGCCCTTG). The nested primers was designed with Xho I and EcoR I enzyme sites, respectively. The amplicons were named as Sa (S for spleen and a for ADC), Ba (B for basal ganglia), Sn (n for non-ADC) and Bn. PCR agents were from Promega (USA) and oligonucleotide primers were synthesized by Shenggong Biological Company (Sangon, China).
**Cloning and Expressing HIV-1 tat Gene**

The PCR amplified tat gene was cloned into pMD19-T vector (TaKaRa, China), transformed into E. coli DH5-α competent cells (TransGen, China). Positive clones were sequenced by Shenggong Biological Company (Sangon, China). The tat gene sequences were confirmed by BLAST on NCBI website. The recombinant pMD19-T/tat was digested with Xho I and EcoR I and the tat gene was ligated into expression vector MSCV-IRES-GFP (MIG) (Addgene, USA). The plasmids were transformed into DH5-α and the recombinants were selected by ampicillin. The recombinant retroviral expressing vectors containing Sa, Ba, Sn, or Bn tat gene were named as Sa/MIG, Ba/MIG, Sn/MIG, and Bn/MIG, respectively.

**Analysis of HIV-1 tat Sequence**

HIV-1 tat sequence alignment was performed by using CLUSTAL W and phylogenetic analysis was performed by using Neighbor-joining with MEGA4 software.

**Packaging Retrovirus and Detecting Infection Unit**

293T cells were cultured in Dulbecco modified Eagle medium (Gibco, USA) with 10% fetal calf serum (FCS) supplemented with 1% penicillin-streptomycin. Four different recombinant retrovirus expression vectors (Sa/MIG, Ba/MIG, Sn/MIG, Bn/MIG) were transfected respectively into 293 T cells together with packaging plasmid pUMVC and auxiliary plasmid pCMV-VSV-G (Addgene, USA) at a ratio of 10:9:1. Three control groups included C: cotransfect plasmid MSCV-IRES-GFP, pUMVC and pCMV-VSV-G; C1: only transfect MSCV-IRES-GFP; C2: cotransfect plasmid pUMVC and pCMV-VSV-G. The supernatant was collected 48 h after transfection and filtered through a 0.22 μm filter to remove cellular debris and detected with fluorescence microscope.

Dilutions of virus supernatants were added to 293T cells during the exponential phase of growth. After 96 h, fluorescent cells were reduced as the virus dilution fact was increasing. The deuto-few number of fluorescent cells multiply dilution fact just was the virus infection unit, and the final effective infection unit was the average of three parallel determination experiments.

**Expressing Tat Protein and Determining Effect of Tat on Cytokine Secretion**

Retrovirus supernatant with the same infection units plus 5 g/mL polybrene, which can enhance the virus infectivity, were applied to U87 cells. U87 cells were cultured at 37 °C with 5% CO₂ for 8 h. After refreshing medium, the cells were cultured for 96 h and U87 cells and the supernatant were harvested to determine Tat protein expression and cytokine concentration.

Immunohistochemistry stain was conducted to determine the expressing level of Tat protein in U87 cells with monoclonal antibody against HIV-1 Tat (Abcam, British) and horseradish peroxidase-conjugated goat anti-mouse IgG as secondary antibody (ZSGB, China). By using an image analysis system (Jieda, China), the Tat-positive area (brown area) was selected in the image, and its integrated optical density (IOD) was measured, which is directly correlated with the expressing level of Tat. Then IOD was analyzed by one-way ANOVA with SPSS statistical package.

The supernatant was harvested by centrifugation at a low speed to remove cell debris, then the cytokines TNF-α, IL-1β were detected with an ELISA kit (R&D, USA). Each test was performed in triplicate, and the data were analyzed by one-way ANOVA with SPSS statistical package.

**RESULTS**

**Cloning and Expressing Vector Construction of HIV-1 tat Genes**

Four HIV-1 tat exon 1 genes (Sa, Ba, Sn, Bn) were amplified with PCR from the ADC patient and the non-ADC patient (Figure 1). The gene was 230 bp

![Figure 1](image-url)

Figure 1. PCR amplification of HIV-1 tat exon 1. M: DL2000 DNA Marker; Sa and Ba: HIV-1 tat isolated from spleen and basal ganglia tissues of the ADC patient; Sn and Bn: HIV-1 tat isolated from spleen and basal ganglia tissues of the non ADC patient.
as expected. DNA sequencing results confirmed that they were HIV-1 tat genes. The PCR products were successfully cloned into cloning vector pMD19-T/tat and subsequently expression vector MSCV-IRES-GFP/tat.

**Phylogenetic Analysis of tat Gene Sequences**

Phylogenetic tree constructed by using sequences of Sa, Ba, Sn, and Bn, and HXB2 tat gene showed that Sa, Sn, and Ba were in one clad with short branches, and Bn in another clad by itself (Figure 2).

**Analysis of Amino Acid Sequences of Tat Proteins from Different Tissues**

The amino acid sequences encoded by Sa, Ba, Sn, and Bn of HIV-1 tat exon 1 genes are shown in Figure 3. Compared with HXB2, amino acid variation sites existed in all the four sequences; I39T, T40K, and A42G existed in Sa, Ba, and Sn; L69I existed in Sa, Sn, and Bn. Moreover, the 4 sequences had their own variation sites respectively, Sa was at T23N; Ba was at K19R, Q54R; Sn was at R53G; and Bn was at I39M, N61D.

**Detection of Retrovirus Packaging and Infection Unit**

**Retrovirus Packaging** Circular green fluorescence was observed in Sa, Sn, Ba, and Bn transformed 293T cells groups, indicating that retrovirus was packaged successfully. CI was only transfected with MSCV-IRES-GFP, failed to package complete retrovirus, so the fluorescence showed a normal cell morphology (Figure 4).

**Retrovirus infection unit** The retrovirus infection unit in the supernatant harvested from Sa, Sn, Ba, Bn, and C groups were from 5x10⁶ to 06.33 x 10⁶ IU/mL. There was no difference among these groups.

**Expression of Tat Protein and Its Effects on Secretion of TNF-α and IL-1β by U87 Cells**

**Tat Expression in U87 Cells Infected with Retroviruses** Immunohistochemical stain showed that Tat protein was expressed in U87 cells cotransfected with Sa, Ba, Sn, and Bn groups, except for the control group C (Figure 5). IOD of the Tat proteins was from 149.95 to 175.34, and there was no significant difference in protein expressing level among the four groups (Sa, Ba, Sn, Bn) (P>0.05).

**Effects of Tat Protein on Secretion of TNF-α and IL-1β** There was no difference in the level of TNF-α and IL-1β between the negative control group (C) and U87 cells control group (U), which indicated that the empty recombinant retrovirus could not affect the TNF-α, IL-1β production (P>0.05); Compared with C, TNF-α and IL-1β levels of Sa, Ba, Sn, and Bn in the 4 Tat protein positive groups were significantly higher (P<0.05), indicating that Tat protein could induce U87 cells to secrete TNF-α and IL-1β. The 4 Tat protein groups had no significant differences in TNF-α levels (P>0.05), while the level of IL-1β in Sa, Ba and Sn in the 3 Tat positive groups

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**Figure 2.** Phylogenetic tree of HIV-1 tat genes. Sa and Ba: HIV-1 tat isolated from spleen and basal ganglia tissues of the ADC patient; Sn and Bn: HIV-1 tat isolated from spleen and basal ganglia tissues of the non ADC patient. HXB2: tat gene of HIV-1B standard strain. Branch lengths are drawn to scale, with the bar at the bottom indicating 0.01 nucleotide substitution per site. The number along a branch represents bootstrap value and the value is hided if it's <70%.

**Figure 3.** Amino acid sequences of HIV-1 Tat. Sa and Ba: amino acid sequence deduced from HIV-1 tat isolated from spleen and basal ganglia tissues of the ADC patient; Sn and Bn: amino acid sequence deduced from HIV-1 tat isolated from spleen and basal ganglia tissues of the non ADC patient; Underlined capital letter: amino acids in core function area of Tat.

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Figure 4. Detection of Retrovirus Packaging in 293T cells Sa, Sn, Ba, Bn, and C represents 293T cells cotransfected with Sa/MIG, Sn/MIG, Ba/MIG, Bn/MIG, MSCV-IRES-GFP, and pUMVC, pCMV-VSV-G, respectively; C1 represents 293T cells transfected with MSCV-IRES-GFP; C2 represents 293T cells cotransfected with pUMVC and pCMV-VSV-G.

Figure 5. Detection of Tat protein expression in infected U87 cells by immunocytochemistry stain (×400). Sa, Ba, Sn, and Bn represent Tat protein expressed by tat gene isolated from different spleen and basal ganglia tissues of the two AIDS patients respectively, C is the blank retrovirus expression in the vector control group.

Figure 6. Concentration of TNF-α and IL-1β in retrovirus infected U87 cells. U: U87 cells control group; C: blank retrovirus control group; Sa, Ba, Sn, Bn: Positive groups containing Tat expressed by tat gene isolated from different spleen and basal ganglia tissues of the two AIDS patients respectively. *: Compared to C, P<0.05; #: Compared to Bn, P<0.05.

DISCUSSION

In the early stage of HIV-1 infection, HIV-1 cross the blood-brain barrier into the central nervous system with the infected peripheral blood mononuclear cells which is repository to replicate a large number of viruses, resulting in central nervous system damage. HIV-1 Tat can not only promote viral replication in infected cells, but also cause neuronal apoptosis and eventually leads to the occurring of ADC by activating NMDA receptors, destructing the balance of glutamate and calcium, triggering oxidative stress, and inducing macrophages and glial cells to secrete cytokines and chemokines[5,9,14-21].

For lack of 3′→5′exonuclease activity in HIV-1 reverse transcriptase, the virus genome during the replication is with highly variable characteristics. HIV-1 gene mutation in peripheral tissues is different from the mutation in CNS which has its specific environment, and with the development of CNS disease, the genetic variability of HIV-1 in CNS increases gradually[22]. Studies have found that the occurring of ADC is not always related with the load of HIV-1 in brain, and it may also be associated with HIV-1 gene mutations and biological activity changes[15-16].

Induction of cytokines TNF-α and IL-1β by Tat protein is one of the most important mechanism for...
the occurring of ADC, which is related with the inflammation in CNS and blood-brain barrier damage[8,23-24]. The tat gene has its diversity with the HIV-1 genomic variations. In order to explore the effects on the secretion of TNF-α and IL-1β of Tat encoded by different tat genes, we analyzed the diversity of tat genes isolated from peripheral and central tissues of the AIDS patients with ADC and the AIDS patient without ADC and studied the secretion of TNF-α and IL-1β induced by Tat protein with different sequences by using the recombinant retrovirus expressing system.

Our results showed that the level of TNF-α in four Tat protein positive groups are in the order of Sa > Ss > Bn > Ba. For the same patient, the TNF-α level induced by peripheral derived Tat were higher than than the central tissue derived Tat, which is consistent with the finding that the genetic variation in peripheral tissues is greater than in central tissues. Besides, the central tissue derived Tat induced TNF-α level which was higher in the ADC patient than that in the non ADC patient. Wesselingh reported that compared with non ADC patients, ADC patients had higher TNF-α level in the CNS [25]. However, there was no statistical difference among the TNF-α levels in the four Tat positive groups, indicating that the amino acid mutation sites in the four Tat sequences derived from different tissues did not affect TNF-α production by U87 cells, which might be due to small sample size in our study.

Our results showed that the level of IL-1β in Sn, Sa and Ba groups were significantly higher than that in Bn group. Brabers et al. reported that the IL-1β levels increased in serum of HIV-1 patients and cerebrospinal fluid of ADC patients [26]. Sippy also confirmed that the levels of IL-1β in the brain tissue and cerebrospinal fluid of ADC patients were higher than those of non ADC patients [25,27]. Our results also showed that ADC peripheral and central tissue derived and non ADC peripheral derived Tat could induce higher level of IL-1β than non ADC central tissue derived Tat, which is consistent with the reports mentioned above, indicating that Tat protein is one of the factors affecting the IL-1β levels in AIDS patients.

The phylogenetic tree showed that the tat genes from ADC spleen, basal ganglia and non ADC spleen were in the same clade, and the tat gene from non ADC basal ganglia was in another clade; in addition, with the p-distance analysis, we found that the non ADC basal ganglia derived tat gene was the closest to the standard HXB2 tat gene, and its gene variation was the smallest. All of these are consistent with the capacity of Tat inducing IL-1β secretion, which suggested that the genetic diversity of tat gene could affect the biological functions of Tat protein in inducing IL-1β secretion.

Through the amino acid sites analysis, we found that the amino acid mutation sites in non ADC basal ganglia derived Tat were the smallest, the universal mutation sites in other three Tat sequences were I39T, T40K, and A42G, which are concentrated in the core functional areas (38-47aa) of Tat proteins, indicating that the core area is not only the necessary peptide to link TAR and to result in viral replication, and that the mutation in this area also could enhance the U87 cells to secrete IL-1β. In the meantime, we cannot rule out the influence of the mutation sites occurred only in the non ADC basal ganglia derived Tat sequence. I39M, N61D mutation sites may diminish the U87 cells to secrete IL-1β.

Although we only studied four Tat proteins isolated from spleen and basal ganglia of one AIDS patient with ADC and the other AIDS patient without ADC, our results confirmed that Tat protein could induce the secretion of TNF-α and IL-1β by U87 cells. We also found that the capacity of Tat protein to induce the secretion of IL-1β could be affected by the genetic variation and amino acid mutation sites, and the core functional area of Tat protein might be the key amino acid sequence of enhancing the secretion of IL-1β.

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