Evaluation of the role of *TAX*, *HBZ*, and HTLV-1 proviral load on the survival of ATLL patients

Mohammad Mehdi Akbarin[1], Abbas Shirdel[2], Alireza Bari[2], Seyedeh Tahereh Mohaddes[2], Houshang Rafatpanah[1], Ehsan Ghayour Karimani[3], Kobra Etminani[4], Amin Golabpour[4], Reza Torshizi[5]

[1]Inflammation and Inflammatory Diseases Research Center, Faculty of Medicine, [2]Hematology Department, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, [3]Molecular Diagnostic Unit, Research and Education Department, Razavi Hospital, [4]Department of Medical Informatics, [5]Department of Modern Sciences and Technologies, Molecular Medicine Department, Faculty of Medicine, Mashhad University of Medical sciences, Mashhad, Iran

Background

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive malignancy with very poor prognosis and short survival, caused by the human T-lymphotropic virus type-1 (HTLV-1). The HTLV-1 biomarkers trans-activator x (TAX) and HTLV-1 basic leucine zipper factor (HBZ) are main oncogenes and life-threatening elements. This study aimed to assess the role of the *TAX* and *HBZ* genes and HTLV-1 proviral load (PVL) in the survival of patients with ATLL.

Methods

Forty-three HTLV-1-infected individuals, including 18 asymptomatic carriers (AC) and 25 ATLL patients (ATLL), were evaluated between 2011 and 2015. The mRNA expression of *TAX* and *HBZ* and the HTLV-1 PVL were measured by quantitative PCR.

Results

Significant differences in the mean expression levels of *TAX* and *HBZ* were observed between the two study groups (ATLL and AC, $P=0.014$ and $P=0.000$, respectively). In addition, the ATLL group showed a significantly higher PVL than AC ($P=0.000$). There was a significant negative relationship between PVL and survival among all study groups ($P=0.047$).

Conclusion

The HTLV-1 PVL and expression of *TAX* and *HBZ* were higher in the ATLL group than in the AC group. Moreover, a higher PVL was associated with shorter survival time among all ATLL subjects. Therefore, measurement of PVL, *TAX*, and *HBZ* may be beneficial for monitoring and predicting HTLV-1-infection outcomes, and PVL may be useful for prognosis assessment of ATLL patients. This research demonstrates the possible correlation between these virological markers and survival in ATLL patients.

Key Words   HTLV-1, ATLL, *TAX*, *HBZ*, Proviral load

Correspondence to
Reza Torshizi, Ph.D.
Department of Modern Sciences and Technologies, Mashhad University of Medical Sciences, Mashhad, Razavi Khorasan 91388-13944, Iran
E-mail: torshiziR901@mums.ac.ir

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INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a lentivirus that belongs to the Retroviridae family in the genus Deltaretrovirus [1]. About 20 million infected people live in Africa, the Caribbean, Asia, and Central and South America [2].

The *Gag*, *pro*, *pol*, and *env* are the main genes of HTLV-1and also contains two long terminal repeat (LTR) regions. These genes encode the viral integrase, reverse transcriptase, and regulatory proteins (e.g., TAX and superficial glycoprotein) [3]. While HTLV-1 can transform and immortalize cells *in vitro*, only 2–5% of infected individuals go on to develop one of the two major pathogenic conditions, which occurs after a long-term period of dormancy [4]. The two major diseases associated with HTLV-1 infection are the antagonistic CD4+CD25+ T cell malignancy called adult T-cell leu-

kemia/lymphoma (ATLL) and a host autoreactive inflammatory disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5, 6].

According to the Revised European American Lymphoma (REAL) classification and World Health Organization (WHO), ATLL is classified into four subtypes (acute, lymphoma, chronic, and smoldering) based on the following symptoms: the persistence of flower-like cells in peripheral blood smear, elevated lactate dehydrogenase (LDH), hypercalcemia, skin lesions, organomegaly, and lytic bone injury [7, 8]. The most aggressive ATLL types are the acute and lymphoma types. The acute type has the highest prevalence rate (60%) and shortest survival time (4–6 mo) among all ATLL types, and it shows some of the most aggressive symptoms (e.g., hepatosplenomegaly, lymphadenopathy, a high frequency of atypical lymphocytes with nuclear hypersegmentation, as well as high levels of LDH and calcium in serum). The lymphoma type has a prevalence rate of 20% and a 9–10-month survival period, which demonstrates the aggressiveness of ATLL. The chronic and smoldering types have a survival period of 2–5 years and are less frequently associated with aggressive clinical conditions [2, 9]. The primary infection is followed by a long-term latency period (20–40 yrs). Then, leukemogenesis of infected T-cells occurs in a multistep process via genetic and epigenetic alterations [10].

Although the exact pathogenic mechanism of ATLL is unknown, it is found that clonal expansion of infected T-cells increases the number of viral progeny, leading to plus-strand transcription of the HTLV-1 genome. In addition to the major gene described above, the genome also encodes other factors including accessory genes (open reading frames I and II), regulatory genes (*tax* and *rex*), and P12, P13, and P30, which are necessary for viral persistence and infection. These viral regulatory proteins (e.g., TAX and HBZ) are thought to initiate transformation and promote genetic instability and transactivation in this T-cell malignancy [8].

Transactivation of the viral LTR and functional cellular genes is synchronized by TAX; however, TAX does not bind to DNA, but may upregulate the expression of cell survival factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which function as anti-apoptotic elements [11]. Previous studies have suggested that TAX protein promotes downregulation of the expression of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and activating transcription factor (ATF) signaling proteins, as well as DNA repair proteins (e.g., P53 and retinoblastoma [Rb]). Therefore, TAX may play a critical role in malignant T-cell proliferation [12, 13]. Regarding the TAX antigen structure, a strong immune response in HTLV-1-infected patients can eliminate or suppress TAX expression [14]. However, genetic alteration of HTLV-1 by methylation, deletion, or mutation in the 3′ LTR region can lead to low TAX protein expression [15]. These mechanisms may allow ATLL cells to escape detection by the host immune system, enabling latent activation of the HTLV-1 genome [16]. The *HBZ* gene is preserved in and expressed from the antisense

strand of the LTR region in HTLV-1-infected cells, and both HBZ mRNA and protein play pivotal roles in cross-reactions with intracellular proteins such as JunB, c-Jun, JunD, and CREB-2 in the development of ATLL [17]. This study aimed to demonstrate the possible correlation between virological markers (PVL and *TAX* and *HBZ* expression) and the survival time of patients with ATLL.

MATERIALS AND METHODS

Study population

A case series study was conducted on 43 HTLV-1-infected individuals in the Departments of Hematology and Oncology of Mashhad University of Medical Sciences, Mashhad, Iran, and in Ghaem and Imam Reza Hospitals from July 2011 to November 2015. Patients with suspected ATLL were investigated, and on the basis of symptoms and laboratory test results, 25 patients (12 males and 13 females) were diagnosed with ATLL and placed in the ATLL group. The remaining 18 patients (7 male and 11 female) were considered healthy, asymptomatic HTLV-1-infected carriers, and placed in the control group. HTLV-1 seropositive reactivity was checked for all subjects, and the presence of HTLV-1 was confirmed by conventional PCR. ATLL patients were further divided into four groups: acute, lymphoma, chronic, and smoldering, based on the criteria reported by Shimoyama [18].

Ethical approval

The study was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran. All procedures were conducted in accordance with the Declaration of Helsinki (1964) and its later amendments or comparable ethical standards.

Isolation of peripheral blood mononuclear cells (PBMCs) and extraction of RNA

Ficoll density gradient centrifugation (Inno-train, Germany) was performed to sort cells from peripheral blood to isolate PBMCs. Total RNA was extracted from PBMCs, using Tripure isolation reagent (Roche, Germany) according to the manufacturer's instructions. Purified RNA was treated with DNase I to remove genomic DNA contamination. RNA integrity was confirmed by gel electrophoresis.

cDNA synthesis

cDNA was synthesized using random primers and reverse transcriptase (Bioneer, South Korea) according to the manufacturer's instructions. The reaction conditions were 12 cycles of 30 seconds at $24^{\circ}C$, 4 minutes at $44^{\circ}C$, and 30 seconds at $55^{\circ}C$. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was included as a positive control for cDNA synthesis. The *GAPDH* primers were: forward, 5′-CAAGGTCATCCATGA CAACTTTG-3′ and reverse, 5′-GTCCACCACCCTGTTGCT GTAG-3′.

Table 1. Primers and probes for *TAX, HBZ,* and *β2-microglobulin*.

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Probe (5´-3´) Fam-BHQ1
TAX	CCCTAATAATTCTACCCGAGGACTG	GCCATCGGTAAATGTCCAAATAAGG	TTGCCCACCACCTTTTCCAGCCTG
HBZ	AATTGGTGGACGGGCTATTATCC	CGCGGCTTTCCTCTTCTAAGG	ACCGTCAAGCACAGCCTCCTCCTCC
β2-microglobulin	TTGTCTTTCAGCAAGGACTGG	CCACTTAACTATCTTGGGCTGTG	TCACATGGTTCACACGGCAGGCAT

Primer design

After obtaining the desired sequences for the *TAX* and *HBZ* genes from the National Center for Biotechnology Information (NCBI) GenBank, primers and probes were designed using AlleleID version 5.

Real-time PCR

Real-time PCR (TaqMan method) was performed in a Rotor-Gene 6000 cycler (Corbett, Hilden, Germany). Duplicate samples were included in the analysis, and expression is reported relative to that of a housekeeping gene (β2-microglobulin). The primers and probes used to amplify the corresponding genes are shown in Table 1. The PCR conditions were as follows: $95^{\circ}C$ for 4 minutes, followed by 45 cycles of denaturation at $94^{\circ}C$ for 15 seconds, annealing at the optimum temperature 20 seconds, and extension at $72^{\circ}C$ for 20 seconds.

PVL measurement

To evaluate the HTLV-1 PVL, PBMCs were isolated from ethylenediaminetetraacetic acid (EDTA)-treated blood samples, as described above under "Isolation of PBMCs and extraction of RNA," and DNA was extracted from the PBMCs, using a DNA extraction kit. Real-time PCR was performed using a commercial absolute quantification kit (Novin Gene, Iran) to measure the PVL of HTLV-1, using specific primers and a fluorogenic probe. HTLV-1 copy number was reported as the actual amount of cellular DNA quantified, using the albumin gene (*ALB*) as a reference. There are two copies of *ALB* in one cell. HTLV-1 and *ALB* concentrations were calculated from two 5-point standard curves. For normalization, the PVL was calculated as the ratio of HTLV-1 DNA copy number/one-half of the *ALB* copy number $\times 10^4$, and is expressed as the number of HTLV-1 proviruses per 10^4 PBMCs.

Therapeutic procedures

Different therapies were administered to the study participants according to the type of disease. The most common first-line treatment for T-cell lymphoma was combination chemotherapy, such as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), or cyclophosphamide, vincristine, doxorubicin, and dexamethasone (CVAD), which was administered to most patients [19, 20].

Statistical analysis

Data were analyzed using SPSS version 18, and the results are reported as mean±standard deviation (SD). The Kolmogorov-Smirnov test was conducted to check variable for nor-
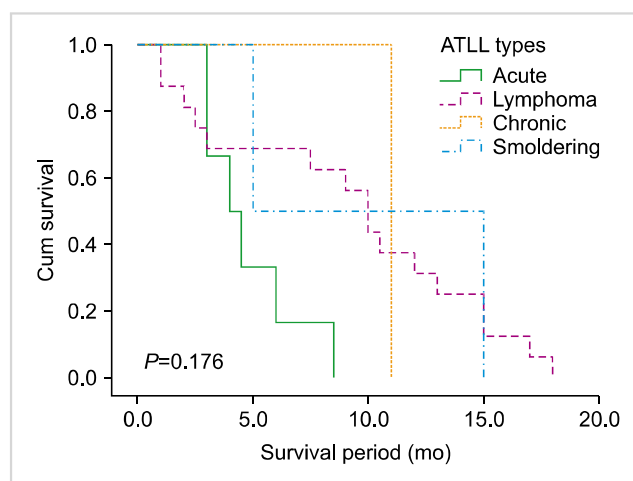

Fig. 1. Survival time of adult T-cell leukemia/lymphoma (ATLL) patients according to subtype.

mal distribution. The Mann–Whitney U test was performed to analyze the two groups. Spearman's rank correlation coefficient was used to determine the relationship between study parameters. Kaplan-Meier analysis was performed to evaluate survival. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Patient demographics

In this study, 25 ATLL patients (13 female and 12 male) and 18 HTLV-1 carriers (11 female and 7 male) were evaluated. The mean age of the subjects in the ATLL group was 53.92±2.17 years (95% confidence interval [CI], 35.42–88.29), while the mean of the carrier group was 38.94±2.04 years (95% CI, 25.13–53.43).

Clinical and paraclinical findings and survival time of ATLL patients

The frequencies of various clinical findings in ATLL patients were as follows: hepatomegaly (12%), splenomegaly (24%), lymphadenopathy (80%), edema (40%), and skin lesions (60%). The ATLL patients were further classified into four subtypes: acute (6), lymphoma (16), chronic (2), and smoldering (1). The mean calcium level was 10.44±0.56 mg/dL (95% CI, 7.23–16.83) in the ATLL group and 9.52±0.34 mg/dL (95% CI, 8.64–10.31) in carriers. Blood calcium levels in the acute, lymphoma, and smoldering groups were

13.13±0.98 (95% CI, 10.24–16.81), 9.14±0.31 (95% CI, 7.45–11.25), and 8.43, respectively. These data showed a significantly higher calcium blood level in patients with acute ATLL than in patients with other types of ATLL (P=0.000).

White blood cell (WBC) counts in ATLL patients and carriers were 191,340±6,789 cells/mL (95% CI, 1,200–132,000) and 4,933±392.3 cells/mL (95% CI, 4,400–5,700; P=0.015), respectively. The health of the participants in the carrier group was confirmed by a physician's examination. All the ATLL patients expired during this study, with a median survival time of 8.26±1.05 months (95% CI, 1–18 mo). The mean survival time was 4.83±0.86 months (95% CI, 3–8.5) for the acute group, 9.1±1.44 months (95% CI, 1–18) for the lymphoma group, and 10±5 months (95% CI, 5–15) for the smoldering group. The survival time of the only patient in the chronic group was 11 months. The

Kaplan-Meier test results did not show a significant relationship of survival time according to ATLL type (Fig. 1). The clinical symptoms and survival times of the patients are provided in Table 2.

HTLV-1 PVL and viral gene expression

The HTLV-1 PVL was investigated in all 43 study subjects (18 carriers and 25 ATLL patients). The mean HTLV-1 PVL in ATLL patients was 18,100±4,918.9 copies/10^4 cells (95% CI, 53–91,183), and the percentage of HTLV-1-infected PBMCs in ATLL group was 118±49% (95% CI, 0.5–911). The mean HTLV-1 PVL in the carrier group was 531.23±119.2 copies/10^4 (95% CI, 2–1,329) in the carrier group, which indicated that 5.3±1.1% (CI: 95% 0.02-13.1) of PBMCs were infected with HTLV-1 (P=0.000).

The expression of each gene was divided by the expression

Table 2. Clinical manifestations and survival time of adult T-cell leukemia/lymphoma (ATLL) patients.

Age (yrs)	Gender	Hepatomegaly	Splenomegaly	Lymphadenopathy	Edema	Skin lesions	Survival time (mo)
62	Male	-	-	-	-	+	15
42	Male	+	+	+	+	-	4.5
51	Female	-	-	+	-	-	7.5
58	Female	-	+	+	-	+	4
45	Male	+	-	+	+	-	13
40	Male	-	-	+	-	+	10
38	Female	-	-	+	-	+	2.5
62	Female	-	-	+	-	-	18
60	Female	-	+	-	+	+	11
52	Male	-	-	+	-	-	15
68	Female	-	-	+	-	-	10.5
55	Female	-	-	-	-	+	5
53	Male	-	+	+	-	-	1
51	Male	-	-	+	+	+	6
64	Male	-	-	+	+	-	1
45	Female	-	-	+	-	-	8.5
53	Female	-	-	+	-	+	10
55	Female	-	-	+	+	+	15
59	Male	-	-	+	-	+	17
55	Male	-	-	+	-	+	12
35	Male	-	-	+	-	-	3
88	Male	-	-	+	+	+	2
55	Female	-	-	+	+	+	9
49	Female	+	+	-	+	+	3
53	Female	-	+	-	+	+	3

Table 3. *TAX* and *HBZ* expression in adult T-cell leukemia/lymphoma (ATLL) patients and asymptomatic carriers.

	Group	Fold change ATLL/Carrier	Mean	Minimum	Maximum	SEM	SD	$P^{a)}$
TAX	ATLL	320	0.2378	4.767283E-6	2.3331108	0.21	0.6971	0.014
	Carrier		7.460417 E-4	7.349814E-7	0.006898022	6.1676E-4	0.0020	
HBZ	ATLL	937	0.1096649	0.00231	0.83824676	0.0777	0.2579	0.000
	Carrier		1.172605E-4	1.3782E-8	8.608E-4	7.7134E-5	0.99925	

a) P<0.05.
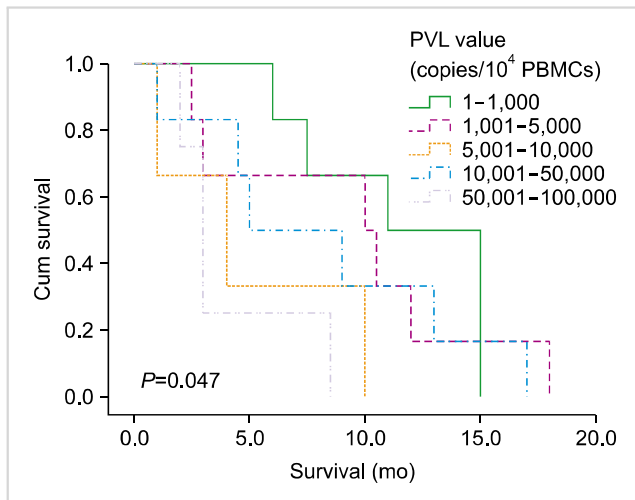Abbreviations: SD, standard deviation; SEM, standard error of the mean.

Fig. 2. Association between survival time and proviral load (PVL) in adult T-cell leukemia/lymphoma (ATLL) patients (five PVL groups were defined to examine the impact of HTLV-1 PVL on survival among patients with ATLL).

of the β2-microglobulin gene to normalize the gene expression values. The mean *TAX* gene expression level was 0.2378±0.21 (95% CI, 4.7672834E-6–2.3331108) in the ATLL group and 7.460417 E-4±6.1676E-4 (95% CI, 7.349814E-7–0.006898022) in the carrier group (*P*=0.014). The mean *HBZ* gene expression level was 0.1096649±0.0777 (95% CI, 0.00231–0.83824676) in the ATLL group and 1.17260526E-4±7.7134E-5 (95% CI, 1.3782E-8–8.608E-4) in the carrier group (*P*=0.000; Table 3).

Correlations between HTLV-1 PVL and oncogenic gene expression

A significant correlation was observed between the HTLV-1 PVL and *HBZ* gene expression, improving the persistence and constant presentation among ATLL patients (r=0.479, *P*=0.001). Furthermore, a direct positive correlation was found between *HBZ* gene expression and *TAX* gene expression (r=0.636, *P*=0.001), which was intensified by the *TAX* gene. In contrast, no positive or negative correlation was observed between *TAX* gene expression and HTLV-1 PVL (r=0.203, *P*=0.354).

Significant relationship between survival time and HTLV-1 PVL

Survival was investigated in all 25 ATLL patients. The variables evaluated in this analysis were age, gender, treatment (such as CHOP or CVAD), and viral gene expression (*TAX* and *HBZ*). According to our findings, no significant positive or negative relationship was observed between any of these variables and survival time by Kaplan-Meier analysis. However, the Log Rank test results showed a significant relationship between low PVL and longer survival (*P*=0.047; Fig. 2).

DISCUSSION

The progression of ATLL is a polyphasic, long-term proc-

ess, and the outcome of HTLV-1 infection is affected by host-virus interaction as well as cellular division and function [21]. The TAX and HBZ proteins are the two main viral components that interfere with the immune response and cause malignancy [16]. The HTLV-1 *TAX* and *HBZ* genes encode highly motivated proteins that disrupt the cell cycle by interfering with apoptosis, DNA repair mechanisms, cell signaling, and control checkpoints and activating transcription factors that regulate cell proliferation [22]. In the present study, we observed higher *TAX* gene expression in ATLL patients than in the healthy HTLV-1 carriers.

As previously mentioned, ATLL is a multi-step malignancy, and it seems that TAX is used by the virus to initiate disease progression. This process is followed by transactivation of infected T-cells, during which *TAX* expression can be downregulated by other transcription factors, such as HBZ, to evade the host immune response [5]. Despite the higher *TAX* gene expression in ATLL patients, there was no direct correlation between *TAX* gene expression and PVL, which is indicative of the initiating role of this protein.

Constant expression of the *HBZ* gene might play a pivotal role in the progression of ATLL through activation of anti-apoptotic elements and CREB-dependent cellular proliferation [23]. As HBZ does not contain any immunologically active sites, it can avoid cytotoxic T-cell (CTL) responses in ATLL patients. In the current study, the mean *HBZ* mRNA level was higher in the ATLL group, and was significantly positively correlated with PVL, which confirmed its essential function in oncogenesis.

Most infected T-cells are resistant to common chemotherapy combinations, and they continue to proliferate, with the exception of cells infected with the HIV retrovirus [24, 25]. In the present study, the mean WBC count was higher in ATLL patients than in HTLV-1 carriers, which is indicative of aggressive HTLV-1-induced leukemogenesis.

A significant positive correlation was observed between PVL and *HBZ* mRNA levels, which suggested that HBZ plays a more important role in ATLL cell division, viral replication, or viral assembly than TAX. According to our findings, the progression of ATLL may occur as follows: ATLL is initiated by *TAX* gene expression, which increases viral replication and TAX protein levels, followed by downregulation of *TAX* gene expression and upregulation of *HBZ* gene expression and HTLV-1 PVL in the final stage of ATLL.

Two major life-threatening events occur in ATLL caused by the high levels of blood calcium, which can lead to heart attacks and opportunistic infections [26, 27]. Hypercalcemia in acute ATLL patients might result from osteolytic bone marrow metastases through hyperactivation of osteoclastogenesis and resorption. It was previously shown that over-expression and secretion of parathyroid hormone-related protein (PTHrP) and macrophage inflammatory protein 1 alpha (MIP-1α) from infected T-cells leads to osteoclast development in bone marrow, resulting in the an osteolytic condition in ATLL patients with high numbers of infected lymphocytes [28].

In this study, a negative relationship was observed between

the survival time of ATLL patients and HTLV-1 PVL. This result suggests the prognostic potential of PVL for monitoring ATLL patients. In addition, *TAX* and *HBZ* mRNA expression levels, in association with the HTLV-1 PVL, could be used to monitor and predict HTLV-1 infection outcomes.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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