

Evaluation of HTLV-1 activity in HAM/TSP patients using proviral load and Tax mRNA expression after *In Vitro* lymphocyte activation

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ABSTRACT

Objective(s): HTLV-1 is the first human retrovirus that has been recognized and is associated with HAM/TSP and ATLL. Studies have shown that less than five percent of HTLV-1 infected carriers develop HAM/TSP or ATLL and about ninety-five percent remain asymptomatic. Therefore, the proviral load with Tax may affect cellular genes such as cytokines and oncogenes, as well as involve in pathogenicity.

Materials and Methods: Thirty HAM/TSP patients, thirty HTLV-1 healthy carriers, and MT-2 cell line were evaluated for HTLV-1 activity. PBMCs were isolated and activated using PMA and ionomycin. Real-time PCR and TaqMan methods were performed using specific primers and fluorescence probes for Tax expression and proviral load assessment. B2microglobulin (β_2m) and albumin were used as controls in Tax expression and in proviral load, respectively.

Results: An insignificant increase in Tax expression was observed in rest PBMCs of HAM/TSP patients compared to healthy carriers. However, after lymphocyte activation there was a significant increase in Tax expression in HAM/TSP patients ($P=0.042$). The Proviral load in patients was significantly higher than in carriers. Moreover, there was a significant correlation between Tax mRNA expression in activated PBMCs and proviral load ($R=0.37$, $P=0.012$).

Conclusion: Although proviral load had been addressed as a valuable index for monitoring HTLV-1 infected subjects, the results of this study demonstrated that Tax expression in activated PBMCs along with proviral load assessment in HAM/TSP patients are a more reliable factor for determining the prognosis and monitoring healthy carriers and HAM/TSP patients.

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Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is an oncogenic human retrovirus that causes diseases with different clinical outcomes such as adult T-cell leukemia/lymphoma (1, 2), and inflammatory neurologic disease, mainly HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) (3, 4). Other autoimmune diseases including sjogern's syndrome, polymyositis, inflammatory arthritis and uveitis might be associated with HTLV-1 infection (5). Although the pathophysiology of these disorders has not been fully understood, it seems that immunovirological factors should play major roles.

HTLV-1 infects 10-20 million people all over the world (5). It is endemic in parts of the world

including the Caribbean, parts of central Africa, Brazil, southern parts of the United States (6) and Northeast of Iran. Northeast of Iran is an endemic area for HTLV-1 and 2.1 percent of the residents of Mashhad, the largest city in northeast of Iran, are HTLV-1 carriers (7).

Studies have shown that less than five percent of HTLV-1 infected carriers develop HAM/TSP or ATL while about 95 percent remain asymptomatic carriers (8). Patients who develop ATL are often infected in childhood with a clinical latency period of 20-60 years; in contrast, HAM/TSP patients have shorter clinical latency periods (9). Typically, patients with HAM/TSP have more HTLV-1 infected lymphocytes than asymptomatic carriers (10, 11).

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A common reason for the pathogenesis of HTLV-1-associated diseases is T-cell growth activity and induction of T-cell proliferation by HTLV-1 transactivator (Tax) (12). Tax is a regulatory gene in HTLV-1 that is essential for cell transformation and replication. HTLV-1 transactivator encodes a phosphoprotein that increases HTLV-1 Long Terminal Repeat (LTR) transcription by activation of nuclear factors NF- κ B. Tax does not bind directly to DNA fragments but activates other transcription factors such as NF- κ B (13). It can activate many cell promoters such as promoters of IL-2, IL-2R, GM-CSF. Activation of these genes lead to continuous proliferation of HTLV-1 infected cells (14, 15). It has been reported that expression of Tax mRNA is increased after culturing peripheral blood mononuclear cells from HAM/TSP patients (16, 17).

HTLV-1 proviral load has been evaluated as predictor of HAM/TSP development. Some studies have shown that HTLV-1 proviral load is higher in HAM/TSP patients than in asymptomatic carriers (18, 19). Although proviral load has possibly an important role in the development of HAM/TSP, association between this factor and the disease remains unclear (23) and it seems this index is not able to differentiate HTLV-I in latent phase. Therefore, it is estimated that assessment of proviral load along with Tax expression (an index of activity) should be a better indicator of developing HTLV-I associated diseases. In this study, the HTLV-1 Tax mRNA expression and proviral loads in PBMCs of HAM/TSP patients and asymptomatic carriers were evaluated in two different conditions, base line and activated cells (20).

Material and Methods

Subjects

The study was approved by the Ethics Committee of Tehran University of Medical Sciences. Patients were admitted to the Department of Neurology, Ghaem Hospital, Mashhad University of Medical Sciences (MUMS, Mashhad, Iran).

The patients had no history of previous treatment for HAM/TSP. Informed consents were obtained from the participants or their legal guardian at the first visit. The inclusion criterion was the definitive diagnosis of HAM/TSP according to the World Health Organization criteria (21-24). Thirty HAM/TSP

patients, thirty HTLV-1 healthy carriers, and MT-2 cells were evaluated for HTLV-1 activity. Contamination of subjects with HTLV-1 were identified by serological methods and confirmed by PCR technique (7). MT-2 cell line is a human T-cell line that is infected with HTLV-1 (25, 26).

Oligonucleotide designing and complementary DNA synthesis

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated blood samples by Ficoll density gradient (Sigma, Germany). A quantitative real-time PCR assay (TaqMan method) was carried out to measure Tax expression and proviral load of HTLV-1 in PBMCs using specific primers and a fluorogenic probe by a Rotor Gene Q real-time PCR machine. RNA was extracted from fresh PBMCs using viral RNA extraction Mini Kit (Qiagen, Germany) and complementary DNA (cDNA) was synthesized using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). Table 1 shows the nucleotide sequence of primers and probes. The procedure for conventional PCR was as follows: 1 μ l cDNA (100 ng/ μ l), 2.5 μ l reaction buffer 10x, 0.5 μ l dNTP (10 mM, GeNet Bio), 2 μ l MgCl₂ (25 mM, Fermentas), 0.5 μ l each of primers (10 pM), 0.2 μ l prime Taq DNA polymerase (5 U/ μ l, Fermentas), 12.8 μ l distilled water. β_2m was used as human housekeeping or reference gene in Tax expression measurement. The expression of this gene remains stable within PBMCs from samples (27).

Cell culture

After PBMCs isolation, the cells were washed with RPMI medium. Cells were then introduced into an enriched RPMI 1640 medium (containing 12% inactivated bovine serum albumin) in the presence of 1 mg / μ l PMA (phorbol 12-myristate13-acetate), 25 ng/ml ionomycin, and incubated in 5% CO₂, 95% humidity for 6 hr. Afterwards, samples were collected for RNA extraction. Moreover, a cell line (MT-2) culture with ionomycin and PMA was used as control.

Real-time PCR

HTLV-I Proviral Load

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated blood samples by Ficoll density gradient (Sigma, Germany). DNA was extracted using Ge Net kit (Korea). To assess the

Table 1. The nucleotide sequence of specific primers and probes for HTLV-I Tax and Beta 2- Microglobulin

HTLV-I Tax:	
The forward primer:	(5096-5115); 5'-ATCCCGTGGAGACTCCTCAA-3'
The reverse primer:	(5180-5183, 7302-7312); 5'-CCTGGGAAGTGGGCCATG-3'
The probe:	(5123-5145); 5'-CATGCCCAAGACCCGTCGGAGG-3'
Beta 2- Microglobulin	
The forward primer:	TTGTCTTTCAGCAAGGACTGG
The reverse primer:	CCACTTAACATCTTGGGCTGTG
The probe:	TCACATGGTTCACACGGCAGGCAT

HTLV-1 proviral load, a real-time TaqMan PCR absolute method was carried out using HTLV-1 RG kit (Novin Gene, Iran) (28). Briefly, the HTLV-1 copy number was referred to the actual amount of cellular DNA by quantifying albumin gene as the reference gene. HTLV-1 and albumin DNA concentrations were calculated using two 5-point standard curves. The normalized value of the HTLV-1 proviral load was calculated as the ratio of (HTLV-1 DNA copies number /albumin DNA copies number/2) $\times 10^4$ and expressed as the number of HTLV-1 proviruses per 10^4 PBMCs (7).

Tax measurement

After cDNA synthesis, serial dilutions of standards (5 for each gene) were applied to relatively quantify the mRNA copy number of each single gene. Two standard curve methods were used for target and reference genes quantification by a Rotor Gene Q real-time PCR machine (Qiagen, Germany). The Rotor Gene 6000 software was used to analyze the standards and the unknown mRNA copy numbers. The relative quantity of each mRNA was normalized to the relative quantity of β_2m mRNA. Then the relative Tax expression levels for each sample were calculated by an equation: Tax Normalized Index = copy number of gene of interest (Tax)/copy number of reference gene (β_2m). Then the fold change in expression for each group was calculated by the following equation: mean of Tax normalized index of test group/ mean of Tax normalized index of healthy carriers.

Results

Tax mRNA in HTLV-1 infected subjects before stimulation of PBMCs

The HTLV-1 Tax mRNA load was measured in PBMCs of HAM/TSP patients and asymptomatic carriers (ACs). The mean \pm SEM of Tax mRNA load was 31.24 ± 21.21 copies/ml in patients with HAM/TSP, while the value was 5.63 ± 2.5 copies/ml in the ACs. An increase of 5.54-fold was observed in the patients group with HAM/TSP compared with the ACs, which was statistically insignificant ($P=0.671$, Mann Whitney).

Tax mRNA load in HAM/TSP patients and ACs after stimulation of PBMCs

After isolating the PBMCs, the cells were treated with PMA and ionomycin for 6 hr, cDNA was synthesized, and real-time PCR performed. The treatment of T-cells infected with HTLV-1 resulted in the induction of Tax mRNA expression in the stimulated cells. Tax mRNA load was 2658 ± 2005 copies/ml in HAM/TSP patients and it was 99.72 ± 27.37 copies/ml in ACs. The expression level of Tax mRNA in the stimulated cells of HAM/TSP patients was 26.65 higher than ACs cells, which was

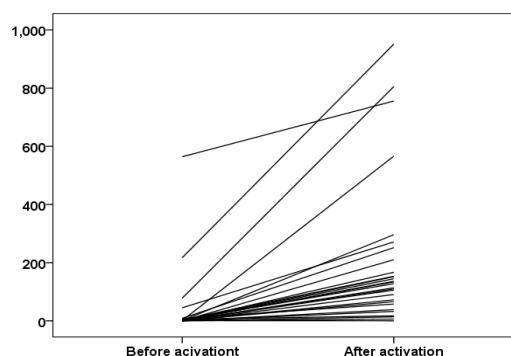
statistically insignificant ($P=0.042$, Mann Whitney). Figure 1. Difference between Tax gene expression before and after cells stimulation was statistically significant ($P=0.000$, Wilcoxon's test).

Demographic results

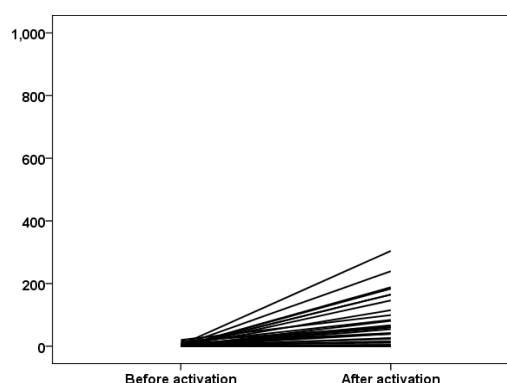
HAM/TSP patients and control group showed no significant difference in gender ($P=0.292$) Figure 2. The mean of age in carriers was 42.15, and in patients was 45.04. Analyzing the data showed there was no significant difference between two groups in respect of age ($P=0.34$). It needs to be noted that the HTLV-1-associated diseases such as HAM/TSP and HTLV-1 infection are more prevalent in women than in men in Iran (2). No correlation was found between Tax mRNA expression and gender (Spearman's rho test).

Proviral load

Real-time PCR was performed on DNA that was extracted from peripheral blood mononuclear cells as previously described. Men and women were significantly different in proviral load ($P=0.009$).



a)



b)

Figure 1. mRNA Tax expression was assessed before and after activation with PMA and ionomycin. Tax markedly increased after activation in HAM/TSP patients (a) compared to carriers (b) ionomycin and PMA were used as lymphocyte activators

Table 2. Laboratory findings in HAM/TSP patients and carriers

Characteristics	Asymptomatic carriers	HAM/TSP patients	P-value
HTLV-1 Proviral load/ 10^4 PBMCs	460 \pm 153	2052 \pm 579	0.004
Tax expression copy/ml (before stimulation)	5.63 \pm 2.5	31.24 \pm 21.21	0.671
Tax expression copy/ml (after stimulation)	99.72 \pm 27.37	2658 \pm 2005	0.042

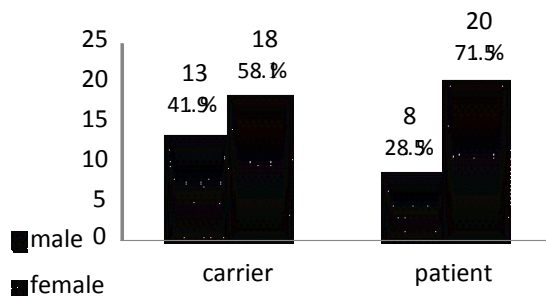


Figure 2. The gender distribution in HAM/TSP patients and carriers

Proviral load in patients was significantly higher than in carriers ($P=0.004$, Mann Whitney). The mean \pm SD proviral load in asymptomatic carriers was 460 \pm 153 copy/ 10^4 PBMCs and in patients with HAM/TSP was 2052 \pm 579 copy/ 10^4 PBMCs Table 2. Results confirmed that proviral load in patients with HAM/TSP was 4.46 times higher than in asymptomatic carriers. To investigate the relationship between Tax gene expression and proviral load Spearman's rho test was used and there was a significant correlation between Tax mRNA expression in activated PBMCs and proviral load ($R=0.32$, $P=0.03$).

Discussion

A nationwide survey by Iranian Blood Transfusion Organization (IBTO) in 1996 and other epidemiological studies demonstrated that Razavi and north Khorasan and Golestan provinces in northeast of Iran are endemic for HTLV-I infection. These studies suggested that the highest prevalence of the virus was observed in Razavi Khorasan and southeast of Golestan provinces mainly in Mashhad, Neyshabour and Kalalah (7, 29). Therefore the study of virus activities in Iran is an urgent priority.

In spite of the fact that HTLV-1 and its associated diseases such as ATLL and HAM/TSP were studied for several years, the pathogenesis of HTLV-1-associated diseases and the interaction between virus, infected cells and the host immune system yet to be understood. Previous studies have provided evidence that T cell stimulation lead to viral protein expression in infected cells (30, 31). HTLV-I Tax is one of such protein that can induce pro-inflammatory cytokine production, mainly IL-2, GM-CSF and IFN- γ , in CD4 $^+$ infected T cells (32). Pro-inflammatory cytokine production may affect on host immune responses results in forming inappropriate

inflammatory reactions toward HAM/TSP (29). We have previously argued that the virological and host immune modulators might play the main roles in the pathophysiology of HAM/TSP (2).

This study showed that HTLV-I Tax mRNA expression dramatically increased in HAM/TSP patients compare to healthy carriers after *in vitro* PBMCs stimulation with PMA-ionomycine. However, Tax mRNA expression in HAM/TSP and healthy carriers in inactivated PBMCs did not show any differences.

This finding shows that HTLV-I infected T cells in HAM/TSP subjects maybe more sensitive to stimulatory signal and produces more Tax protein than healthy carries. Moreover, these changes in Tax expression, as the immune-dominant antigen, of HTLV-I infected CD4 $^+$ cells make them more suitable to killing activities of specific CTLs too.

There are many controversial studies regarding the prognostic value of proviral load in healthy carriers or HAM/TSP patients. HTLV-I provirus load is generally higher in patients with HAM/TSP than ACs, however, there are ACs who have comparable HTLV-I proviral load with HAM/TSPs. Moreover, there have been conflicting reports on Tax expression and HAM/TSP progression. For instance, the results of a study indicated that Tax mRNA expression in HTLV-I-infected cells of HAM/TSP subjects after 12hr culture did not show any differences with ACs with high proviral load. Whereas others demonstrated that the Tax mRNA expression in HTLV-I infected cells was higher in patients with HAM/TSP than in ACs (33, 34).

Our study demonstrated that neither proviral load nor Tax in inactivated PBMCs are useful prognostic markers for monitoring of HTLV-I infected subjects. Tax expression may act as an index for viral activity and as an important index in predicting long-term status of HAM/TSP patients. The researchers still do not know why the small number of HTLV-I infected people develop associated diseases (35, 36).

The finding of the present study indicated that proviral load in HAM/TSP patients was statistically higher than asymptomatic carriers ($P=0.004$). Although, Tax expression in inactivated PBMCs of HAM/TSP patients was more than asymptomatic carriers, it was not significant. Activated PBMCs of HAM/TSPs had 26.65 fold tax expressions than healthy carriers which was statistically meaningful. Therefore, if HTLV-I proviral load and Tax expression in activated PBMCs of HTLV-I infected

individuals taken into account, they may act as a new strong prognostic index for monitoring of healthy carriers and HAM/TSP patients.

However, there are some limitations for this study; it can be assessed at the same time the HTLV-I HBZ expression as an important regulator of HTLV-I replication. Moreover, assessment of these viral protein and host immune response such as T cell population or T cell transcription factor make the study very strong.

Conclusion

Proviral load and Tax production in infected subjects are clearly affect on development of HTLV-I associated diseases (37, 38). However, it seems that in the presence of T cell stimulatory signals the amount of Tax expression from infected CD4⁺ cells is dramatically increase which may have two different effects, it makes these cells more susceptible to CTLs killing activities or induces pro-inflammatory cytokines production toward forming HTLV-I associated diseases.

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