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ORIGINAL INVESTIGATION

HTLV-1-infected asymptomatic carriers compared to HAM/TSP patients over-express the apoptosis- and cytotoxicity-related molecules

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Abstract

HTLV-1 infection causes a chronic progressive debilitating neuroinflammatory disease which is called, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). One of the host defense mechanisms against viral infection is apoptosis which may control HTLV-1 infection. Therefore, we aimed to investigate this process and its interaction with viral factors in HTLV-1-infected asymptomatic carriers (ACs) compared to HAM/TSP patients. Fas, FasL, TRAIL, perforin, granzyme A, granzyme B, and granulysin gene expression and serum levels of Fas, FasL, TRAIL, and granulysin in the peripheral blood of 21 sex- and age-matched healthy controls (HCs), ACs, and HAM/TSP patients were evaluated. Also, the level of granulysin secretion in the cell culture supernatant was measured. Finally, the correlation of the expression of these molecules with HTLV-1 proviral load (PVL), Tax, and HBZ mRNA expression was analyzed. ACs compared to HAM/TSP patients significantly over-expressed the Fas, FasL, TRAIL, perforin, and granzyme B molecules. Fas, FasL, TRAIL, and granulysin serum levels were not different among studied groups; whereas, the secretion of granulysin was significantly decreased in ACs and HAM/TSP patients compared to HCs. Also, HAM/TSP patients expressed higher levels of HTLV-1 PVL, Tax, and HBZ mRNA. In addition, in ACs, inverse correlations between the Fas, FasL, TRAIL, perforin, granzyme B, and granulysin levels with HBZ mRNA expression were seen. ACs compared to HAM/TSP patients over-expressed the apoptosis- and cytotoxicity-related molecules. It could be concluded that successful control of the HTLV-1 infection and suppression of HAM/TSP development stem from the strong apoptosis and cytotoxic activity in the peripheral blood of ACs.

Keywords HTLV-1 · Viral neuroinflammation · HAM/TSP · Apoptosis · Cytotoxicity

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Introduction

Human T cell lymphotropic virus type-1 (HTLV-1) was discovered by Robert Gallo et al. [1]. HTLV-1 worldwide infects between 5 and 10 million people [2], and Mashhad in the north-east of Iran is considered as an endemic area [3]. HTLV-1 causes a type of chronic progressive debilitating neuroinflammatory disease which is called, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4, 5]. Whereas the majority of HTLV-1-infected subjects lifelong remain asymptomatic carriers (ACs), about 2–3% of the infected subjects develop HAM/TSP [4, 5]. Currently, the mechanisms of this phenomenon are unknown but several factors have been proposed to be associated with the pathogenesis of this disease. These factors including virological factors such as HTLV-1 proviral load (PVL), Tax and HTLV-1 bZIP factor (HBZ) mRNA expression [6–8], immunological and inflammatory factors, and cytotoxic factors of cytotoxic cells such as T lymphocytes (CTLs) and Natural killer (NK) cells [8–14].

Cytotoxic activities may constitute an important part of the host immune defenses against viral infections such as HTLV-1 [15–17]. NK cells and CTLs play an important role in the control or elimination of virus-infected cells [15–17]. They have very potent direct antiviral activities. These activities require direct cell contact and involve the receptor-mediated pathway or cytotoxic granules exocytosis pathway [15–17]. In the receptor-mediated pathway, the interaction between membrane Fas-ligand (mFasL) and membrane tumor necrosis factor (TNF)-related apoptosis inducing ligand (mTRAIL) on CTLs and NK cells with membrane Fas (mFas) and TRAIL receptors on target cells induces apoptosis [15–17]. Perforin, granzymes, and granulysin are cytolytic molecules store in the cytoplasmic granules of effector cells and after exocytosis are secreted into the intercellular junction between cytotoxic cells and target cells which finally activate the caspase cascade and lead to the death of the target cells [15–17].

However, whether expression of the apoptosis and cytotoxic-related molecules in the peripheral blood mononuclear cells (PBMCs) relates to HTLV-1 PVL, Tax, and HBZ mRNA expression, and HAM/TSP disease development remains unknown. In this study, we evaluated the expression of these molecules in the PBMCs and serums of ACs and HAM/TSP patients, compared to each other and to normal healthy controls (HCs). Also, HTLV-1 PVL, Tax and HBZ mRNA expression in ACs and HAM/TSP patients was evaluated, and their interaction with apoptosis and cytotoxic-related molecules were analyzed. Subsequently, the association between these molecules with HAM/TSP disease development was investigated.

Materials and methods

Patients and controls selection

This study population consists of 63 subjects: 21 HCs, 21 ACs, and 21 HAM/TSP patients. Infection with HTLV-1 was confirmed by Enzyme-linked immunosorbent assay (ELISA) (Diapro, Italy), Western Blotting (WB) and/or polymerase chain reaction (PCR) (Diagnostic Biotechnology HTLV WB 2.4, Genelabs Technologies, Singapore) [18, 19]. The HCs were blood donors who attended a blood transfusion center in Mashhad, Iran. All the HTLV-1 infected individuals and HCs were excluded from the study, if they were positive for blood-borne pathogens and other bacterial and viral infections or had used immunosuppressant in the 1 year before to the start of the study. Also, the HCs of this study were negative for HTLV-1 virus-screening tests. The groups were similar in terms of age and sex, with a predominance of older females' subjects in each group. Based on the clinical and neurological findings, the HTLV-1-infected individuals were classified into two different groups: ACs who did not show any clinical manifestations and HAM/TSP patients who show the clinical manifestations of HAM/TSP disease. This study protocol was approved by the ethics committee of the Mashhad University of Medical Sciences (MUMS), Mashhad, Iran, and written informed consent was obtained from all participants before sample collection. The demographic characteristics of all participants are summarized in Table 1.

Sample collection and serum separation

Blood samples were collected in venepuncture vials containing Ethylenediaminetetraacetic acid (EDTA). PBMCs were prepared immediately after collection, by separating through Ficoll–Hypaque density gradient (Lympholyte®-H from Cedarlane, Burlington, Ontario, Canada) centrifugation. PBMCs were then harvested and washed twice in cold phosphate buffer saline (PBS) (1X). For serum separation, blood samples were clotted at room temperature for 30 min, centrifuged for 15 min at 1000×*g*, and then serum samples were gathered and kept at – 70 °C until analysis.

Cell culture, PBMCs stimulation, and granulysin secretion

Separated PBMCs of all participants were washed twice in cold PBS (1X) and resuspended in complete culture medium RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% FBS and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). Degranulation was induced by adding phorbol

Table 1 Demographic characteristic of study population (Mann–Whitney *U* test)

Demographic and laboratory findings	HAM/TSP patients ($n=21$)	ACs ($n=21$)	HCs ($n=21$)	*p* values
Age (years) [mean ± SD]	47.38 ± 12.67	46.19 ± 10.82	47.38 ± 12.49	0.999
Sex (male/female)	2/19	2/19	2/19	–
HTLV-1 proviral load/10^4 PBMCs	3719 ± 495	1939 ± 595	–	0.02
Tax mRNA qPCR expression level	25.19 ± 5.76	9.8 ± 2.89	–	0.014
HBZ mRNA qPCR expression level	91.14 ± 18.65	14.09 ± 2.48	–	0.001

HAM/TSP HTLV-1-associated myelopathy/tropical spastic paraparesis, *ACs* asymptomatic carriers, *HCs* healthy controls, *SD* standard deviation of mean, *qPCR* quantitative polymerase chain reaction

12-myristate 13-acetate plus ionomycin (PMA/Ionomycin) (Sigma-Aldrich, St. Louis, MO, USA) to final concentrations of 100 ng/ml and 0.5 µg/ml, respectively. The content of each well mixed completely with a pipette after the ingredients were combined, followed by incubation for 6 h in a CO_2 incubator (5% CO_2, 37 °C). The cells were then harvested and centrifuged at 400×*g* for 10 min [20]. Subsequently, the cell culture supernatant was removed, aliquoted, and stored at – 70 °C until analysis.

ELISA for determination the soluble forms of Fas, FasL, TRAIL, and granulysin

The serum-soluble forms of Fas (sFas), sFasL, and sTRAIL were assayed by commercially available ELISA kits (R&D System, Minneapolis, MN, USA); as well as serum and cell culture supernatant levels of granulysin were evaluated by Pre-coated ELISA kit (Granulysin Pre-coated ELISA kit, BioLegend, San Diego, CA, USA) based on the manufacturer's instruction. All standards and controls were measured and calculated according to the manufacturer protocols described previously [12].

Quantitative TaqMan probe real-time PCR

Using the Tripure reagent (Roche Diagnostic GmbH, Mannheim, Germany) and based on the manufacturer's protocol, total RNA was extracted from the PBMCs. The quality of extracted RNA samples was examined by agarose gel electrophoresis and visualizing sharp 18S and 28S ribosomal RNA bands. The concentration and purity of extracted RNA were estimated by UV absorbance at 260/280 nm. Subsequently, about 1–5-µg total RNA from each sample was converted into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), following the manufacturer's protocol.

Sequence-specific primers and fluorogenic probes were designed using the Beacon designer software (Version 7.9) (PREMIER Biosoft; Palo Alto, CA, USA) based on the mRNA sequences taken from the National Center for Biological Information website (http://www.ncbi.nlm.nih.gov) as described previously [12]. Table 2 shows the nucleotide sequence of the gene-specific primers and fluorogenic probes [12].

The quantitative real-time polymerase chain reaction (real-time PCR) was done by TaqMan probe real-time PCR master mix (Takara Bio, Inc., Kyoto, Japan), and Rotor-Gene 6000 machine (QIAGEN, Hilden, Germany) in a total volume of 10 µL as described previously [12]. mRNA relative quantification was done using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Finally, target gene expression for each sample was normalized to the GAPDH expression and data were expressed as differences with respect to mean levels in normal subjects.

Quantification of the HTLV-1 DNA PVL

The extraction of genomic DNA was performed from the PBMCs using the PrimePrep Genomic DNA Isolation Kit (GeNet Bio, Korea) and QIAamp DNA Blood kit (QIAGEN, Hilden, Germany). To assess the HTLV-1 PVL, TaqMan probe real-time PCR was carried out by a commercially available absolute quantification kit (Novin Gene, Tehran, Iran) and Rotor-Gene 6000 instrument (QIAGEN, Hilden, Germany). The HTLV-1 PVL in each sample was determined based on the method of the previous study [21].

Statistical analysis

The statistical analyses were performed by an SPSS software (Version 20) (SPSS Inc., Chicago, IL, USA), and graphs were plotted using GraphPad Prism software (Version 6.0) (San Diego, CA, USA). To determine the use of parametric versus nonparametric tests, the one-sample Kolmogorov–Smirnov test for normality was used. The results of the Kolmogorov–Smirnov test indicated that the data distribution deviates significantly from a normal distribution. The significance of the differences between multiple groups of interests was analyzed using the Kruskal–Wallis test. For comparing the variables between two independent groups,

Table 2 The oligonucleotide sequences of specific primers and probes

Name	Primer sequence	Accession number	Size of amplicon (bp)
Total Fas (tFas)	Forward primer: 5′-GTGTGATGAAGGACATGGCTTAG-3′ Reverse primer: 5′-GGTCACAGTGTTCACATACAGTAG-3′ Probe: FAM-CGGACCCAGAATACCAAGTGCAGATGT-BHQ-I	NM_152871.3	116
Membrane Fas (mFas)	Forward primer: 5′-CCAAGTGCAAAGAGGAAGGATC-3′ Reverse primer: 5′-CTTCCTTTCTCTTCACCCAAACA-3′ Probe: FAM-TGGCAAAAGAAGAAGACAAAGCCACCC-BHQ-I	NM_000043.5	96
Fas-Ligand (FasL)	Forward primer: 5′-GTGGCCCATTTAACAGGCAAG-3′ Reverse primer: 5′-CACTCCAGAAAGCAGGACAATTC-3′ Probe: FAM-AACTCAAGGTCCATGCCTCTGGAATGG-BHQ-I	NM_001302746.1	87
TRAIL	Forward primer: 5′-CCAACGAGCTGAAGCAGATG-3′ Reverse primer: 5′-CGGAGTTGCCACTTGACTTG-3′ Probe: FAM-CAGCAGGGGCTGTTCATACTCTCTTCG-BHQ-I	NM_001190942.1	139
Perforin	Forward primer: 5′-AGTGCCGCTTCTACAGTTTC-3′ Reverse primer: 5′-GGTGCCGTAGTTGGAGATAAG-3′ Probe: FAM-CACCACTTCAACGCCTCCACCCAGC-BHQ-I	NM_001083116.1	140
Granzyme A	Forward primer: 5′-TGCATCTTGGTCCGATACTCTG-3′ Reverse primer: 5′-GGCTTCCAGAATCTCCATTGC-3′ Probe: FAM-AGTCTCTTCCACCTCGGAGGCTTCCAG-BHQ-I	NM_006144.3	167
Granzyme B	Forward primer: 5′-GCTCACTGTTGGGGAAGCTC-3′ Reverse primer: 5′-GGCTGCACAGCTCTGGTC-3′ Probe: FAM -TCAAAGAACAGGAGCCGACCCAGCAGT-BHQ-I	NM_004131.5	191
Granulysin	Forward primer: 5′-ACAGGACCTGTCTGACGATAG-3′ Reverse primer: 5′-GACCTCCCCGTCCTACAC-3′ Probe: FAM-CGGGTCGCAGCATTGGAAACACTTCTC-BHQ-I	NM_001302758.1	106
HBZ	Forward primer: 5′-CTCGACCTGAGCTTTAAACTTACC-3′ Reverse primer: 5′-CATGACACAG GCAAGCATCG-3′ Probe: FAM-CGGACGCAGTTCAGGAGGCACCAC-BHQ-I	DQ273132.1	133
Tax	Forward primer: 5′-ATCCCGTGGAGACTCCTCAA-3′ Reverse primer: 5′-CCTGGGAAGTGGGCCATG-3′ Probe: FAM-CATGCCCAAGACCCGTCGGAGG-BHQ-I	M32165.1	102
GAPDH	Forward primer: 5′-AGCCTCAAGATCATCAGCAATG-3′ Reverse primer: 5′-GAGTCCTTCCACGATACCAAAG-3′ Probe: FAM-CTCCTGCACCACCAACTGCTTAGCACC-BHQ-I	NM_002046.5	96

the Mann–Whitney *U* test was used. Also, for correlations, data were analyzed using the Spearman's rank correlation coefficient test. Data were expressed as mean ± standard deviation (SD) and *p* values less than 0.05 were considered as statistically significant.

Results

Increases in the viral factors in HAM/TSP patients compared to ACs

The HTLV-1 DNA PVL, and Tax and HBZ mRNA expression values of the infected groups are shown in Table 1. The results showed that HAM/TSP patients PVL is significantly higher than the ACs (Table 1). The mRNA expression of Tax and HBZ were investigated in infected individuals: the HBZ mRNA was found to be expressed at a higher level than the Tax mRNA (Table 1). Additionally, the correlation among expression of Tax and HBZ mRNA with PVL and with each other was analyzed (Table 3). The Tax mRNA level showed a significant positive correlation with PVL in all individuals and also in ACs and HAM/TSP patients. Conversely, the results indicate that there was no significant correlation between HBZ mRNA expression and PVL in all infected individuals or in ACs and HAM/TSP patients. The Tax mRNA level showed a significant positive correlation with HBZ mRNA expression in all of the HTLV-1-infected subjects and also in HAM/TSP patients. Additionally, a significant positive correlation between Tax and HBZ mRNA expression with an age in HAM/TSP patients were observed (Table 3).

Table 3 Analysis of the correlation among HTLV-1 DNA proviral load, Tax and HBZ mRNA expression levels in asymptomatic carriers (ACs) and HAM/TSP patients (Spearman's rank correlation coefficient test)

Individuals	mRNA level	Correlation with		
		Proviral load	HBZ mRNA level	Age
All infected individuals ($n=42$)	Tax	$p=0.002$; $r=0.385^{**}$	$p < 0.0001$; $r=0.536^{**}$	$p=0.15$; $r=0.222$
	HBZ	$p=0.217$; $r=0.158$	–	$p=0.11$; $r=0.246$
ACs ($n=21$)	Tax	$p<0.0001$; $r=0.611^{**}$	$p=0.289$; $r=0.243$	$p=0.59$; $r=-0.124$
	HBZ	$p=0.227$; $r=0.276$	–	$p=0.57$; $r=-0.13$
HAM/TSP patients ($n=21$)	Tax	$p=0.018$; $r=0.459^{*}$	$p=0.021$; $r=0.501^{*}$	$p=0.04$; $r=0.452^{*}$
	HBZ	$p=0.325$; $r=0.226$	–	$p=0.03$; $r=0.473^{*}$

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Significant increases in the Fas, FasL, and TRAIL mRNA expression in ACs

First, we analyzed the expression of total Fas (tFas), mFas, FasL, and TRAIL mRNA in PBMCs of HCs, ACs, and HAM/TSP patients. A significant increase in tFas mRNA levels in ACs was found, but there was no significant difference between HCs and HAM/TSP patients (Fig. 1a). This tendency did not reflect in the expression of mRNA encoding for mFas. In fact, mFas levels were not different among HCs, ACs, and HAM/TSP patients (Fig. 1b). The expression of FasL mRNA showed significant increases in ACs, but there was no significant difference between HCs and HAM/TSP patients (Fig. 1c). Next, we analyzed the amount of TRAIL mRNA expression. The results showed that the level of TRAIL mRNA was increased in ACs compared with HAM/TSP patients, but there was no considerable difference between HCs and HAM/TSP patients or ACs with HCs (Fig. 1d).

Increases expression of the perforin and granzyme B in ACs

The mRNA expression of perforin, granzyme A, granzyme B, and granulysin was also investigated. We observed a significant increase in perforin mRNA levels in ACs compared with HAM/TSP patients, but there was no significant difference between HCs and HAM/TSP patients and between ACs and HCs (Fig. 2a). Furthermore, granzyme B mRNA expression showed a significant increase in ACs, but there was no significant difference between HCs and HAM/TSP patients (Fig. 2c). In contrast, the expression of granzyme A mRNA showed that the levels of this molecule were not significantly different among the studied groups (Fig. 2b). Similarly, in granulysin mRNA expression no significant difference was observed among the studied groups (Fig. 2d).

Decreases granulysin secretion in ACs and HAM/TSP patients

We checked the serum level of soluble forms of Fas, FasL, and TRAIL proteins which act as decoy receptors and apoptosis inhibitors [22]. Among the studied groups, no differences were observed in the mean serum levels of sFas (Fig. 3a), sFasL (Fig. 3b), and sTRAIL (Fig. 3c) proteins.

Serum granulysin levels in ACs, HAM/TSP patients and HCs were similar and showed no significant difference (Fig. 3d). Also, the results showed that granulysin secretion levels form stimulated PBMCs of both ACs and HAM/TSP patients were significantly decreased compared to HCs, but the secretion of granulysin was similar between ACs and HAM/TSP patients (Fig. 3e).

Analysis of the correlation of the immunological factors with the virological factors

The correlation between apoptosis and cytotoxic-related molecules with virological factors was examined in all HTLV-1-infected subjects, including both ACs and HAM/TSP patients. The tFas, FasL, TRAIL, perforin, granzyme B, and granulysin mRNA expression were negatively correlated with the HBZ mRNA expression, but no significant correlations among apoptosis and cytotoxic-related molecules with the amount of PVL and Tax mRNA expression were found (Table 4).

Discussion

HAM/TSP is a chronic debilitating viral neuroinflammatory disease that typically has a slow progression. The PVL of HTLV-1 in infected individuals strongly correlates with the risk of HAM/TSP development. In HAM/TSP patients, the PVL of HTLV-1 is higher than that in ACs and the high HTLV-1 PVL commonly coincides with HAM/TSP disease

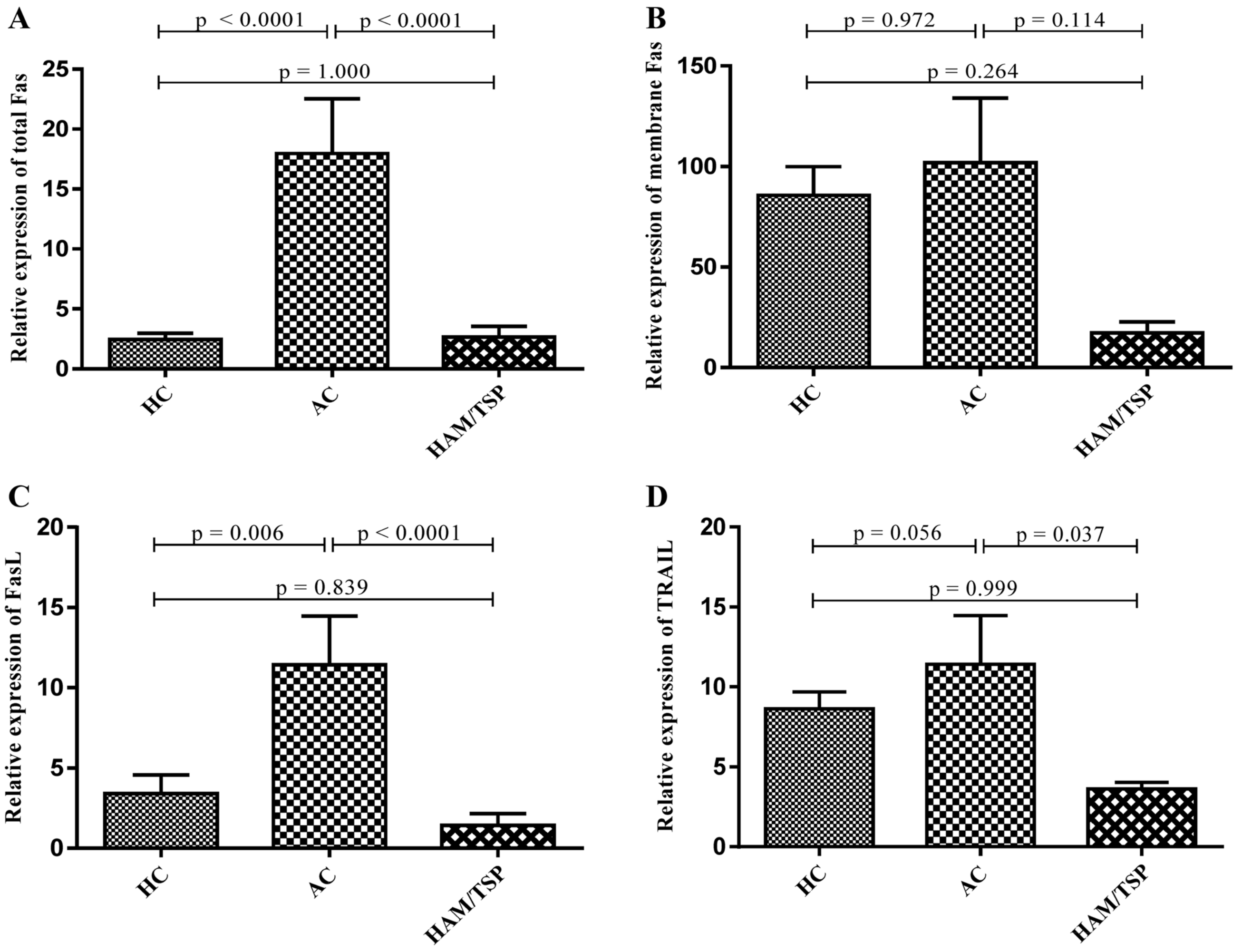

Fig. 1 Profile of apoptotic mediator's gene expression. mRNA expression of total Fas (tFas) (**a**), membrane-bound Fas (mFas) (**b**), Fas-Ligand (FasL) (**c**) and TRAIL (**d**) gene expression mRNA levels in (PBMCs) from 21 healthy controls (HCs), asymptomatic carriers (ACs) and (HAM/TSP) patients determined by TaqMan probe quantitative Real-Time PCR (TaqMan probe qPCR). The copy number values, calculated as the difference between the values of the target genes and the house-keeping gene (GAPDH) (Kruskal–Wallis test)

development and also clinical symptoms worsening [7, 9–11]. Although high PVL of HTLV-1 is a risk factor for disease development, it is insufficient to determine the outcome of infection. Two main HTLV-1 factors by which the virus develops the diseases are Tax and HBZ proteins that cause cellular immortalization, oncogenesis, and inflammation and have been proposed to be implicated in the HAM/TSP disease pathogenesis [8, 23, 24]. These factors play a crucial role in regulating viral transcription, modulating apoptosis pathways in their host cells, and finally HTLV-1 pathogenicity [8, 23, 24]. In the present study, there are significant differences in HAM/TSP patients and ACs in the amount of HTLV-1 PVL, Tax, and HBZ mRNA expression. Also, there was a significant direct correlation between HTLV-1 PVL and Tax mRNA expression in both ACs and HAM/TSP patients, while HBZ mRNA expression showed no significant correlation with the PVL in ACs and HAM/TSP patients. Also, we showed that there is a direct significant correlation between the Tax and HBZ mRNA expression in HAM/TSP patients and in all HTLV-1-infected individuals. In addition, we found that the Tax and HBZ mRNA expression positively correlates with the age in the HAM/TSP patients. These results imply that Tax and HBZ expression plays a crucial role in the HAM/TSP pathogenesis and this risk increases with increase in age.

An apoptosis failure in infected CD4$^+$ T cells could lead to an increase in the infected cells proliferation or migration to the central nervous system (CNS), resulting in damage to oligodendrocytes and neurons [25–27]. Since the presence of apoptosis-related molecules in peripheral blood may reflect apoptotic events takes place in the CNS [25–27], the determination of such molecules may be useful in evaluating the apoptosis rate, as well as disease activity and severity. mFas, mFasL, and mTRAIL are extrinsic pathway

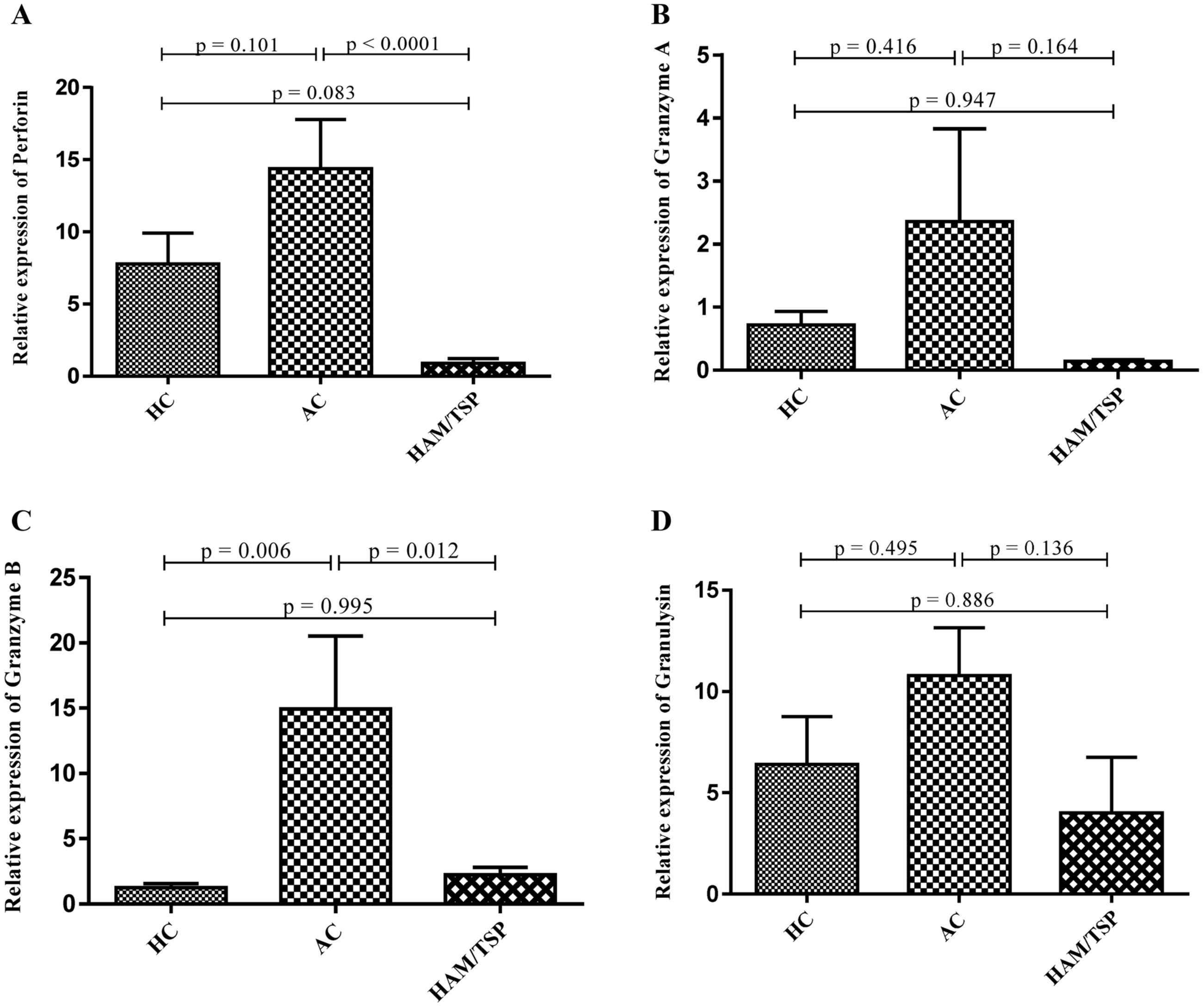

Fig. 2 Profile of cytotoxic mediator's gene expression. mRNA expression of perforin (**a**), granzyme A (**b**), granzyme B (**c**) and granulysin (**d**) in freshly PBMCs from 21 healthy controls (HCs), asymptomatic carriers (ACs) and HAM/TSP patients determined by TaqMan probe quantitative Real-Time PCR (TaqMan probe qPCR). The copy number values, calculated as the difference between the values of the target genes and the house-keeping gene (GAPDH) (Kruskal–Wallis test)

member of the death ligands which expresses on cytotoxic lymphocytes and known to induce apoptosis during viral infections [28–31]. sFas, sFasL, and sTRAIL arising from their mRNA transcripts which act as decoy receptors and an inhibitor of membrane Fas-, FasL-, and TRAIL-inducing apoptosis [22]. In the present study, high levels of Fas, FasL, and TRAIL mRNA expression in ACs compared to the HAM/TSP patients were observed, but no variation among the protein levels of these molecules which act as decoy receptors and apoptosis inhibitors was found. Previous studies have showed that sFas levels increased in the serums of HAM/TSP patients, compared to HCs [32]. sFasL levels are higher in either the serum [33] or cerebrospinal fluid (CSF) of HAM/TSP patients [34]. The serum levels of both sFas and sFasL were significantly higher in HTLV-1 carriers than in the controls. Increased sFasL levels in the lungs of ACs were associated with an accumulation of CD4$^+$ T cells and resistance to apoptosis in HTLV-1-infected T cells. Therefore, the over-production of sFasL could contribute to T-lymphocytic alveolitis via the down-regulation of Fas/FasL-mediated apoptosis [35]. FasL mRNA is predominantly expressed in the CD8$^+$ T lymphocytes of HAM/TSP patients, and it is preferentially expressed in HAM/TSP patients with increased PVL and a longer disease duration [36]. Umehara et al. [37] showed that Fas was preferentially expressed on infiltrating T cells in the active–chronic lesions of HAM/TSP patients, while FasL expression was up-regulated on various cells, but mainly on microglia/

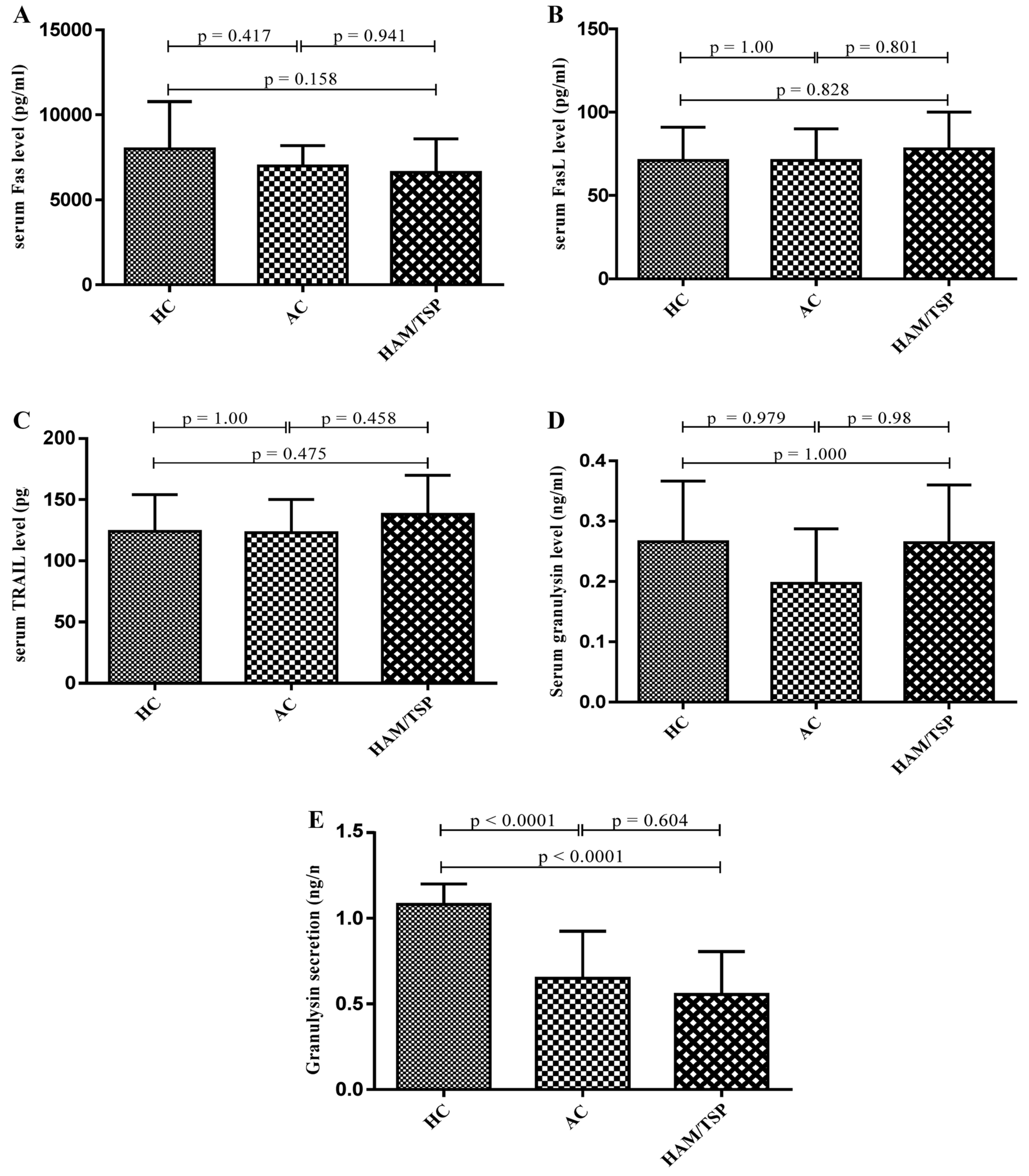

Fig. 3 Serum levels of soluble Fas (sFas), sFasL, sTRAIL and granulysin in serum and granulysin protein secretion in the cell culture supernatant. Serum levels of sFas, sFasL, sTRAIL, granulysin in serum and granulysin protein secretion in cell culture supernatant after PBMC stimulation with PMA/Ionomycin in HAM/TSP patients, asymptomatic carriers (ACs) and healthy controls (HCs) were determined by ELISA (mean ± SD). The levels of sFas (**a**), sFasL (**b**), sTRAIL (**c**) and granulysin (**d**) showed no difference between HAM/TSP patients, ACs and HCs but, the level of granulysin (**e**) protein secretion were significantly decreased in HAM/TSP patients and ACs compared to HCs (Kruskal–Wallis test)

Table 4 Analysis of the correlation of the apoptosis and cytotoxic-related molecules with HBZ mRNA expression (Spearman's rank correlation coefficient test)

mRNA expression level	Correlation with HBZ mRNA level
tFas	$p=0.005$; $r=-0.347$
FasL	$p < 0.0001$; $r=-0.513$
TRAIL	$p=0.004$; $r=-0.354$
Perforin	$p=0.001$; $r=-0.424$
Granzyme B	$p < 0.0001$; $r=-0.589$
Granulysin	$p=0.004$; $r=-0.362$

macrophages in active–chronic lesions. Furthermore, the constitutive expression of both Fas and FasL mRNA in the spinal cords of HAM/TSP patients suggested that the Fas/FasL system is involved in the inflammatory process in the CNS of HAM/TSP patients [37]. Although Fas/FasL is not the cause of disease development, it is more likely to play a role in HAM/TSP pathogenesis in the CNS through the Fas-dependent death of CNS resident cells, especially microglia that express FasL [37]. These studies collectively suggest that Fas/FasL system may be actively involved in the pathogenesis of HAM/TSP. Therefore, it seems that the Fas, FasL, and TRAIL may have protective anti-inflammatory and antiviral properties in ACs and may act as pathways against viral replication and disease progression.

We also assessed the mRNA expression of perforin, granzyme A, granzyme B, and granulysin. The results showed that perforin and granzyme B mRNA levels are significantly higher in ACs. However, no significant differences were observed among the groups in terms of granzyme A and granulysin mRNA levels. Furthermore, we observed that the secretion of granulysin following the stimulation of PBMCs significantly decreased in all HTLV-1-infected subjects including ACs and HAM/TSP patients. This abnormal degranulation and secretion of granulysin in ACs and HAM/TSP patients might be due to the dysregulation of several mechanisms that control cytolytic granules turn-over due to HTLV-1 infection. Our study confirmed the results of previous studies which reported that there is a correlation between the expressions of granule-associated cytolytic effector molecules and the control of HTLV-1 infection [9, 11]. These studies provide the evidence that the increased expression of perforin, granzymes, and granulysin in $CD8^+$ T cells is associated with the inhibition of HTLV-1 replication in ACs that have lower PVL, compared to HAM/TSP patients who have higher PVL.

In this study, the correlation between apoptosis and cytotoxic-related molecules expression with virological factors such as HTLV-1 PVL, Tax, and HBZ mRNA expression was also examined in the both of ACs and HAM/TSP patients. The tFas, FasL, TRAIL, perforin, granzyme B, and granulysin mRNA expression were negatively correlated with the HBZ mRNA expression, but no significant correlations among apoptosis and cytotoxic-related molecules with the amount of HTLV-1 PVL and Tax mRNA expression were found. A load of HBZ mRNA is a valid predictor of progression of HAM/TSP disease [23, 24]. Expression of HBZ mRNA plays an important role in inducing inflammatory complications of HAM/TSP patients [23, 24]. Our results may support the hypothesis that ACs by over-expression of apoptosis and cytotoxic-related molecules controls HBZ mRNA expression and subsequently suppresses HAM/TSP disease progression.

In summary, our results demonstrated that there are significant increases in Fas, FasL, TRAIL, perforin, and granzyme B gene expression in ACs and a significant increase in PVL, Tax and HBZ mRNA expression in HAM/TSP patients. In addition, our results demonstrated that HBZ mRNA expression was suppressed by the apoptosis pathways in ACs. In conclusion, we showed that the Tax and HBZ expression plays a crucial role in HAM/TSP pathogenesis. As a consequence, apoptosis pathways are successfully controlled HBZ mRNA expression, and finally disease progression in ACs. Therefore, apoptosis pathways could act as inhibitors of viral replication, infected-$CD4^+$ T lymphocyte proliferation and migration to the CNS, pro-inflammatory immune responses induction, and subsequently HAM/TSP disease progression. Additionally, viral factors, including PVL, Tax, and HBZ, could be used in the future as predictive factors for evaluating the risk of disease.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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