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Possible Trace of HTLV-1 Virus in Modulation of Cbl-b, ITCH, and PP2A Suppressor Genes

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For almost 40 years, human T-lymphotropic virus type 1 (HTLV-1) has posed a persistent challenge in managing the major diseases associated with HTLV-1. Intracellular inhibitors are critical regulators of T cell activation, and their function can be influenced by viruses. Because of less studied aspects of HTLV-1 in T cell activation, we evaluated three suppressor genes in patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and asymptomatic carriers (ACs). Thirty samples were collected from three groups from 10/09/2022 to 03/27/2024. To confirm all the samples, ELISA and PCR tests were done. The isolation of peripheral blood mononuclear cells (PBMCs), RNA extraction, and cDNA synthesis were conducted. Subsequently, the expression of *Tax trans-activator, HTLV-1 bZIP factor (HBZ), protein phosphatase 2 A (PP2A),* and two E3 ligases, including *Casitas B lymphoma–b (Cbl-b)* and *itchy E3 Ubiquitin protein ligase (ITCH),* was measured via Real-time PCR. This survey showed a significant increase in ITCH among individuals with HAM/TSP and ACs compared to the healthy group. The *PP2A* mRNA expression level was upregulated in the ACs; in contrast, the expression levels were approximately similar in the HAM/TSP and healthy groups. Also, the mean expression level of *Cbl-b* was higher in the ACs than in the other groups; however, it was not statistically significant. Our findings demonstrated that the intercellular suppressor genes could be dysregulated during the HTLV-1 infection, probably as part of the virus's strategic goals. The findings can be helpful for future investigation in the diagnosis and treatment area.

Keywords: HTLV-1, E3 ligase, protein phosphatase 2, suppressor genes

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Introduction

The only approved human oncogenic member of the Retroviridae family is the human T-lymphotropic virus type 1 (HTLV-1). Despite being discovered four decades ago, the virus has been distributed at an unclear speed globally (1, 2). It is reported that roughly 20 million HTLV-1-infected people exist worldwide. The most frequent ways to transmit the virus are through infected body fluids during breastfeeding, unprotected sex, blood transfusions, and organ transplants (1, 3).

Asymptomatic carriers (ACs) of HTLV-1 are the most infected individuals. The virus has a considerable role in several inflammation-related disorders and, more significantly, two life-threatening diseases, like a chronic, slowly progressive neurological disorder, HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), and a kind of T cell neoplasm well known as adult T cell leukemia/lymphoma (ATL) (3-5). Currently, no approved vaccine or treatment has been introduced for HTLV-1 (6).

HTLV-1 frequently infects $CD4^+$ T lymphocytes as cellular hosts. These cells, with a highly heterogeneous cell group, have crucial functions in specific immunity and regulating the activity of other cells. HTLV-1 can induce immune dysfunction in the target cell by altering intracellular signaling to escape the immune system and also increase the proliferation rate of the infected T cell for its maintenance (7, 8). However, the exact molecular and cellular processes of these various appearances of HTLV-1 infection are not definitively known. Two main viral regulatory factors, Tax trans-activator and HTLV-1 bZIP factor (HBZ), are the leading players in various aspects of pathogenesis, such as cell transformation, proliferation, immune responses, and inflammatory reactions (5, 9).

As suppressors of immune responses, protein phosphatase 2A (PP2A) and two E3 ligases, including Casitas B lymphoma-b (Cbl-b) and itchy E3 Ubiquitin protein ligase (ITCH), play crucial functions in controlling many cell activities, like signaling transduction cascades, T cell activation threshold, tolerance regulation, cell cycling, differentiation, and transformation (10-12). Indeed, negative regulatory factors lead to the termination of TCR signals to avoid improper activation of T cells. Besides, reduced responsiveness to Treg was observed in Cbl-b-deficient T cells (13, 14), and dysfunction of PP2A mediated many diseases, including cancer and neurodegenerative disorders (15, 16). ITCH deficiency is usually associated with autoimmunity disorders and human malignancies, while tumorigenic roles related to overexpression of ITCH were also reported in anaplastic thyroid carcinoma and breast cancer (17-20)

Obligate intracellular parasites, such as viruses have created several strategies to manipulate host immune defenses (21). Evidence manifests upregulation of T cell activation-related genes like *LCK*, *ZAP70*, and *VAV* in the HAM/TSP patients (22). Programmed cell death-1, also known as PD-1, the cell surface negative regulator, was significantly enhanced in the cytolytic T cells (CTLs) of individuals suffering from ATL and ACs. Another investigation confirmed the finding specifically in the ATL and HAM/TSP groups, except for the ACs (10, 23, 24).

Unlike T cell activating signaling pathways that were relatively more studied as an approach method for following immune response to viruses and tumors, especially in understanding the pathogenesis of HTLV-1, intracellular inhibitor proteins that prevent T cell antigen receptor (TCR) signaling, remain under-examined (10, 22, 24-26). Because inhibitor proteins have an excellent capacity to be clinical targets for immunotherapy of viral diseases, this research focused on perceiving the expression of intracellular inhibitor proteins of *Cbl-*

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b, ITCH, and *PP2A* in the HAM/TSP group in comparison to the ACs and healthy individuals to reveal a more comprehensive molecular pathogenesis of the HTLV-1.

Materials and methods

Population and Sample Collection

In this study, 30 specimens were gathered from 10/09/2022 to 03/27/2024. The target population included three groups of ten people, according to the study sample size: the group of patients (HAM/TSP), ACs, and healthy individuals from the Tehran and Alborz Blood Transfusion Organizations as well as the Imam-Khomeini and Shariati Hospitals. The inclusion criteria consisted of preliminary neurologist diagnosis for HAM/TSP, only the new cases, and then all the patients and the ACs were rescreened and confirmed via serological (ELISA; Dia. Pro, Italy) and molecular (polymerase chain reaction, PCR) test. At the same time, the groups should not have autoimmune and other viral diseases, immune deficiency disorders, use of any specific drugs, or recent vaccination history. The healthy controls were negative for HTLV-1 and met other inclusion criteria. In contrast, factors contradicting the inclusion criteria led to exclusion from the study.

After obtaining informed consent from the individuals, about 6 to 10 ml of whole blood was collected in EDTA-containing tubes to isolate the plasma and peripheral blood mononuclear cells (PBMCs). Then, these specimens were transported to the Microbiology Department of Alborz University of Medical Sciences under cold chain conditions for further supplementary tests. This study was approved by the Ethics Committee of Golestan University of Medical Sciences, Golestan, Iran (IR.GOUMS.REC.1401.277).

Isolation of PBMCs

First, plasma was separated from whole blood via centrifugation to the ELISA test. The sedimented cells were diluted in two-fold parts of Phosphate-buffered saline (PBS) and gently inverted. The mixed blood was slowly layered onto Ficoll-Paque lymphocyte separation medium (Capricorn Scientific, Germany), in a 50 mL Falcon tube and centrifuged at 800 g for approximately 20 min at 18 °C to facilitate the separation. The collected cell layer was twice washed for hemocytometer cell counting to determine cell concentration.

RNA extraction and cDNA synthesis

Mononuclear cell RNA extraction was performed using a commercial RNJia RNA kit (ROJE, Iran) and then treated with DNase to eliminate DNA contamination. Subsequently, cDNA synthesis was accomplished using the RT-ROSET kit (ROJE, Iran), following the company's operation manual. All products were stored at -20°C for real-time PCR.

Quantitative Real-time polymerase chain reaction (qPCR)

For this study, the sequences of internal control RPLP0, Ribosomal protein lateral stalk subunit P0, Tax, and HBZ primers were derived from the sequence of the previous research to access more consistent and reliable findings (27). In addition, three specific primers of target genes, including Cbl-b, ITCH, and PP2A, were meticulously designed for this study (Table 1).

Gene Runner version 5.0, the Oligo Analyzer Tool, and the GenBank database were utilized for primer design. Also, primer quality control, such as accuracy and sensitivity, was tested via gel electrophoresis and Sanger sequencing. The measurement of mRNA expression level was assessed by QIAGEN's Rotor-Gene Q cycler, based on 40 cycles of three steps: denaturing step at 94 °C for 40 sec, optimum annealing temperature

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of primers (Table 1) in 40 sec, and extension step at 72 °C for 25 sec.

Table 1. The details of the primers designed for this study

Gene name		Sequence (5`-3`)	Product length	TM	T_a Opt
ITCH	Forward	GGGATAGACCAGAACCTCTACCT	156	57	58
	Reverse	GACTACGCTGTAGCTGCCATT		57.2	
PP2A	Forward	ACCGTGAACGCATCACCATTC	157	58.1	61
	Reverse	ACCAAGGCAGTGAGAGGAAGA		58	
Cbl-b	Forward	CAGCACCACCTCCTCCCTTA	134	58.8	58
	Reverse	AGGCATTGGCGGGTCTCT		59.5	
HBZ	Forward	ACGTCGCCCGGAGAAAACA	152	59.6	61.8
	Reverse	CTCCACCTCGCCTTCCAACT		59.4	
Tax	Forward	AGCACCTCCCAACCCTGTCT	118	60.9	59
	Reverse	CAGGTGATGGGGGGGGAAAG		60.4	
RPLP0	Forward	GACAAAGTGGGAGCCAGCGA	164	60.1	61.2
	Reverse	ACACCCTCCAGGAAGCGAGA		60.5	

The optimal annealing temperature (T_a Opt), ITCH (itchy E3 ubiquitin protein ligase), PP2A (protein phosphatase 2, catalytic subunit, alpha isoform), Cbl-b (Casitas B lymphoma –b), Tax (transactivator from the X-gene region), HBZ (HTLV-1 basic leucine zipper factor), LTR (long terminal repeat), RPLP0 (ribosomal protein lateral stalk subunit P0).

Statistical analysis

All data, such as demographics and the results of the Real-time PCR, were entered into Microsoft Excel (2016). The statistical assessments, including the Kolmogorov-Smirnov to normality and descriptive statistics (mean and standard deviation, SD) and nonparametric tests, such as Mann-Whitney U test and Kruskal Wallis test, were performed using GraphPad Prism software version 8.0.2 at a 95% confidence level. A p-value of less than 0.05 was considered statistically significant.

Results

Population data

The patient group included (seven males and three females), the healthy carriers (eight males and two females), and the control group (ten males and zero female) with mean ages of 51.8 ± 6.40, 54.7 ± 8.32, and 56.9 ± 5.2, respectively, and no history of the exclusion criteria.

ITCH mRNA expression

The comparison test among the three groups revealed that HAM/TSP individuals showed a significantly higher mRNA expression level of the ITCH gene compared to the other two groups, with a p-value of 0.0001 and a 95% confidence interval. In addition, the ITCH mRNA expression level increased in the healthy carriers compared to the healthy individuals (Figure 1). The mean±SD expression for the HAM/TSP, ACs, and healthy groups were 0.017±0.008, 0.013±0.011, and 0.002±0.001, respectively. The Dunn's multiple comparisons test displayed the increased *ITCH* mRNA expression in two comparative classes, including the

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healthy group compared to ACs, and the healthy group compared to the HAM/TSP group, which were statistically significant (P-values=0.007 and 0.0002, respectively) (Table 2).

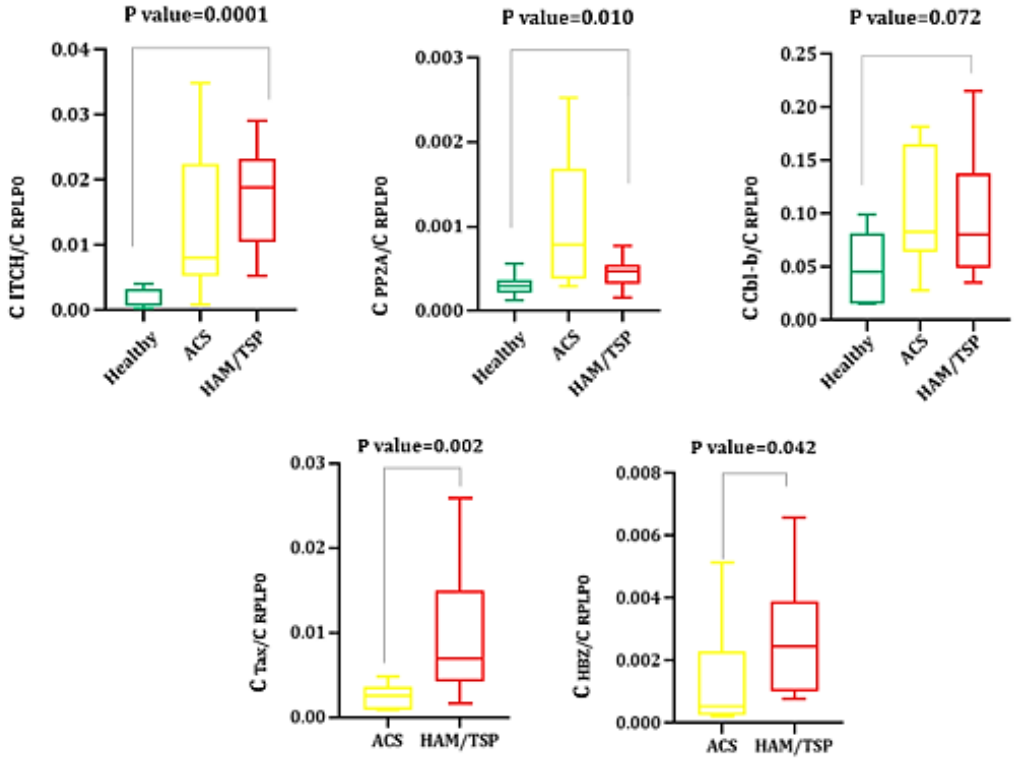


Fig. 1. The cellular and viral gene expression levels; ITCH E3 ligase (A), Protein phosphatase 2 A, PP2A, (B), Cbl-b E3 ligase (C), Tax (D), and HBZ (E) in the three sample groups; healthy individuals, ACs, and HAM/TSP.

Table 2. The results of Dunn's multiple comparisons test for the study of gene expression in healthy individuals, ACs, and HAM/TSP

Dunn's multiple comparisons test

Gene	Comparison group	Significant	Summery	Adjusted P Value
ITCH	Healthy vs. ACs	Yes	**	0.0069
	Healthy vs. HAM/TSP	Yes	***	0.0002
	ACs vs. HAM/TSP	No	ns	0.9656
PP2A	Healthy vs. ACs	Yes	**	0.0078
	Healthy vs. HAM/TSP	No	ns	0.2022
	ACs vs. HAM/TSP	No	ns	0.7125
Cbl-b	Healthy vs. ACs	No	ns	0.1189
	Healthy vs. HAM/TSP	No	ns	0.1703
	ACs vs. HAM/TSP	No	ns	>0.9999

Abbreviations: Non-Significant (ns)

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PP2A mRNA expression

Our result demonstrated a significant accumulation in the *PP2A* expression in the ACs, with a P-value of 0.010. The mean±SD expression level was 0.0004±0.0002 and 0.0003±0.0001 in the HAM/TSP and the healthy groups, respectively, representing the increased level (Fig. 1). Also, the expression of *PP2A* was statistically significant only in the healthy group vs. the ACs (P-value = 0.007) (Table 2).

Cbl-b mRNA expression

The *Cbl-b* mRNA expression level increased in the ACs compared to HAM/TSP and healthy groups, but it was not statistically significant (P-value = 0.072) (Fig. 1). The mean±SD expression level was 0.101±0.053 in the ACs, 0.097±0.058 in the HAM/TSP group, and 0.050±0.032 in the healthy group.

Tax and HBZ mRNA expression in sample groups

The outcomes of the expression of *Tax* and *HBZ* genes are shown in Fig. 1. *Tax* and *HBZ* expression levels were elevated in the HAM/TSP group compared to the ACs (P-values= 0.002 and 0.042, respectively). The mean±SD of *Tax* expression level was 0.010±0.008 in the HAM/TSP group and 0.002±0.001 in the ACs. Moreover, the mean±SD of *HBZ* expression level was 0.003±0.002 in the HAM/TSP group and 0.001±0.002 in the ACs.

Correlations between cellular genes, Tax, and HBZ

The results of Spearman's correlation test in the ACs and HAM/TSP groups are summarized in Tables 3 and 4. The findings indicated a significant negative correlation between the expression levels of *Tax* with *ITCH* and *PP2A* in the ACs, specifically, as the levels of *ITCH* and *PP2A* decreased, the expression of Tax increased (P-values= 0.012 and 0.017). Additionally, a significant positive correlation was observed between the expression levels of *ITCH* and *PP2A* in the ACs (P-value= 0.020). Besides, a significant positive correlation was observed between HBZ and *ITCH* expression levels in the HAM/TSP group (P-value= 0.035).

Table 3. The results of the nonparametric correlation test in the ACs group.

Gene	Correlation & P-value	Tax	HBZ	ITCH	PP2A	Cbl-b
Tax	Correlation P-value	1	0.249241 0.483	-0.7697 0.012	-0.74545 0.017	-0.04242 0.918
HBZ	Correlation P-value	0.249241 0.483	1	0.19453 0.588	-0.04255 0.911	0.395139 0.257
ITCH	Correlation P-value	-0.7697 0.012	0.19453 0.588	1	0.733333 0.020	0.224242 0.537
PP2A	Correlation P-value	-0.74545 0.017	-0.04255 0.911	0.733333 0.020	1	0.466667 0.179
Cbl-b	Correlation P-value	-0.04242 0.918	0.395139 0.257	0.224242 0.257	0.466667 0.179	1

Spearman's correlation Test (ACs)

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Table 4. The results of the nonparametric correlation test in the HAM/TSP group.

Spearman's correlation Test (HAM/TSP)

Gene	Correlation & P-value	Tax	HBZ	ITCH	PP2A	Cbl-b
Tax	Correlation P-value	1	0.030303 0.946	0.454545 0.191	-0.07879 0.838	0.369697 0.296
HBZ	Correlation P-value	0.030303 0.946	1	0.684848 0.035	0.393939 0.263	-0.22424 0.537
ITCH	Correlation P-value	0.454545 0.191	0.684848 0.035	1	0.490909 0.155	-0.2 0.584
PP2A	Correlation P-value	-0.07879 0.838	0.393939 0.263	0.490909 0.155	1	-0.6 0.073
Cbl-b	Correlation P-value	0.369697 0.296	-0.22424 0.537	-0.2 0.584	-0.6 0.073	1

Discussion

The quest to find the missing pieces of the HTLV-1 pathogenesis puzzle continues. A comprehensive look at the various aspects of T lymphocyte activation as an expanding research area leads to a more correct and accurate insight into this issue and more effective future actions. The intracellular inhibitor factors ITCH, PP2A, and Cbl-b were examined in line with this goal. This research revealed a significant increase in the *ITCH* expression level in the HAM/TSP group and the *PP2A* mRNA expression in the ACs; however, there was no statistically significant increase in the *Cbl-b* mRNA expression in the study groups.

Consistent with other studies, our result showed a significant enhancement of both Tax and HBZ in the patients compared to the ACs (28, 29). Tax and HBZ proteins are the two primary arms of the HTLV-1 in its pathogenesis (30), so many cellular signaling pathways like the Jak/STAT (31), CREB, NF-κB (32), and AP1 (the upregulation of c-Fos, c-Jun, and JunB expression) (33) were influenced by Tax. Furthermore, the induction of RNA synthesis of *Foxp3*, *CCR4*, and *TIGIT* genes takes place via HBZ (34).

The E3 ubiquitin ligase ITCH is classified as the Nedd4 family, which regulates the function of various proteins in the cellular signaling network of diverse processes through ubiquitination (10, 35). For example, in TCR signaling pathways, ITCH protein leads to the degradation of JunB and decreases ZAP-70 phosphorylation in complex with Cbl-b, thereby ending TCR signaling. Moreover, ITCH ubiquitylates PLCγ and PKCθ in the anergy state (35).

How Tax interacts with cellular negative regulators remains unclear. Tax protein is a potential trigger of the NF-kB pathway, which participates in inflammatory responses, proliferation, transformation, and survival (36, 37). For instance, Tax can modulate an inhibitor regulator of NF-κB, the ubiquitin-editing enzyme A20, by linking with TAX1BP from the TAX1BP1-ITCH-A20 complex, thereby destroying the connection (38). Furthermore, the Nedd4 family, like the ITCH protein, primarily affects HTLV-1 budding by linking to the PPXY Gag protein motif at the budding site. Therefore, HTLV-1 release was reduced in overexpression of the ITCH protein through increasing Gag ubiquitination (39).

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In this research, the *ITCH* mRNA expression level increased in the HAM/TSP groups compared with the ACs and healthy group, the two-by-two comparison between the healthy group vs. ACs and the healthy group vs. HAM/TSP group. Moreover, the correlation between Tax and ITCH was negative, while ITCH and HBZ showed a positive correlation. Considering the cell function in terminating signaling in response to the virus, the increase in the *ITCH* mRNA expression during HTLV-1 infection seems reasonable. Nevertheless, the ways in which the virus manages to overcome this challenge are still not well understood. However, the overexpression of ITCH is intriguing due to its function in some cancers. On the other hand, the HTLV-1 employs strategies to overcome cellular limitations during chronic inflammation. Hence, HBZ plays a primary function in infected cells to immune escape through the upregulating cellular coinhibitory factors (40). Altogether, our data showed a positive correlation between HBZ and the ITCH coinhibitory molecule.

Another gene examined is *PP2A*, a ubiquitous Ser/Thr phosphatase concerned with T-cell functions, activation, and differentiation (41, 42). Evidence has shown that PP2A is linked to the element of the mTOR signaling pathway to prevent mTORC1 activity, so PP2A is essential for the Treg functions (43). PP2A is a complex of three subunits that are the primary target for the life cycle and pathogenesis of many viruses (42). PP2A snatching occurs through HTLV-1 Tax and the integrase (IN) protein in different ways. Tax can bind to the unknown part of the PP2A subunit in complex with a regulatory subunit IKKγ to abolish the inhibitory role of PP2A on IκB kinase complex and NF-κB cascade activation. Also, HTLV-1 IN, in connection with the B56 region of the PP2A protein, leads to augmented infection and may be a targeting site for integration (42, 44, 45).

Our study significantly increased in the ACs, so the mRNA levels in the patient group and healthy people were closer to each other. A negative correlation between Tax and PP2A in the ACs was also observed. While, this relation was not seen in the HAM/TSP patients, which may depend on the sample size, the individual's characteristics, and the other gene interactions in the signaling network. Our findings also indicated a negative correlation between PP2A and Cbl-b. Overall, the decrease in mRNA expression level of *PP2A* highlighted the Tax function in modulating the PP2A protein, which may play a notable function in HTLV-1 virulence in infection.

The E3 ligase Cbl-b is one of the central checkpoints of immune cells to access balancing between activating and inhibitory outcomes. Also, Cbl-b is an ever-present regulator in most cells, especially T lymphocytes, modulating signaling cascades to limit activation factors (46). In addition, Cbl-b can interact with Wiskott-Aldrich syndrome protein (WASP), PKC-θ, and PKB (Akt), preventing actin reorganization, TCR clustering, and NF-κB activation (12).

A bulk of evidence shows that dysregulation of the PI3K–AKT signaling pathway or the loss of downregulation factors like Cbl-b can increase susceptibility to autoimmunity. Tran et al. 's investigation revealed that inactivation of GSK-3 by Akt can abolish Cbl-b phosphorylation, decreasing Cbl-b protein (47). Moreover, the microarray results of the ATL and HAM/TSP samples in the dataset and network study output suggested that *CBL* expression level was higher in the ATL than in the HAM/TSP individuals.

Our findings also showed a rise in *Cbl-b* expression only in the ACs, even though it was not statistically relevant. In other words, the mean expression of HAM/TSP was nearly similar to that of the healthy group.

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The reduction of *Cbl-b* expression in the HAM/TSP group can be related to the virus's efforts to overcome the function of Cbl-b protein with an unknown mechanism to immune escape, survival, and even spread.

One of the noteworthy strengths of this research is the evaluation of the expression changes of inhibitory genes involved in the regulatory processes of lymphocytes, as well as the correlation analysis between the viral genes *Tax* and *HBZ* in the three groups of ACs, HAM/TSP, and healthy individuals. This approach greatly enhances our understanding of the virus's pathogenesis and may provide insights into potential therapeutic targets. Additionally, another key strength is the use of PBMCs instead of whole blood, which enables focused analysis of immune cells and enhances the accuracy of gene expression studies.

Despite the mentioned strengths, this study has several limitations. First, focusing on gene expression at the mRNA level may not provide a complete picture of the protein and biological activities; simultaneous evaluation of proteins could have offered deeper insights. Second, the small sample size, along with the imbalance in the number of males and females, may limit the statistical power of the analyses and reduce the generalizability of the results. Finally, the absence of the ATL group may hinder the ability to comprehensively assess changes in gene expression and their relationship with the Tax and HBZ proteins. It is suggested that future studies utilize larger sample sizes and more diverse clinical groups for a deeper exploration of gene expression.

The balanced activity of T cells is connected with diverse activators and inhibitory proteins that can be affected by the HTLV-1 as part of its inevitable life cycle. A deeper understanding of intracellular inhibitory molecules in the context of HTLV-1 infections, alongside T lymphocyte activation, is essential for accurately comprehending HTLV-1 pathogenesis. Consequently, our research concentrated on the cellular suppressors PP2A, ITCH, and Cbl-b, revealing that modulating the target genes in HTLV-1-infected individuals led to a significant increase in *PP2A* mRNA levels among healthy carriers. In contrast, the level of expression in the patient and healthy groups was close to the same. Also, *ITCH* mRNA levels showed a significant increase in the patient group and the carriers compared with the healthy group. In addition, the mean expression of *Cbl-b* mRNA was more significant in the ACs than in the HAM/TSP and healthy groups; however, it was not statistically significant. Our findings indicate that HTLV-1 can effectively dysregulate target genes to further its infectious objectives. Furthermore, more research to investigate the interactions of these genes with HTLV-1 infections and their potential applications in diagnosis and treatment could be both insightful and beneficial.

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