Involvement of the small ncRNAome in fresh peripheral blood mononuclear cells of human T-cell leukemia virus type I- associated myelopathy/tropical spastic paraparesis patients and viral carriers

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a slowly progressive spinal cord disorder with no effective treatment. There is much of interest in developing potential biomarkers for predicting the pathogenesis of HAM/TSP disorder. This study used Illumina massive parallel sequencing (MPS) technology to assess the cellular global noncoding RNAome expression profile in HAM/TSP patients (n = 10), asymptomatic HTLV-1 infected

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carriers (ASP, n = 8), and a second group of healthy controls (n = 5). Using various bioinformatics tools, the sRNA MPS reads were aligned, annotated, and profiled. There were 251 known and 50 potential novel sRNAs among the 402 detected sRNAs in the HAM and ASP groups versus the HC group. Sixty-eight known sRNAs were found to be significantly different between the ASP and HAM groups. In HAM vs ASP subjects, 88 mature miRNAs were downregulated. Three of these miRs (hsa-miR-185-5p, 32-5p, and 192-5p) have the potential to be used as biomarkers for predicting the pathogenesis of HAM/TSP. The top seven deregulated miRS target genes were linked to a variety of biological processes and molecular functions. Relevant reactome pathways to our findings provide a rich source of data and an opportunity to further understand sRNA regulation and function in HTLV-1 pathophysiology.

Keywords: small RNA; HTLV-1; HAM/ TSP; massive parallel sequencing.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is a human retrovirus that infects 5-10 million people worldwide [1; 2]. Most HTLV-I infected individuals remain healthy lifelong asymptomatic carriers (ACs), while 1–5% progress to an aggressive mature T-cell malignancy known as adult T-cell leukemia (ATL) or a chronic neurological disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2] depending on unknown cofactors that may vary by geographical location. HAM/TSP is a chronic inflammatory disease of the central nervous system that causes leg weakness or paralysis, lower back pain, and urinary symptoms [3]. The clinical course of HAM/TSP is most similar to the primary-progressive form of multiple sclerosis in that neurologic functioning steadily deteriorates with no distinct relapses [4]. Although it is not entirely clear why some people develop the disease while others do not, some hosts and virus-

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related factors appear to make some people more likely to develop the disease [5]. In order to gain a better understanding of HTLV-1 pathogenesis, it is necessary to investigate regulatory processes.

Small RNAs (sRNAs) play critical roles in the regulation of many targets, most notably cellular genes, through posttranscriptional gene silencing and chromatin-dependent gene silencing in both the cytoplasm and the nucleus [6]. These molecules are a type of short, noncoding RNA that has been classified into structural and regulatory ncRNAs [7]. Structural ncRNAs include transfer RNA (tRNA) and ribosomal RNA, as well as other small but stable noncoding RNAs, including small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), small cytoplasmic RNA (scRNA), ribonuclease P (RNase P), mitochondrial RNA processing RNA, signal recognition particle RNA and telomerase RNA [8; 9]. Regulatory ncRNAs include microRNAs (miRNAs/miRs), P-element induced wimpy testis-interacting RNAs (piRNAs) and long ncRNAs [10]. miRNAs are perhaps the single most extensively investigated small ncRNAs. miRNAs are typically 18–25 nucleotide long, single-stranded RNAs that have emerged as principal posttranscriptional regulators of gene expression and have a vital role in several cellular processes, including cell proliferation [11], differentiation [12], and apoptosis [13]. Many miRNAs have been found to be abnormally deregulated in a variety of cancers and diseases, including multiple sclerosis [14; 15]. Several studies have demonstrated the importance of cellular miRNAs in virus-host interactions [16; 17; 18; 19]. It has been shown, for example, that upregulation of host miR-9041, miR-9850, miR-29a, and miR-373 during white spot syndrome virus, respiratory syncytial virus, or hepatitis C virus infections enhances virus replication by inhibiting the Jak/STAT signaling pathway. The latest evidence on the expression profiles of miRNAs in HTLV-1/ATLL cell lines and primary ATL cells has identified numerous dysregulated microRNAs. For instance,

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Pichler et al. [20] investigated the expression of miRNAs in HTLV-1-transformed cell lines using miRNA array technology and found overexpression of miR-21, miR-24, miR-146a, and miR-155 and downregulation of miR-223. In a similar study with primary ATLL cells, Bellon et al. [21] reported an upregulation of miR-155 and miR-142-3p and downregulation of miR-181a, miR-132, and miR-125a. Microarray-based studies revealed the roles of HTLV-1 miRNA profiles in asymptomatic carriers and ATL cell lines in controlling T-cell proliferation [21; 22; 23]. Most of the aforementioned studies focused on the expression of specific or diverse miRNAs in HTLV-I-infected ATLL cell lines or patient samples; however, we know very little about how human sRNAs are regulated in HAM/TSP-associated HTLV-1 patients and how they function in disease pathogenesis.

The goal of this study was to identify small RNA (sRNA) signatures in PBMC samples that identify HTLV-1-infected patients with HAM/TSP. To that end, we used Illumina massively parallel sequencing (MPS) technology to characterize sRNA expression in PBMCs of eight HTLV-1-infected asymptomatic subjects with known polyclonal rearrangement of gamma T-cell receptors (designated as ASP), ten HAM/TSP patients (designated HAM), and five healthy controls (designated HC). Real-time PCR was used to confirm the deregulation of the selected miRNAs.

Materials and methods

Enrollment of patients and preparation of samples

Ten HTLV-1-positive patients with HAM/TSP were recruited from the HTLV-1 outpatient clinic at the "Emilio Ribas" Institute of Infectious Diseases. At the time of blood donation, the eight ASP subjects were discovered to be HTLV-1 carriers. Five HCs were chosen from a group of apparently healthy volunteers recruited from the laboratory staff who were free of HTLV-1 infection. As a result, the total sample size was ten HAM/TSP cases, eight ASP

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cases, and five HCs. Viral infection was detected using the Murex HTLV I + II (Abbott/Murex, Wiesbaden, Germany) and Vironostika HTLV-I/II (BioMérieux bv, Boxtel, Netherlands) HTLV enzyme immunoassays, and infection was confirmed using HTLV BLOT 2.4. (HTLV blot 2.4, Genelabs Diagnostics, Science Park, Singapore). The clinical status of HAM/TSP was determined using the WHO criteria for HTLV-1-associated diseases [24]. Each participant provided written informed consent. The samples were collected following approval by the institutional health research ethics authority (CAPPesq; approval no. 56/2012), and all individual participants included in the study signed a written informed consent form. Ficoll-Hypaque (Amersham, Upsala, Sweden) density centrifugation was used to isolate PBMCs, which were then stored as previously described [25]. The characteristics of the subjects in the three groups are presented in **Table 1.**

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Table 1. Demographic and clinical characteristics of ASP, HAM/TSP, and HCs participants subjected to small RNA analysis

Sample	Sex	Age, years	Proviral load (copies/1000 PBMCs)	Total input reads	Total removed reads (%)	Mapped reads
131ASP*	Female	52	17	4.029.633	766,396 (19.02%)	3.263.237
146ASP	Female	59	208	12.757.216	2,880,332 (22.58%)	9.876.884
151ASP	Female	72	1	11.790.906	1,814,813 (15.39%)	9.976.093
152ASP	Female	34	60	6.445.747	885,609 (13.74%)	5.560.138
167ASP	Female	58	32	5.942.283	940,399 (15.83%)	5.001.884
172ASP	Female	31	102	4.441.212	731,908 (16.48%)	3.708.304
182ASP	Female	80	22	7.312.922	1,407,403 (19.25%)	5.905.519
188ASP	Female	49	8	9.207.927	2,312,871 (25.12%)	6.895.056
011HAM#	Female	58	52	2.535.464	740,243 (29.20%)	1.795.221
016HAM	Male	65	614	4.550.381	936,158 (20.57%)	3.614.223
079HAM	Male	63	333	17.430.869	4,975,503 (28.54%)	12.455.366
125HAM	Female	40	210	807.596	475,723 (58.91%)	331.873
142HAM	Female	68	214	7.303.448	1,510,819 (20.69%)	5.792.629
018HAM	Male	62	578	10.371.022	2,874,890 (27.72%)	7.496.132
022HAM	Female	73	100	3.449.215	989,459 (28.69%)	2.459.756
077HAM	Female	60	129	9.513.936	2,144,231 (22.54%)	7.369.705
080HAM	Female	48	95	3.623.765	915,412 (25.26%)	2.708.353
082HAM	Female	50	256	5.107.387	1,324,293 (25.93%)	3.783.094
002HC¥	Female	38	NA	3.707.168	432,109 (11.66%)	3.275.059
003HC	Male	53	NA	8.917.856	910,883 (10.21%)	8.006.973
004HC	Female	37	NA	5.078.632	847,486 (16.69%)	4.231.146
005HC	Male	32	NA	2.969.362	474,233 (15.97%)	2.495.129
006HC	Female	32	NA	6.665.538	868,276 (13.03%)	5.797.262

* ASP, HTLV-1 infected asymptomatic subjects with known polyclonal rearrangement of the gamma T cell receptors
# HAM, ¥ HAM/TSP patients

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Genomic DNA and RNA extraction and HTLV-1 proviral load determination

A QIAamp blood kit was used to extract genomic DNA from PBMCs (QIAGEN, Tokyo, Japan). Total RNA and sRNA were extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and TRIzol (Life Technologies, USA) procedures, as recommended by the manufacturer. The concentrations of DNA and RNA, including sRNA, were measured on a Qubit 2.0 fluorometer using the Qubit® DNA or RNA HS Assay Kit (Thermo Fisher Scientific). The extracted DNA was used as a template to amplify a 97-bp fragment from the HTLV-1 tax region with previously published primers [26] and protocols [27]. The Applied Biosystems 7500 real-time PCR system was used for amplification and analysis.

Library preparation and sRNA sequencing

sRNA libraries were prepared for each sample in each group using the Small RNA v1.5 sample preparation kit according to the manufacturer's instructions (Illumina, San Diego, CA) and the previous protocol [28]. In brief, 5 µl of purified total RNAs was ligated with a 1 µl RNA 3′ Adapter, followed by a 5′ RNA adapter (Illumina, San Diego, CA). The sequencing primer was also included in the 5′ adapter. Following RT-PCR amplification, the products were separated using polyacrylamide gel electrophoresis (PAGE) (6% Novex Tris-borate-EDTA [TBE] PAGE; Invitrogen). Following gel electrophoresis, sRNA bands of 145-150 bp were excised and purified. Finally, each four libraries were pooled, and up to 8-10 pM of the pooled libraries were loaded and sequenced on the Illumina MiSeq platform using a 36-base single-end protocol, as directed by the manufacturer.

sRNA data analysis and interpretation

Base-calling, demultiplexing and trimmed FASTQ files were generated with MiSeq reporter. Only high-quality reads with a Sanger scale score of 30 or higher were considered for further

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analysis. Strand NGS v3.1 was used to align the sequencing reads against the hg19 whole genome build. The latter software package was also used to analyze novel molecule discoveries and interpretations. Using a model previously described by Langenberger *et al.* [29] novel sRNAs were discovered and classified by the decision tree method with threefold validation accuracy. The quantile normalization algorithm was used to compute the distributions of sRNA data in each clinical condition, with a baseline transformation set to the median of all samples. Only sRNA sequences with a minimum read coverage of > 5 were considered novel or known sRNAs and were included in subsequent analyses. sRNAs with a fold change ≥ 2 were considered differentially expressed. All the sRNA raw data generated by MPS have been deposited into the Zenodo repository.

qRT-PCR validation of miRNAs

Two miRNAs, hsa-miR-29b-3p (MI0000105) and hsa-let-7f-5p (MI0000067), that showed a strong dysregulation tendency among samples were chosen and validated using qRT-PCR. These two downregulated miRNAs were validated in PBMC samples from 30 HAM patients (10 from this study and 20 from a previous study) and 18 ASC subjects (8 from this study and 10 prospectively collected samples). Using the TaqManTM MicroRNA Reverse transcription kit, 5 µl of each enriched miRNA was converted into cDNA (REF 4366596, Thermo Fisher Scientific, Inc., Rockford, IL, USA). The TaqMan™ Universal master mix II (REF: 4440040, Thermo Fisher Scientific, Inc.) was used for qRT-PCR on a 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). Each reaction was tested in triplicate, including the no-template negative control. The relative level of miRNA expression was normalized to the internal control miR16 (MI0000070). The PCR conditions were as follows: UNG activation at 50 °C for 2 minutes, denaturation, and hot start Taq activation at 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. For the

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evaluation of the data, we used the dCT (CT) method (−dCT values = −[CT of target miRNA −CT of internal control miRNA])[30].

Functional annotation and pathway analysis of miRNA target genes

The miRWalk 3.0 algorithm predicted target genes from differentially expressed miRNAs in the HAM and ASP groups versus the HC group. We scanned these targets for Gene Ontology (GO) enrichment terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification after obtaining a list of putative and experimentally validated targets relative to each miRNA. The significant deregulated miRNA target genes were interrogated for significant well-curated signaling pathways obtained from Reactome using MirWalk v.3 (Mar/2020 update) sorted by *p* value ranking 0.05 using Benjamini-Hochberg multiple testing corrections to control the false discovery rate (FDR).

Constructing a regulatory network between miRNAs and their targets

The posttranscriptional gene regulatory network is defined as a directed and bipartite network in which miRNA-target gene interacting pairs' expressions are reversely correlated. The miRWalk network algorithm was used to analyze the network for the interaction of the putative target miRNA-messenger RNA (mRNA) [31].

Statistical analysis

Moderated t-test or one-way analysis of variance (ANOVA) followed by FDR was used for statistical analysis with a P(Corr) cutoff <0.05 to indicate statistical significance of sRNA regulation, unless otherwise indicated. Hierarchical clustering was performed using Strand NGS version 3.1 (Strand Life Science). The $2^{-\Delta\Delta}$ CT method was used to determine relative expression levels from the qRT-PCR results, and paired t-tests were used to determine

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whether the differences were statistically significant using GraphPad Software v.8 (GraphPad Software, San Diego, CA, USA).

Results

Characteristics of the Patient

Table 1 shows the characteristics of the subjects in each group. The ASP group consisted of eight female participants, with a median age of 55 years (range, 31-80 years). Seventy percent of the HAM patients were female, with a median age of 61 years and a range of 40–73 years. The healthy controls included three females and two males ranging in age from 32 to 53 years. HTLV-1 proviral load levels in the ASP group ranged from one copy to 208 copies/10^3 PBMCs and from 95 to 614 copies/10^3 PBMCs in the HAM group (two-tailed P value = 0.14).

Description of sRNA-sequencing data from the entire genome from all groups

Small RNA sequencing yielded 153,959,485 raw reads with high diversity and quantity for all groups. After removing low-quality reads that failed the vendor's quality control, 121,799,036 high-quality reads from combined genes were obtained and mapped to the genome (**Table 1**). The Illumina MPS produced 402 sRNA molecules, 330 of which were derived from known sRNA and 72 from novel sRNA, respectively. There were 187 miRNAs, 26 scRNAs, 8 scRNA pseudogenes, 46 snoRNAs, 10 snRNAs, and 53 tRNAs among the 330 known sRNAs (**Table S1**). Eight of the 72 novel genes were novel miRNAs, two were scRNAs, ten were snoRNAs, three were snRNAs, four were tRNAs, and 45 were unknown (**Table S2**). Our analysis revealed 212 differentially expressed mature known miRNAs in all three groups when we considered the -3p and -5p mature forms of miRNA. (**Table S3)**.

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Differential analysis of the known and novel sRNA expression among groups

Of the 330 filtered sequences of known sRNAs on expression in the raw data, 251 (211 upregulated and 40 downregulated sRNAs) were found to differ significantly in their levels between the ASP and HAM vs HC groups (ANOVA p-value 0.05). (**Table S4**). There were 137 miRNAs, 19 scRNAs, 4 scRNA pseudogenes, 42 snoRNAs, 9 snRNAs, 21 tRNAs, and 40 tRNA pseudogenes identified. The top five most expressed sRNAs were hsa-mir-150, 28, 23b, 342, and SNORD3B-1, while the top five most downregulated sRNAs were hsa-mir-183, 451a, 144, 486, and 19a. **Figure 1** depicts the unsupervised hierarchical cluster analysis of the 251 differentially expressed known sRNAs among the three groups. The differential pattern of expression revealed three major sRNA clusters in which HAM and ASP were separated from healthy controls. The first cluster consists of 20 sRNA genes with a higher expression pattern in the HC group, particularly hsa-miR-144, hsa-miR-451a, and hsa-miR-183. This finding indicates that a change in sRNA expression pattern occurred after HTLV-1 infection. The findings support the clinical significance of the PBMC sRNA profile in the diagnosis of HTLV-1 infection. When ASP and HAM patients were compared to the HC group, a differential expression pattern with 83 significantly deregulated entities was observed in the second cluster. Finally, the third cluster consists of 148 sRNAs with expression profiles similar to the second cluster, demonstrating upregulation of all HTLV-1-infected samples except 125HAM, 011HAM, and 022HAM compared to the HC group. The 251 sRNA expression profiles of ASP individuals were mostly separated from those of HAM patients in the 3-D principal component analysis (PCA) plot, while those of HC samples were also distantly separated from the other groups (**Supplementary Figure 1**).

During the comparison of HAM and ASP vs the HC group, 50 of the 72 novel genes reached FDR significant values (p (Corr) cutoff 0.05), with 35 being upregulated and 15 being

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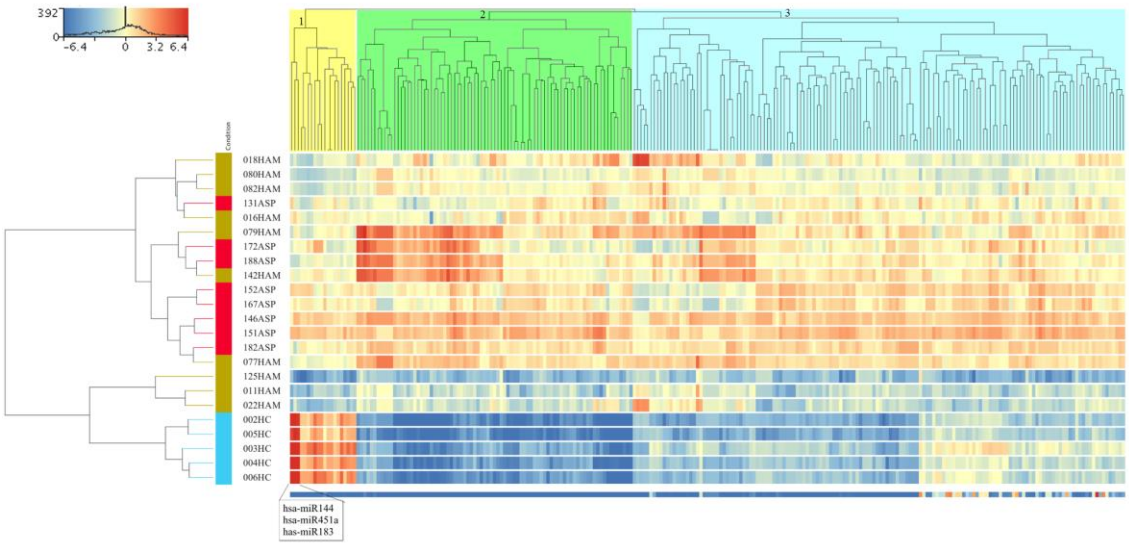
downregulated (**Table S5**). Twenty-eight of the 50 significantly deregulated novel sRNAs (56%) were previously unknown. NEWGENE176 (chr5, 79946806- 79946866) was the most significantly upregulated gene, followed by NEWGENE625 (snoRNA) (chr5, 93905130-93905240). The most downregulated novel genes were NEWGENE407 (chr12, 138513819-138513879) and NEWGENE500 (chr15, 99077146- 99077181). We performed the 3-D PCA plot based on the expression of the 72 novel sRNAs, which showed a similar clustering as seen with the sRNA data (**Supplementary Figure 2**). The HC samples clustered in a separate group, whereas the ASP and HAM showed a partial overlap.



1-Differences in known small RNA (sRNA, n= 251) expression levels among HTLV–I-associated myelopathy (HAM, gold) patients, HTLV–I-infected asymptomatic carriers with the polyclonal T-cell receptor γ gene (ASP, red), and healthy controls (HC, blue) represented by a heatmap. The sample clustering tree is displayed to the left, and the sRNA clustering tree is above, forming 3 clusters selected by yellow, green, and blue colors. The color scale at the top indicates the relative expression levels of sRNA across all samples. Red indicates that the expression levels are higher than the mean, whereas blue indicates that the expression levels are lower than the mean. Each column represents one known sRNA, and each row represents one sample.

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Next, we wanted to determine whether there were any significant differences in known and novel sRNA expression between the HAM and ASP subjects. Sixty-eight known sRNAs (64 upregulated and 4 downregulated sRNAs) were found to have significantly different levels in both groups, with ten of them having a fold change greater than 5. (**Table S6**). The analysis of novel sRNA found no difference between the two groups.

Differential analysis of mature miRNA expression among groups

Of the 212 detected mature miRNAs, 157 were significantly deregulated among the three groups (FDR < 0.05, FC ≥ 2). Thirty-eight of these miRs had low expression levels, while 119 had high expression levels in HTLV-1-infected groups regardless of clinical status (**Table S7**). For clarity, we chose mature miRNAs with FDR ≤ 0.05 and FC of 10 (n =87) to generate the unsupervised hierarchical clustering shown in the heatmap diagram (**Figure 2**). There were two distinct sample groups (sample groups A and B). Sample group A contains two well-separated clusters (A1 and A2), with A1 containing samples from ASP subjects. Sample cluster A2 contained seven HAM patient samples (70% HAM samples) and four ASP subject samples (50% ASP samples). In sample group B, two tightly separated clusters (B1 and B2) were also visible, with three samples from HAM patients (022 HAM, 011HAM, and 125HAM) in cluster B1 and all samples from HC in cluster B2. MiRNA expression was found to be divided into four groups (1-4). With a few exceptions, almost all miRNAs in clusters 2-4 showed lower expression in sample groups B 1 and 2. Finally, in sample cluster B2, which included all HC samples, miRNA cluster 1 was significantly upregulated. To identify prognostic markers for the early detection of HAM/TSP in HTLV-1-infected carriers, the analysis was repeated with a stringent statistical parameter of FDR (p-value of 10^{-4} and FC of 5). The study found three miRNAs, hsa-miR-185-5p, 32-5p, and 192-5p, that could classify the majority of samples based on the patients' clinical status (**Figure 3A**). In

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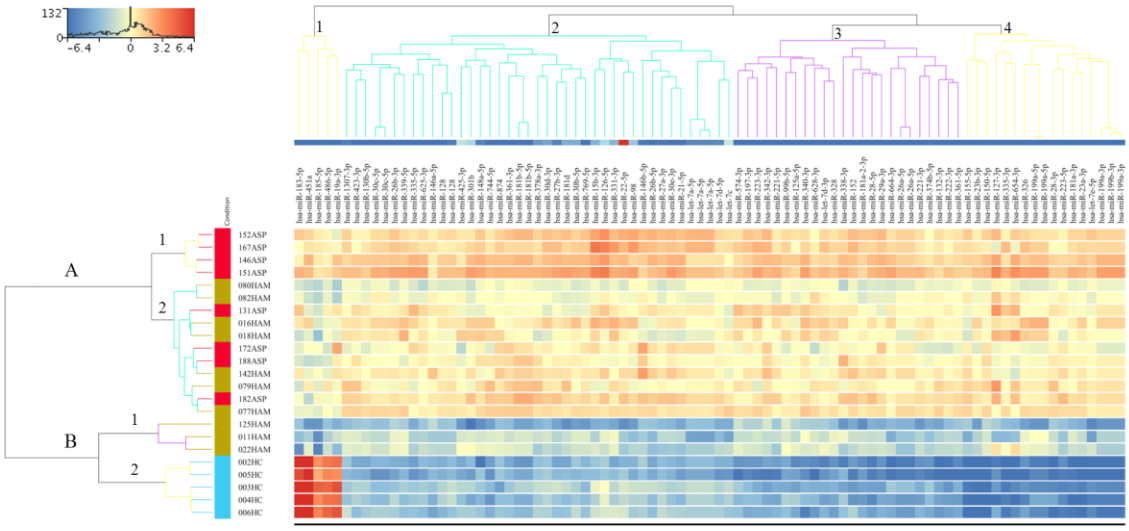
comparison to the HC participants, the expression of these miRNAs was either upregulated or downregulated in the HAM and ASP groups. When compared to the APS or HC groups, most HAM patients had differentially downregulated expression of the three miRs. The ASP samples 188 and 131 were placed in the same cluster as the HAM samples. Both patients' most recent clinical evaluation in 2021 revealed that they were free of HAM symptoms. **Figure 3A** shows that sample 142HAM clustered in the ASP group, but the reason for this unexpected result is unknown. PCA was used to determine how closely related the three groups were in terms of miRNA expression. The findings show that HC patients form a distinct cluster, while HAM patients have only minor overlap with the ASP group (**Figure 3B)**. This visual representation sheds light on the differences in miRNA expression between HAM/TSP patients and ASP groups.



2-Differences in mature microRNA (miRNA, n= 86) expression levels (FDR >0.05 and FC ≥ 10) among HTLV–I-associated myelopathy (HAM, gold) patients, HTLV–I-infected asymptomatic carriers with the polyclonal T-cell receptor γ gene (ASP, red), and healthy controls (HC, blue) represented by a heatmap. The sample clustering tree is displayed to the left, and the miRNA clustering tree is shown above. The color scale at the top indicates the relative expression levels of miRNA across all samples. Red indicates that the expression levels are higher than the mean, whereas blue indicates that the expression levels are lower than the mean. Each column represents one mature miRNA, and each row represents one sample.

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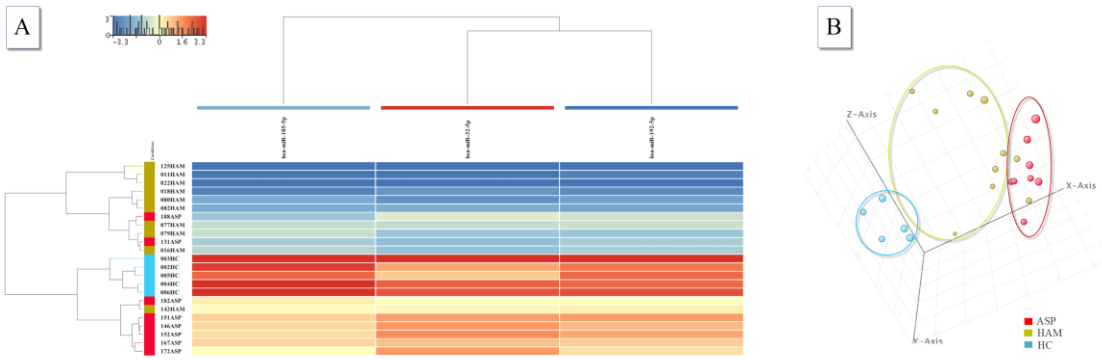

3-Differential miRNA expression analysis between HAM and controls. (A) Differences in mature microRNA (miRNA, n= 3) expression levels (FDR > 10^{-4} and FC = 5) among HTLV–I-associated myelopathy (HAM, gold) patients, HTLV–I-infected asymptomatic carriers with the polyclonal T-cell receptor γ gene (ASP, red), and healthy controls (HC, blue) represented by a heatmap. The sample clustering tree is displayed to the left and the miRNA clustering tree is shown above. The color scale at the top indicates the relative expression levels of miRNA across all samples. Red indicates that the expression levels are higher than the mean, whereas blue indicates that the expression levels are lower than the mean. Each column represents one mature miRNA and each row represents one sample. (B) Principal component analysis (PCA) plot showing distances among the three groups based on miRNA expression.

We then compared the differentially regulated nonredundant miRNAs that we found in our three different miRNAome groups. Of the 74, 15, and 16 miRNAs found to be downregulated 2-fold in ASP vs HAM, ASP vs HC, and HAM vs HC, respectively, 11 overlapped between the data sets (**Figure 4**). A total of 120 and 73 miRNAs were 2-fold upregulated in ASP vs HC and HAM vs HC, respectively. All of the 73 miRNAs in HAM vs HC overlapped between datasets, indicating that in PMBCs, approximately 34% of mature miRNAs studied were upregulated by 2-fold in HAM/TSP.

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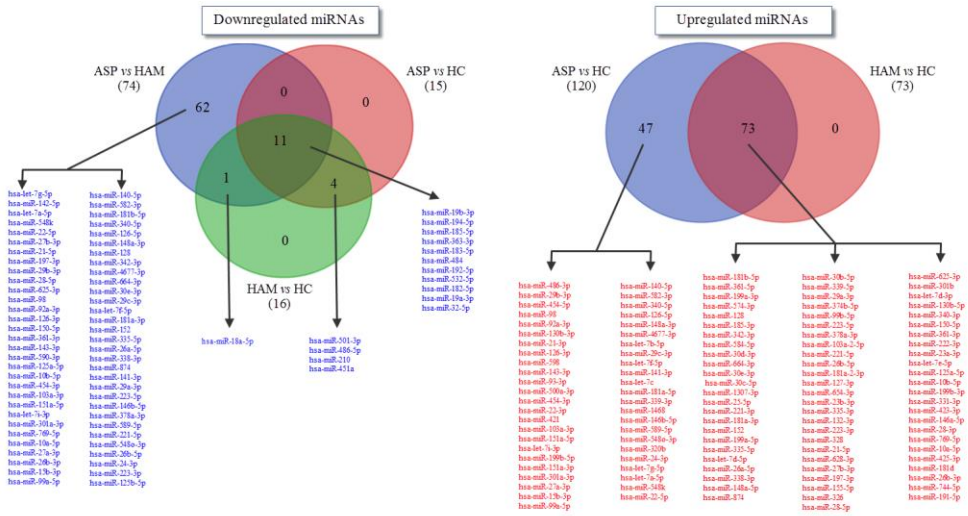

4- A Venn diagram showing miRNAs whose expression was either upregulated or downregulated (>2-fold) in peripheral blood mononuclear cells of patients with HTLV–I-associated myelopathy (HAM, green), HTLV–I infected asymptomatic carriers with the polyclonal T-cell receptor γ gene (ASP, blue), and healthy controls (HC, red).

Individual analysis between the HAM and ASP groups revealed that 89 mature miRNAs, including those with the same accession but different Entrez ID/Ensembl ID and vice and versa (designated here as redundant miRNAs), exhibited lower expression levels in HAM patients than in ASP subjects (**Table S8**). The top seven most deregulated mature miRNAs were hsa-miR-29b-3p, hsa-let-7f-5p, hsa-miR-32-5p, hsa-miR-192-5p, hsa-miR-141-3p, hsa-miR-342-3p, and hsa-miR-140-5p. These miRs were chosen for further analysis.

Validation by qRT-PCR assay

The two most deregulated miRNAs between HAM/ASP patients in our sequencing data (hsa-miR-29b-3p and hsa-let-7f-5p) were selected for validation using qRT-PCR in 10 samples from this study and 20 other independent HAM/TSP PBMC samples. The quantification of hsa-miR-29b-3p revealed a significant correlation with the sequencing data (**Figure 5**). The data also showed that hsa-let-7f-5p was downregulated in HAM patients compared to ASCs, but this difference was not statistically significant.

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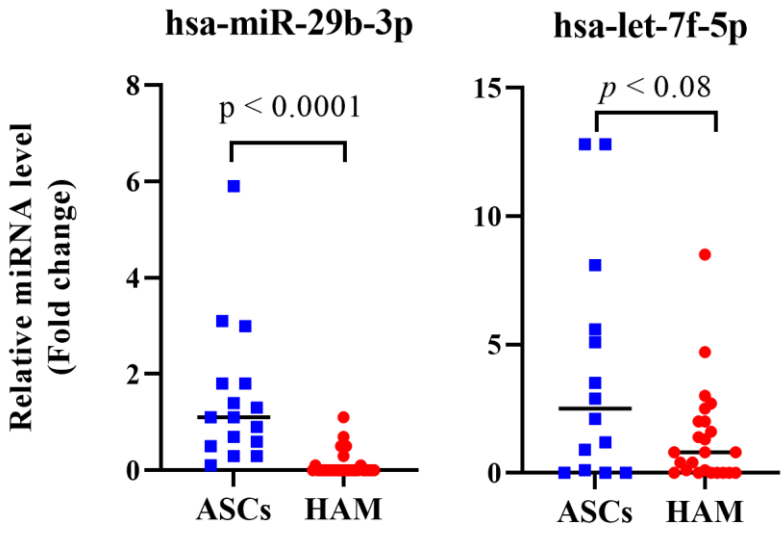

5-Comparison of miRNA (hsa-miR-29b-3p and hsa-let-7f-5p) expression levels (2^ΔΔCt) in peripheral blood mononuclear cells of HTLV-1-associated HAM patients versus HTLV–I infected asymptomatic carriers with polyclonal T cell receptor γ gene. The expression levels of selected miRNAs were analyzed by qRT-PCR.

Target genes, KEGG pathway, and GO enrichment analysis

Data from the top seven nonredundant downregulated miRNAs between the HAM and ASP groups were used to identify target genes of candidate miRNAs. The miRWalk v.3 predicted hybridization patterns between miRNAs and the 3′-UTR of mRNA targets and revealed 61 predicted genes, as shown in **Table S9**. The results showed that hsa-miR-342-3p had two target genes (INO80D and SYNPO2L), whereas hsa-miR-192-5p had three (RB1, DBT, and ZBTB34), hsa-miR-141-3p had five (YAP1, PRELID2, IGF1R, GATA6, and ATXN7L1), and hsa-miR-140-5p had five (GALNT16, KLK10, VEZF1, RALA, and TSC22D2) hsa-miR-32-5p had eight target genes (CPEB2, SPOCK2, FAM126B, CPEB4, CNEP1R1, DUS2, TENT4B, and MIA3), hsa-miR-29b-3p had seventeen target genes (including ENPP2, NREP, and HMGCR), and hsa-let-7f-5p had twenty-three miRNAs (including MDM4, NF200, BZW (**Figure 6**). The 61 target genes for the selected miRNAs were then used in a pathway enrichment analysis to search for molecular pathways related to

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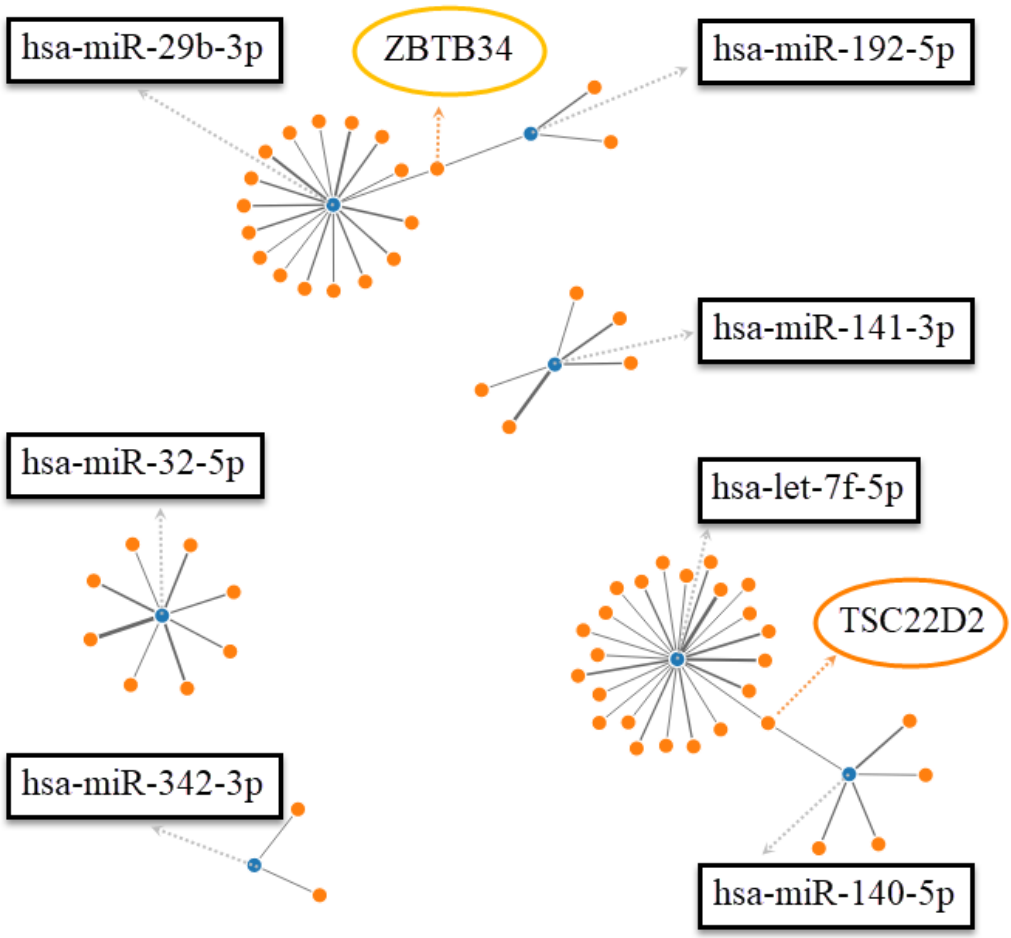
HAM/TSP. As shown in **Table S10**, we found two significantly enriched REACTOME pathways (FDR0.05) (R-HSA-74160 and R-HSA-2559583), two KEGG pathways (hsa05200 and hsa05206), and ten enriched GO terms. Seven and three GO terms, respectively, were associated with biological processes and molecular functions.



6-Interaction network between the top seven most deregulated mature miRNAs and their target genes generated by MirWalk v3. Blue circles represent miRNAs, while orange circles represent mRNAs. The more connections between miRNAs and genes, the more links there are within the network.

Discussion

Although there is no single best treatment for HAM/TSP, early diagnosis allows for the use of treatments that can halt or reverse the disease. Current diagnostic criteria for HAM/TSP

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include recognizing a pattern of clinical symptoms and signs, detecting antibodies against HTLV-I infection, confirming the presence of HTLV-I antibodies in the CSF, and ruling out other conditions with similar symptoms [32]. Techniques based on quantitative PCR assays of the provirus were developed, but they were unable to diagnose HAM/TSP with acceptable sensitivity due to the disease's wide range of symptoms [33]. As a result, there is a clear need to look for biomarkers that not only allow for more sensitive and specific diagnosis and stratification of HAM/TSP but also have the potential to explain the underlying pathogenic mechanism and identify relevant pathways and potential targets for therapy development. It is difficult to find a single biomarker that can fulfill all of these roles; in fact, each of these biomarkers may require distinct characteristics. Small RNAs, including miRNAs, have been identified as possible biomarkers for a variety of human diseases [34]. Circulating miRNAs have also been extensively studied for their potential to serve as an early warning diagnostic or prognostic biosignature in a variety of infection-causing pathogens [35; 36]. Evidence suggests that host miRNAs can bind to a wide range of RNA viruses, affecting replication, limiting antiviral therapy responses, inhibiting apoptosis, and enhancing cellular growth. [37; 38].

MiRNAs play an important role in the interaction of HTLV-1 with host cells. For example, Pichler and colleagues discovered that HTLV-1-infected cell lines have higher levels of miR-21, miR-24, miR-146a, and miR-155 and lower levels of miR-223 than normal CD4+ T cells and uninfected T-cell lines [20]. The viral Tax protein [39], Rex [40], and HBZ [41] proteins have been shown to interfere with the biogenesis of mature miRNAs by promoting the degradation of Drosha [39]. The Rex and HBZ proteins have also been shown to inhibit the production of mature miRNAs by inhibiting Dicer activity [40] and expression [41],

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respectively. Although miRNAs are being studied as prognostic molecular markers in ATLL, their potential as influential biomarkers for HAM/TSP diagnosis and prognosis has been neglected [20; 21; 22; 42; 43]. We observed unique small RNA signatures that can distinguish patients with HAM/TSP from HTLV-1 asymptomatic carriers and healthy participants using PBMC samples and Illumina small RNA high-throughput sequencing technology for the first time. Our study revealed 402 sRNA molecules, with 330 and 72 sequences derived from known and novel sRNAs, respectively. Furthermore, 88 mature miRNAs were found to be less expressed in HAM/TSP patients than in ASP subjects. The top downregulated miRNAs in the PBMCs of patients with HAM/TSP were hsa-miR-29b-3p, hsa-let-7f-5p, hsa-miR-32-5p, hsa-miR-192-5p, hsa-miR-141-3p, hsa-miR-342-3p, and hsa-miR-140-5p. Although those genes showed a significant dysregulation between patients and controls, we were unable to address the correlation between their expression and the severity of HAM/TSP due to our small sample size. In light of our findings, more research is needed to determine whether there is a link between the expression of the aforementioned miRNAs and the severity of HAM/TSP and, if so, how strong that link is.

Deregulation of miR-29, including the family member miR-29b-3p, contributes to tumor pathogenesis, autoimmune disorders, and fibrostic diseases [44; 45]. According to the literature, significant suppression of miR-29b was reported in patients with multiple sclerosis (MS) [46], an autoimmune disease whose certain forms clinically and closely resemble HAM/TSP, implying that miR-29 expression might be deregulated in both diseases and may contribute to their pathogenesis. In contrast, hsa-let-7f-5p and 25 other miRNAs were found to be significantly downregulated in the PBMCs of patients with all subtypes of MS, including primary progressive, secondary progressive, and relapsing-remitting disease [47].

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Low expression of let-7 family miRNAs in HIV-1 infection has been linked to an increase in IL-10 production by CD4+ T cells, providing the virus with an important survival advantage by manipulating the host immune response [48]. A recent study by Kumar et al [49] demonstrated that Let-7f modulates NF-kB activity during *Mycobacterium tuberculosis* infection by targeting A20 (also known as TNFAIP3), a zinc finger protein. It is worth noting that ectopic expression of the HTLV-1 Tax protein, which is significantly overexpressed in HAM/TSP, has been shown to disrupt the A20 ubiquitin-editing complex [50]. Consistent with our sequencing results, we found a close to significant (p=0.08) decrease in miR-let-7f in HAM/TSP patients in our validation experiments. Small sample sizes for RT-qPCR validation and technological errors may partially account for variations in miR-let-7f quantification. Deregulation of hsa-miR-32-5p effectively limited the accumulation of the primate foamy virus type 1 (PFV-1) retrovirus in human cells [51]. To the best of our knowledge, the biological role of hsa-miR-32-5p in HAM/TSP has not been investigated. Regarding miR-192-5p, a plethora of evidence supports the potential role of transforming growth factor-β (TGF-β) signaling in increasing the expression of miR-192-5p in human, mouse, rat tubular epithelial, mouse mesangial, and rat proximal tubular epithelial cells [52; 53; 54; 55]. Because TGF- β signaling is downregulated in HAM/TSP patients, suppressing this pathway may result in low expression of miR-192-5p in these patients [56].

To identify pathways relevant to HAM/TSP, we searched the MirWalk database for target genes for the top seven downregulated miRNAs and found a relevant Reactome pathway, "R-HSA-2559583 Cellular Senescence." This pathway was observed by searching for the target genes for hsa-miR-192-5p, hsa-let-7f-5p, and hsa-miR-29b-3p. As a result, cellular senescence, an irreversible state of cell cycle arrest, is a potentially related molecular pathway

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to HAM/TSP. Merling and colleagues [57] previously proposed that the tax protein induces cellular senescence via a mechanism other than IKK/NF-κB activation. An additional study revealed that tax hyperactivation of NF-κB signaling could result in cellular senescence, contrary to the common perception that NF-B activity increases the proliferation of HTLV-1-infected cells [58]. The data presented above suggest that HTLV-1-infected cells must counteract this senescence phenotype in order to proliferate. Previous studies have reported the involvement of these miRNAs in regulating cellular senescence [59; 60]. For instance, evidence from a previous study indicated that an increase in IL-17 directly represses miR-192 and that IL-17 activation induces the transcription factors NF-κB [61] and MAPK [62]. NF-κB is an inducer of proinflammatory cytokines, thereby promoting senescence. In our study, we showed downregulation of hsa-miR-192-5p in HAM/TSP patients. Although speculative, a possible scenario is that the downregulation of hsa-miR-192-5p would result in the upregulation of the NF-κB pathway, as NF-κB is a direct target for miR-192, which would then promote senescence. The exact mechanism driving hsa-miR-192-5p, hsa-let-7f-5p, and hsa-miR-29b-3-mediated selective modulation of inflammation in HAM/TSP warrants additional investigation.

Currently, no specific therapies have been developed to prevent or reduce the risk of developing HAM/TSP complications in HTLV-1 asymptomatic carriers, and it has proven difficult to predict who will develop the disease [63]. Predictive biomarkers of disease activity have previously been identified in PBMCs using the proviral load level, which is positively correlated with the progression of motor impairment and associated with a long-term prognosis [64]. Increased cell counts, anti-HTLV-1 antibody titers, protein levels, C-X-C motif chemokine 10, and neopterin in cerebrospinal fluid all significantly correlated with

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the rate of HAM/TSP progression [65; 66; 67; 68]. Despite the small sample size, our findings indicated that the differentially expressed miRNAs could be potential biomarkers in HAM/TSP. Based on our findings, more research is needed to develop an integrative approach to infer candidate HAM/TSP miRNA biomarkers described here by linking paired miRNA and gene expression data, as well as significantly reliable miRNA-mRNA target data, that can be applied in a large sample size of HAM/TSP patients.

The novelty of our study was its main strength. To the best of our knowledge, this was the first study to examine the role of small RNAs, specifically miRNAs, in HTLV-1 infected patients with HAM/TSP. Furthermore, we found two significant REACTOMES shared pathways among the top seven significantly downregulated miRNAs, namely "R-HSA-74160 Gene expression" and "R-HSA-2559583 Cellular Senescence."

Although our study is the first to identify several PBMC miRNAs associated with HAM/TSP disorder in HTLV-1 infected patients, it has some limitations that must be addressed. The main limitation of our study is the small number of patients and controls. This limitation may reduce the significance of the presented findings regarding the role of the presented miRNAs in HAM/TSP patients and their prognostic value as potential markers in the disorder. Large prospective studies are needed in the future to confirm and investigate the true role of these miRNAs in HAM/TSP disease. Another limitation of the study is that we only looked at miRNAs. The information on other small RNAs discovered in this study will be used in future research aimed at developing and improving the analytical methods.

Conclusion

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In conclusion, our findings revealed a variety of miRNAs with the potential to predict HAM/TSP patients. These miRNAs and their targets are involved in a known pathogenesis pathway for HAM/TSP. Our findings pave the way for the identification of novel molecular miRNA signatures capable of predicting not only HAM/TSP diagnosis but also carriers who will develop the disease. To the best of our knowledge, this is the first study to investigate the differential expression of miRNAs in PBMC from HAM/TSP patients, with the goal of determining their clinical utility as early prediction markers.

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