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ABSTRACT
Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropic spastic paraparesis
(HAM/TSP) is an insidiously progressive spinal cord disease for which there is no effective
treatment. There is great interest in developing potential biomarkers to predict the pathogenesis
of HAM/TSP disease. In this study, Illumina Massive Parallel Sequencing (MPS) technology was
used to investigate the cellular global noncoding RNAome expression profile in HAM/TSP patients
($n = 10$), asymptomatic HTLV-1-infected carriers (ASP, $n = 8$), and a second group of healthy
controls ($n = 5$). Various bioinformatics tools were used to align, annotate, and profile the sRNA-
MPS reads. Among the 402 sRNAs detected, 251 were known and 50 were potentially novel sRNAs
in the HAM and ASP groups compared with the HC group. Sixty-eight known sRNAs were
significantly different between the ASP and HAM groups. Eighty-eight mature miRNAs were
downregulated in subjects from HAM compared with ASP. 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 seven most deregulated miRs target genes have been associated with a variety of
biological processes and molecular functions. The reactome pathways relevant to our findings
provide a rich source of data and offer the opportunity to better understand sRNA regulation and
function in HTLV-1 pathophysiology. To the best of our knowledge, this study is the first to
demonstrate evaluates sRNAs in HTLV-1 patients with HAM/TSP.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is
a retrovirus that infects between 5 and 10 million peo-
ple worldwide [1,2]. The vast majority of people
infected with HTLV-1 spend their entire lives as
healthy, asymptomatic carriers, but between 1% and
5% develop an aggressive, mature T-cell cancer called
adult T-cell leukaemia (ATL) or a chronic neurologic
disease called HTLV-1-associated myelopathy/tropical
spastic paraparesis (HAM/TSP) [2]. HAM/TSP is
a chronic inflammation of the central nervous system
manifested by weakness or paralysis of the legs, lower
back pain, and problems with urination [3]. Clinically,
HAM/TSP most closely resembles the primary progres-
sive form of multiple sclerosis, in which neurologic
function steadily deteriorates without obvious relapses
[4]. Although it is not known why some people develop

the disease and others do not, a number of host- and
virus-related factors appear to increase the likelihood
that some people will develop the disease [5]. The
regulatory mechanisms of HTLV-1 remain to be
explored to better understand the pathophysiology.

Small RNAs (sRNAs) play important regulatory
roles for a variety of targets, especially cell genes,
through post-transcriptional gene silencing and chro-
matin-dependent gene silencing both in the cyto-
plasm and in the cell nucleus [6]. These molecules
are a type of short, non-coding RNA that has been
divided into structural and regulatory ncRNAs [7].
Ribosomal RNA, transfer RNA (tRNA), small nuclear
RNAs (snRNAs), small cytoplasmic RNAs (scRNAs),
small nucleolar RNAs (snoRNAs), signal recognition
particle RNA, ribonuclease P (RNase P), mitochon-
drial RNA processing RNA, and telomerase RNA are

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examples of small but stable noncoding structural RNAs [8,9]. MicroRNAs (miRNAs/miRs), long ncRNAs, and P-element induced weak testis-interacting RNAs (piRNAs) are examples of regulatory ncRNAs [10]. The most thoroughly studied small ncRNAs are undoubtedly miRNAs. These are single-stranded RNAs with a typical length of 18 to 25 nucleotides that are essential for a number of biological processes such as cell proliferation [11], differentiation [12], and apoptosis [13]. Abnormally deregulated levels of many miRNAs have been found in numerous cancers and diseases, such as multiple sclerosis [14,15]. Several studies have demonstrated the importance of cellular miRNAs in virus-host interactions [16–19]. For example, upregulation of miR-9041, miR-9850, miR-29a, and miR-373 has been shown to promote viral replication during infection with white spot syndrome virus, respiratory syncytial virus, or hepatitis C virus by inhibiting the Jak/STAT pathway. Recent findings on the expression profiles of miRNAs in HTLV-1/ATLL cell lines and primary ATL cells have identified numerous dysregulated microRNAs. For example, Pichler et al. [20] used miRNA array technology to investigate the expression of miRNAs in HTLV-1-transformed cell lines. They observed overexpression of miR-21, miR-24, miR-146a, and miR-155 and downregulation of miR-223. In a similar study using primary ATLL cells, Bellon et al. [21] reported upregulation of miR-155 and miR-142-3p and downregulation of miR-181a, miR-132, and miR-125a. Microarray-based studies reveal the role of HTLV-1 miRNA profiles in asymptomatic carriers and ATLL cell lines in controlling T-cell proliferation [21–23]. While most of the above studies focused on the expression of specific or different miRNAs in HTLV-I-infected ATLL cell lines or patient samples, little is known about how human sRNAs are regulated in HAM/TSP-associated HTLV-1 patients and how they contribute to the pathogenesis of the disease.

The aim of the present study was to identify sRNA signatures in peripheral blood mononuclear cells (PBMCs) samples that would allow identification of HTLV-1-infected HAM/TSP patients. To this end, we used the deep-sequencing capabilities of the Illumina MiSeq platform to analyse the expression of sRNA in PBMCs from eight HTLV-1-infected asymptomatic carriers with known polyclonal rearrangement of gamma T-cell receptors (designated ASP), ten HAM/TSP patients (designated HAM), and five healthy controls (designated HC). Deregulation of the selected miRNAs was confirmed by quantitative real-time PCR (qRT-PCR). To the best of our knowledge, this study is the first to demonstrate evaluates sRNAs in HTLV-1 patients with HAM/TSP.

Materials and methods

Enrollment of patients and preparation of samples

Ten HAM/TSP patients who tested positive for the virus were recruited from the HTLV-1 outpatient clinic of the Emilio Ribas Institute for Infectious Diseases. At the time of blood donation, the eight individuals of the ASP group were found to be HTLV-1 carriers. Five HCs were selected from a group of apparently healthy volunteers recruited by laboratory personnel who did not have HTLV-1 infection. Thus, the total sample included ten HAM/TSP cases, eight ASP cases, and five HCs. The enzyme immunoassays Murex HTLV I + II (Abbott/Murex, Wiesbaden, Germany) and Vironostika HTLV-I/II (BioMérieux bv, Boxtel, The Netherlands) were used to diagnose HTLV-1 infection and HTLV BLOT 2.4 (Genelabs Diagnostics, Science Park, Singapore) to confirm infection. The world health organization (WHO) criteria for HTLV-1-associated disease were used to determine the clinical status of HAM/TSP patients [24]. Whole blood samples were collected and processed after approval by the Ethics Committee of the Institute of Health (CAPPesq, approval number 56/2012). All subjects who participated in the study also signed a written informed consent form. PBMCs were isolated by Ficoll-Hypaque density centrifugation (Amersham, Upsala, Sweden) and subsequently stored as previously described [25].

Extraction of genomic DNA/RNA and quantification of proviral load (PvL)

A QIAamp blood kit was used to extract genomic DNA from PBMCs (QIAGEN, Tokyo, Japan). The MiRNeasy Mini Kit (Qiagen, Hilden, Germany) and TRIzol (Life Technologies, USA) recommended protocols were used to extract total RNA and sRNA, respectively. 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). Using the extracted DNA as a template, previously published primers [26] and protocols [27] were utilized to amplify a 97-bp *tax* fragment. Amplification, acquisition of data, and analysis were performed using the Applied Biosystems 7500 real-time PCR system.

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 previous protocol [28]. Libraries from all four samples were combined, and 8–10 pM of the pool was added and sequenced on the Illumina MiSeq platform according to the manufacturer's protocol.

sRNA data analysis and interpretation

MiSeq Reporter was used for preliminary analysis of base calling, demultiplexing, and trimming of FASTQ files. Only reads with a Q score of 30 or higher were considered for the following analysis. Strand NGS v3.1 was used for alignment of sequencing reads against the hg19 whole genome build. The same software package was used to analyse the expression of known and unknown sRNAs and active miRNAs, and the results were visualized using a volcano plot. The decision tree technique based on the model of Langenberger *et al.* [29] was used to identify new sRNAs. To find out how the sRNA data were distributed in each group, the quantile normalization procedure was used. The median of all samples was used as the base transformation. sRNAs were considered for differential expression analysis if they had a minimum read coverage of > 5 and a fold change ≥ 2. The three groups were compared for differential expression of known sRNA, novel sRNA, and mature miRNAs by forming the following three pairs: ASP vs. HC, HAM vs. HC, and ASP vs. HAM.

qRT-PCR validation of miRnas

Two miRNAs, hsa-miR-29b-3p (MI0000105) and hsa-let-7f-5p (MI0000067), that showed a strong tendency to dysregulate in the samples were selected and validated by qRT-PCR. These two downregulated miRNAs were validated in PBMC samples from 24 HAM patients (9 from this study and 15 from a previous study) and 14 ASP subjects (3 from this study and 11 prospectively collected samples). The main characteristics of the 16 patients from HAM and the 11 subjects from ASP used for validation are shown in **Table S1**. cDNA was reverse transcribed from 5 μl of each enriched miRNA using the TaqManTM MicroRNA Reverse Transcription Kit (REF 4,366,596, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Subsequently, PCR products from the cDNA samples were amplified with a Thermo Fisher Scientific, Inc. 7500 Real-Time PCR Instrument using the TaqMan MicroRNA Assay along with the TaqMan Universal PCR Master Mix. All assays were performed in triplicate according to the manufacturer's protocols. The dCT method (CT) was used to analyse the data [30].

Potential miRNA target genes prediction and regulatory network construction

The miRWalk 3.0 algorithm predicted target genes from differentially expressed miRNAs in the HAM and ASP groups compared with the HC group. Target genes were calculated for each miRNA and analysed for GO enrichment terms and KEGG pathway classification. MirWalk v.3 (March 2020 update) was used to search for significantly deregulated miRNA target genes in Reactome pathways, with p-values in the range of 0.05 using the Benjamini-Hochberg multiple testing correction algorithm to reduce the FDR-inducing false discovery rate (FDR). The miRWalk network algorithm was used to search for possible interactions between target miRNA and mRNA [31].

QIAGEN's Ingenuity Pathway Analysis (IPA) was then used to examine the target genes of the seven miRNAs that differed most between ASP and HAM/TSP patients. The putative target genes were filtered for experimentally validated and putative predicted target genes with high confidence levels. IPA resulted in a total of 97 target genes. By IPA Pathway analysis, the drug pathways and target genes associated with the differentially expressed miRNAs were deduced.

Statistical analysis

Unless otherwise indicated, statistical significance of sRNA regulation was determined using a moderated t test or a one-way test (ANOVA) followed by FDR, with a P (corr) cut-off ≤ 0.05. Hierarchical cluster analysis (HCA) was performed using the default hierarchical cluster algorithm implemented in the Strand NGS package. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression levels from the qRT-PCR results, and paired t tests were used to determine whether the differences were statistically significant (GraphPad software v.8).

Based on the qRT-PCR test results, the $2^{-\Delta\Delta CT}$ formula was used to determine the relative changes in miRNA expression levels, and paired t tests were calculated using GraphPad Software v.8 (GraphPad Software, San Diego, CA, USA) to determine whether or not the differences were statistically significant.

Results

Participants' characteristics

The characteristics of the categorized participants are shown in Table 1. The ages of the eight women in the ASP group ranged from 31 to 80 years, and the median age was 55 years. HAM patients were predominantly female (70%), with median age of 61 years and an age range of 40 to 73 years. Five participants (3 females and 2 males) aged 32 to 53 years were recruited as a HC group. The HC participants were younger than HAM patients at sample recruitment (median age: 37 years versus 61 years; P < 0.05). No significant age difference was found between HC and ASP or between HAM and ASP (p > 0.05). ASP group had one to 208 copies/10 [3] PBMCs of HTLV-1 PvL, whereas the HAM group had 95 to 614 copies/10 [3] (Two-tailed P value ≤ 0.005).

Whole-genome sRNA sequencing data

Sequencing of small RNAs yielded 153.959.485 raw reads with high diversity and quantity for all groups. After removal of low-quality reads that did not pass the Vendor's quality control, 121.799.036 high-quality reads of combined genes were obtained and assigned to the genome (Table 1). Illumina massively parallel sequencing (MPS) yielded 402 sRNA molecules, of which 330 were from known sRNA and 72 were from new sRNA. Among the 330 known sRNAs, there were 187 miRNAs, 26 scRNAs, 8 scRNA pseudogenes, 46 snoRNAs, 10 snRNAs, and 53 tRNAs (**Table S2**).

Eight of the 72 novel genes were novel miRNAs, two scRNAs, 10 snoRNAs, three snRNAs, four tRNAs, and 45 were unknown (**Table S3**). Our initial analysis revealed 212 filtered mature known miRNAs in all three groups (**Table S4**).

### Differential analysis of expression of known sRnas between groups

Of the 330 filtered sequences of known sRNAs in the raw data, 251 (231 upregulated and 20 downregulated sRNAs), 238 (196 upregulated and 42 downregulated sRNAs), and 182 (176 upregulated and 6 downregulated sRNAs) differed significantly (ANOVA p-value 0.05) in their levels between the ASP vs. HC, HAM vs. HC, and ASP vs HAM groups, respectively (Figure 1, Table S2). Of the 251 known sRNAs, 137 miRNAs, 19 scRNAs, 4 scRNA pseudogenes, 42 snoRNAs, 9 snRNAs, and 40 tRNAs were identified. As shown in Figure 1A, the five most highly expressed sRNAs between ASP and HC were hsa-mir-150, 155, 23b, SNORD78, and SNORD29, whereas the five most downregulated sRNAs were hsa-mir-451a, 144, 183, 486, and 19a. The five most highly expressed sRNAs between HAM and HC were hsa-mir-150, SNORD29, SNORD3A, SNORD3B–1, and hsa-mir-23b, whereas the five most downregulated sRNAs were hsa-mir-451a, 144, 183, 486, and 19a (Figure 1B), similar to ASP versus HC. Of the 182 differentially expressed sRNAs between ASP and HAM, shown in Figure 1C, 176 were upregulated and 6 were downregulated in the ASP group compared with the HAM group. The most

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.
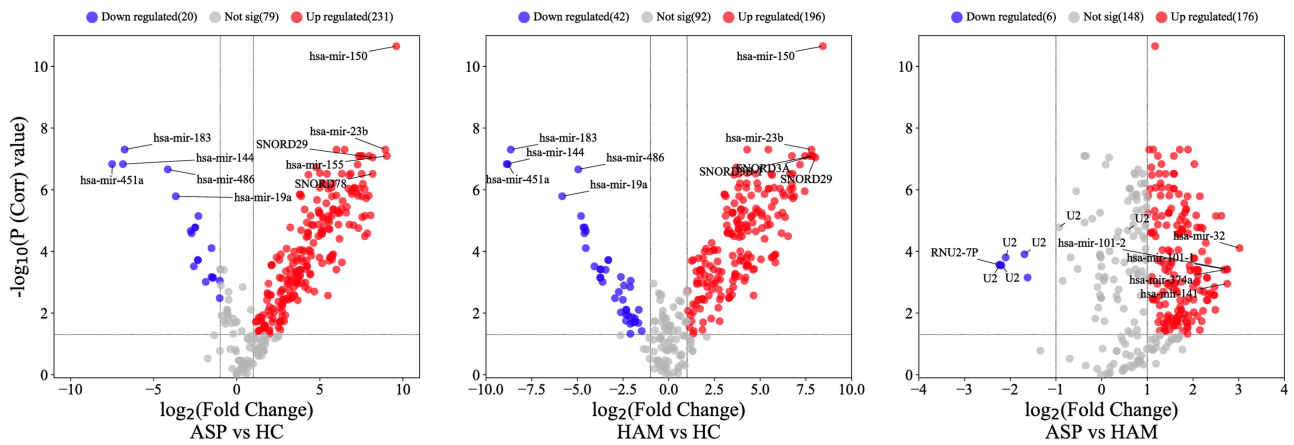
Figure 1. Volcano plots for expressed known sRNA in peripheral blood mononuclear cells (PBMCs) in the three comparison groups. Red (upregulated) and blue (downregulated) circles indicate genes with significant differences, whereas grey circles represent genes without significant differences.

highly expressed sRNAs were hsa-mir-32-5p, followed by hsa-mir-101-3p, 374a, and 141-3p, whereas U2 and RNU2-7P were downregulated.

The unsupervised HCA of 182 known sRNAs with differential regulation in all three pairs group is shown in Figure 2. The differential expression pattern revealed three large sRNA clusters in which ASP and HAM patients were separated from healthy controls, except for 3 HAM samples (125HAM, 011HAM, and 022HAM) that were separated from HC samples in a subcluster. The first cluster consists of 38 sRNA genes (highlighted by yellow colour) with a higher

expression pattern in the HC group, especially hsa-miR-183, 451a, and 144. This result suggests that the sRNA expression pattern changed after HTLV-1 infection. The results support the clinical significance of PBMC sRNA profile in the diagnosis of HTLV-1 infection. Although the three samples from HAM patients (125HAM, 011HAM, and 022HAM) are in the same main branch as the HC samples, their sRNA expression in this cluster differs significantly from that of the HC group but is closest in the tree to most HAM patients. A closer look at the same figure shows that neither sRNA clusters two (34 sRNAs, highlighted by light
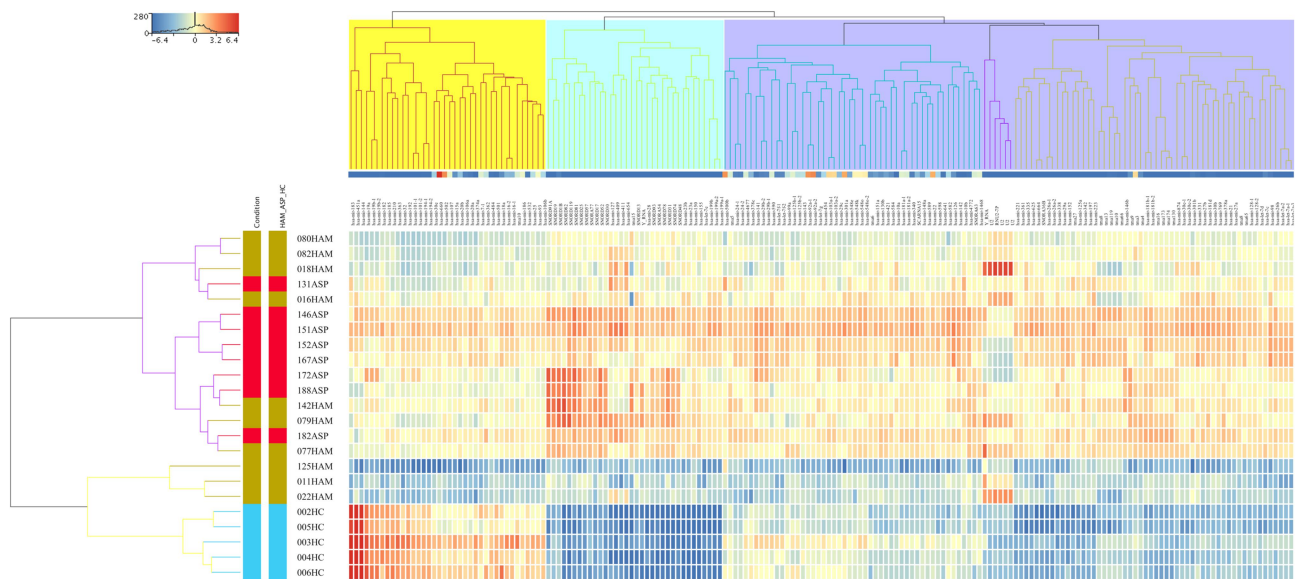
Figure 2. Heatmap showing the results of a hierarchical cluster analysis (HCA) performed independently for the three sample groups and the 182 significantly dysregulated known sRnas. The sample cluster tree is shown on the left, with the sRNA cluster tree above, forming 3 clusters selected by yellow, light green, and violet colours. The colour scale at the top indicates the relative expression level of sRNA in all samples. Red means that the expression levels are higher than the mean, while blue means that the expression levels are lower than the mean. Each column represents a known sRNA, and each row represents a sample.
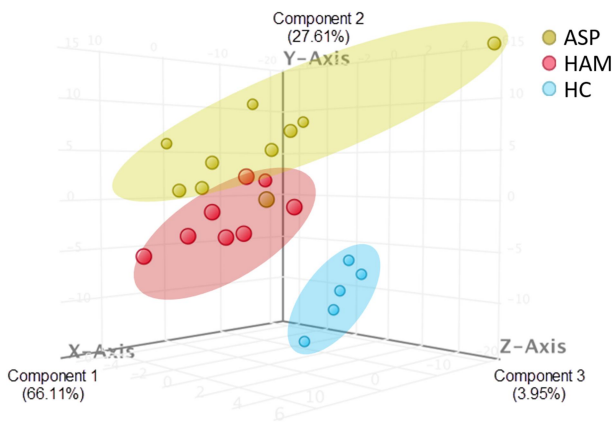
Figure 3. 3-D representation of principal component analysis (PCA) showing the distances between the three groups based on the 182 identified sRNA with significant differential regulation in all three groups.

green colour) nor three (110, highlighted by violet colour) were able to clearly distinguish these samples based on their clinical condition. The 182 sRNA expression profiles of most subjects from ASP were slightly separated from those of patients from HAM in 3-D principal component analysis (PCA), whereas the HC samples were also strongly separated from the other groups (Figure 3).
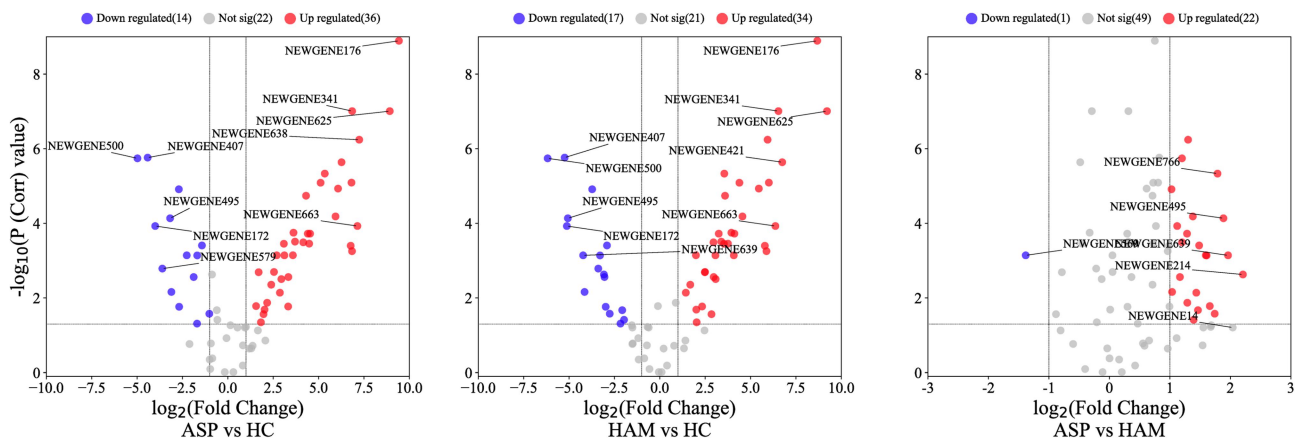
Differential analysis of expression of novel sRNA between groups

Of the 72 novel genes, 50 reached significant FDR values (p (Corr) cut-off 0.05) between the ASP and HC groups, with 36 genes upregulated and 14 downregulated (Figure 4A and **Table S3**). On the other hand, the analysis between HAM and HC revealed 34 upregulated genes and 17 downregulated new genes
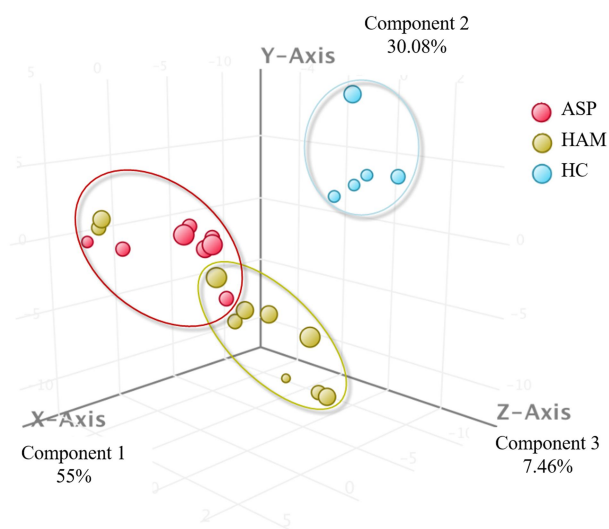
(Figure 4B). Twenty-eight of the 50 significantly deregulated new sRNAs (56%) were previously unknown. NEWGENE176 (chr5, 79946806–79946866) and NEWGENE625 (snoRNA) (chr5, 93905130– 93905240) were the most upregulated novel genes in the ASP and HAM groups compared with the HC group. The most downregulated novel genes were NEWGENE407 (chr12, 138513819–138513879) and NEWGENE500 (chr15, 99077146–99077181). We generated a 3D PCA plot based on the expression of the 50 significantly deregulated novel sRNAs, which showed similar clustering to the sRNA data (Figure 5). The HC samples were clustered separately, whereas the samples from ASP and HAM partially overlapped. No significant difference in the expression of new sRNA was observed between the ASP and HAM groups.

Differential analysis of mature miRNA expression between groups

Of the 212 mature miRNAs filtered, 157, 144, and 109 mature miRNAs were significantly deregulated between ASP and HC, HAM and HC, and ASP and HAM, respectively (Figure 6, Table S4). Of the 157 miRNAs differentially expressed between ASP and HC, 139 were upregulated and 18 were downregulated (Figure 6A). The five most upregulated miRNAs were hsa-miR-150-5p, 155-5p, 23b-3p, 23a-3p, and 654-3p (Log FC > 7.6), whereas the five most downregulated miRNAs were hsa-miR-451a, 183-5p, 486-5p, 19a-3p, and 185-5p (Log FC < −3.0) (**Table S4**). Of the 144 deregulated mature miRNA between HAM and HC, 100 were upregulated and 44 were downregulated (Figure 6B). The five most upregulated and downregulated miRNAs

Figure 4. Volcano plots for expressed novel sRNA in peripheral blood mononuclear cells (PBMCs) in the three comparison groups. Red (upregulated) and blue (downregulated) circles indicate genes with significant differences, whereas grey circles represent genes without significant differences.

Figure 5. 3-D representation of principal component analysis (PCA) showing the distances between the three groups based on 50 significantly deregulated new sRnas in all three groups.

differentially upregulated and downregulated in ASP and HAM patients, respectively. The ASP samples 188 and 131 were classified in the same cluster as the HAM samples. The last clinical examinations of both patients in 2021 revealed that they were free of HAM symptoms. Sample 142HAM was assigned to the ASP cluster, but the reason for this unexpected result is unknown. According to the medical report of the last visit, the patient was apparently healthy and complained of no symptoms. She was and is being treated with sertraline, prednisone, baclofen, and dipyrone. PCA analysis of the 20 differentially expressed miRNA shows that the HC patients form their own cluster, whereas the HAM patients have little overlap with the ASP group (Figure 8). This visual representation provides insight into the different pattern of miRNA expression between HAM patients and ASP participants.

We then compared the differentially regulated miRNAs in each pair of samples using the moderated T test to determine the miRNAome groups associated with HTLV-1 infection and the pathogenesis of HAM/ TSP disease. The analysis revealed 44 miRNAs that were differentially expressed in the three comparison groups (entity list 1–3), as shown by the intersection of the expression profiles in the central part of the Venn diagram in Figure 9. Of these, 13 miRNAs (in green) were downregulated in entity lists 1 and 2, whereas they were upregulated in entity list 3. 51 miRNAs were common in entity lists 1 and 2, 16 in entity lists 2 and 3, and 38 in entity lists 3 and 1. 33, 3, and 11 miRNAs were found to distinguish entity lists 1, 2, and 3, respectively.

Because there are consistent data in the literature linking disease severity in HAM/TSP patients to increased PvL [32], and because HTLV-1-infected individuals may present with a variety of neurological
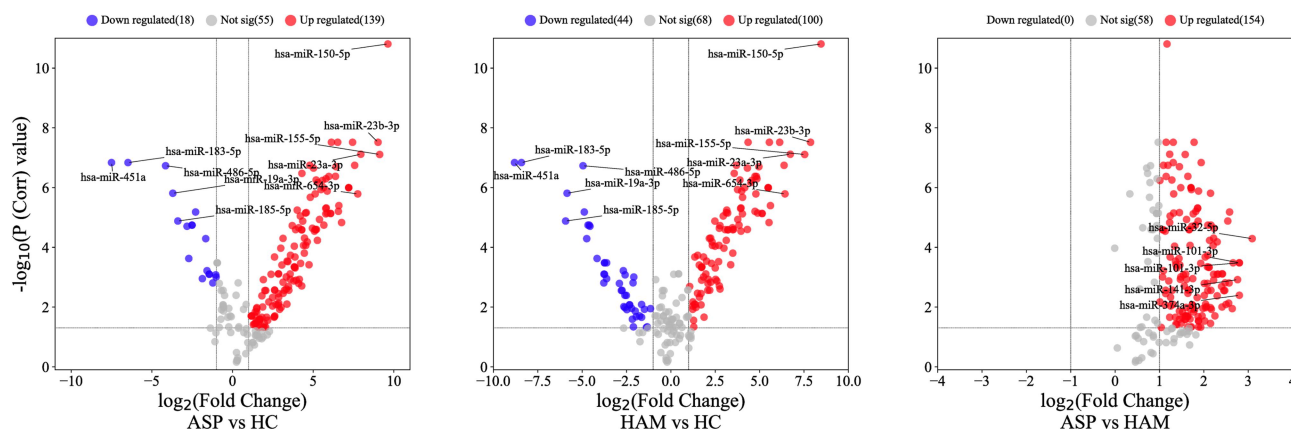
between the two groups were identical to those detected between ASP and HC. Differential expression of mature miRNA between ASP and HAM patients revealed no downregulation but only upregulation of 154 miRNAs in ASP subjects, of which hsa-miR-32-5p, 101-3p, 141-3p, and 374a-3p were the most highly regulated miRNAs (Figure 6C)

Next, we aimed to identify miRNAs as predictive markers for early detection of HTLV-1-infected carriers who are highly likely to develop HAM/TSP disease. To this end, we selected mature miRNAs with FDR ≤ 0.05 and FC of ≥ 5 ($n = 20$) to create the unsupervised hierarchical clustering shown in the heatmap in Figure 7. The miRNAs selected in this analysis were able to classify the majority of the samples according to their clinical characteristics. Most of these miRNAs were


Figure 6. Volcano plots for expressed mature miRNA in peripheral blood mononuclear cells (PBMCs) in the three comparison groups. Red (upregulated) and blue (downregulated) circles indicate genes with significant differences, whereas grey circles represent genes without significant differences.
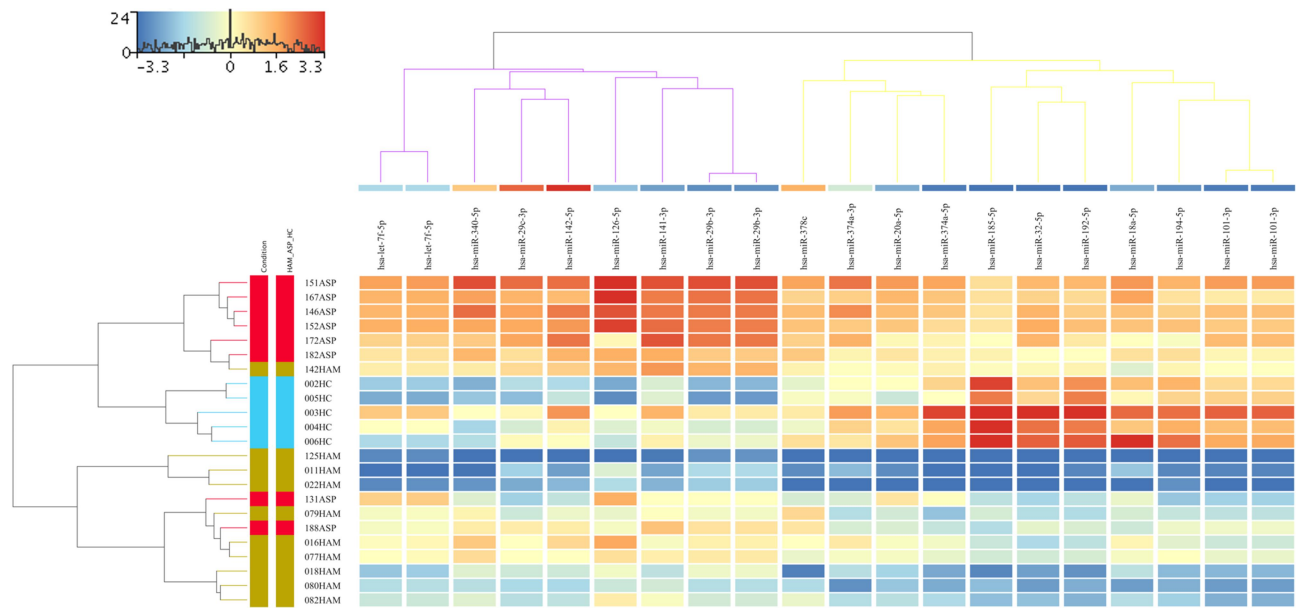
Figure 7. Heatmap showing the results of hierarchical cluster analysis (HCA) performed independently for the three sample groups and the 20 rigorously dysregulated (FDR ≤0.05 and FC of ≥ 5) mature miRnas. The sample cluster tree is shown on the left, with the miRNA cluster tree above forming two major clusters. The colour scale at the top indicates the relative expression level of sRNA in all samples. Red means that the expression levels are higher than the mean, while blue means that the expression levels are lower than the mean. Each column represents a known sRNA, and each row represents a sample.
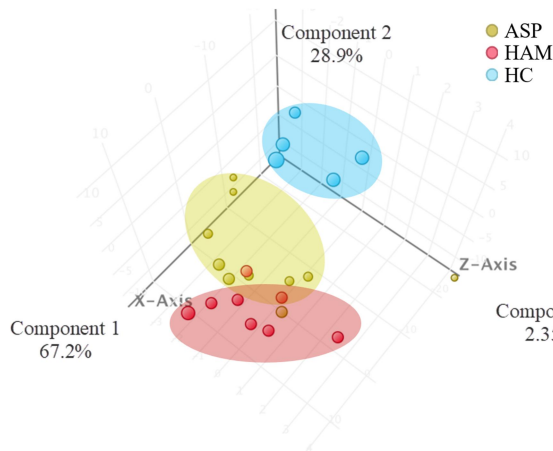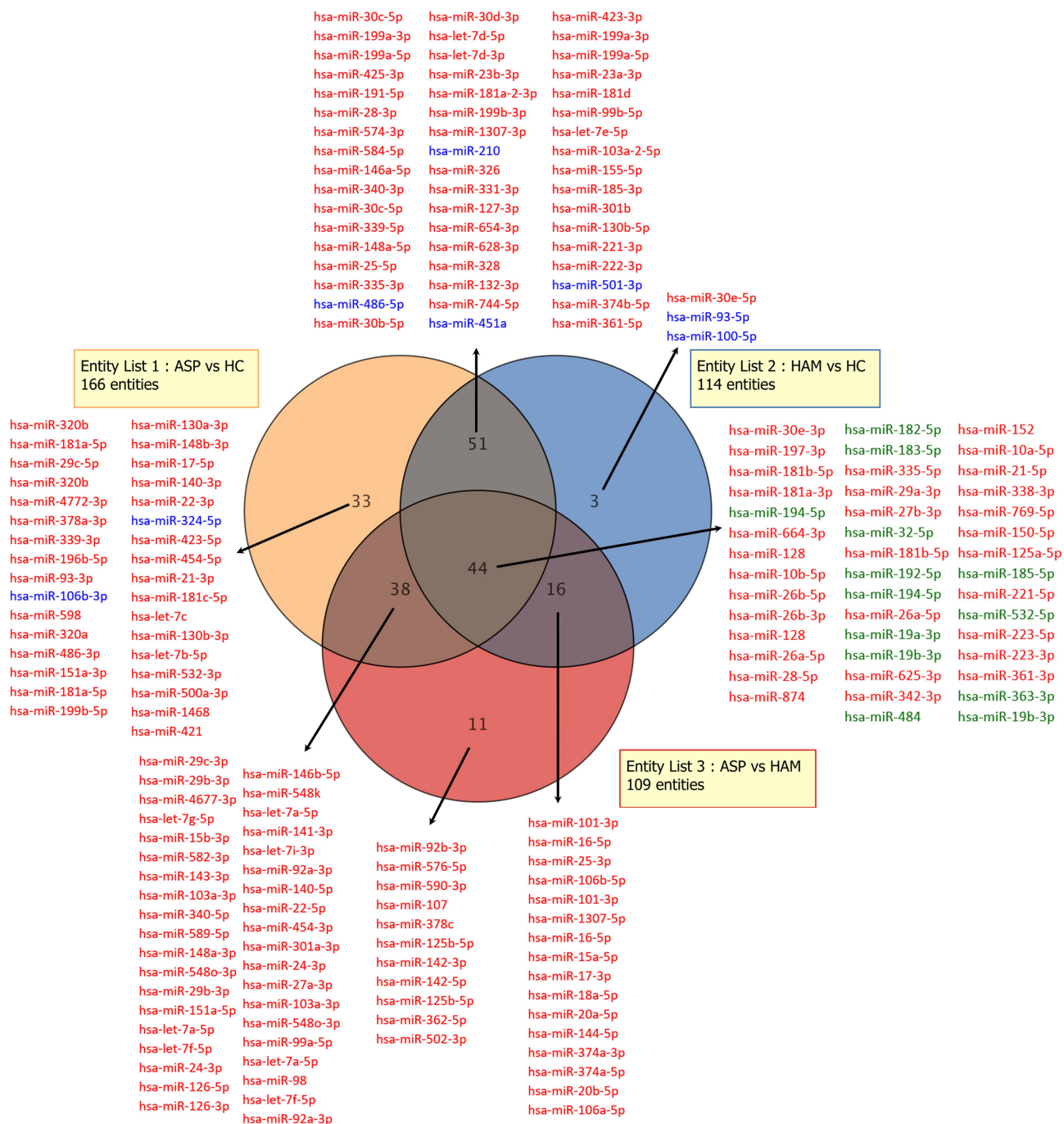

Figure 8. 3-D representation of principal component analysis (PCA) showing the distances between the three groups based on the 20 differentially expressed miRNA in all three groups.

manifestations that differ from the classic picture of HAM/TSP [33], we decided to perform an additional analysis to investigate whether there is an association between the expression of mature miRNAs and PvL independent of clinical status. Therefore, the 18 HTLV-1-infected subjects were divided into low (≤15 copies/1000 cells, $n = 2$) and high PvL profiles (>15 copies/1000 cells, $n = 16$) based on their PvL profile, using previously published criteria [34]. Pairwise comparison of mature miRNA expression between the two groups was performed using a moderated t-test with

FDR-corrected $P \leq 0.05$. The results showed no significant difference between the two groups (data not shown). However, this analysis is likely underpowered by the small sample size, especially in the low PvL group. Therefore, further studies with a large sample size are needed in the future to determine the expression of mature miRNA in patients with different PvL levels and relate it to their clinical status.

To determine whether sex might confound the results, we sorted the data by sex and compared the expression of all miRNAs in PBMC from male and female subjects regardless of their clinical status (**Figure S1**). We also searched the stratified data for the seven significantly dysregulated mature miRNAs that we had distinguished in our previous analysis to investigate whether their expression was affected by sex. We found no statistically significant difference in the expression of these miRs between males and females as shown in supplemental **Figure S2** (Wilcoxon test p value > 0.05).

Individual comparisons between the HAM and ASP groups revealed that 109 mature miRNAs were expressed at a lower level in HAM patients than in ASP patients (**Table S5**). The seven most significantly down regulated mature miRNAs were hsa-miR-29b-3p, 32-5p, 192-5p, 141-3p, 342-3p, 140-5p, and hsa-let-7f-5p (sorted by P (Corr) Value). These miRs were selected for further analysis.

hsa-miR-30c-5p hsa-miR-30d-3p hsa-miR-423-3p
hsa-miR-199a-3p hsa-let-7d-5p hsa-miR-199a-3p
hsa-miR-199a-5p hsa-let-7d-3p hsa-miR-199a-5p
hsa-miR-425-3p hsa-miR-23b-3p hsa-miR-23a-3p
hsa-miR-191-5p hsa-miR-181a-2-3p hsa-miR-181d
hsa-miR-28-3p hsa-miR-199b-3p hsa-miR-99b-5p
hsa-miR-574-3p hsa-miR-1307-3p hsa-let-7e-5p
hsa-miR-584-5p hsa-miR-210 hsa-miR-103a-2-5p
hsa-miR-146a-5p hsa-miR-326 hsa-miR-155-5p
hsa-miR-340-3p hsa-miR-331-3p hsa-miR-185-3p
hsa-miR-30c-5p hsa-miR-127-3p hsa-miR-301b
hsa-miR-339-5p hsa-miR-654-3p hsa-miR-130b-5p
hsa-miR-148a-5p hsa-miR-628-3p hsa-miR-221-3p
hsa-miR-25-5p hsa-miR-328 hsa-miR-222-3p
hsa-miR-335-3p hsa-miR-132-3p hsa-miR-501-3p
hsa-miR-486-5p hsa-miR-744-5p hsa-miR-374b-5p
hsa-miR-30b-5p hsa-miR-451a hsa-miR-361-5p

hsa-miR-30e-5p
hsa-miR-93-5p
hsa-miR-100-5p

Entity List 1 : ASP vs HC
166 entities

Entity List 2 : HAM vs HC
114 entities

hsa-miR-320b hsa-miR-130a-3p
hsa-miR-181a-5p hsa-miR-148b-3p
hsa-miR-29c-5p hsa-miR-17-5p
hsa-miR-320b hsa-miR-140-3p
hsa-miR-4772-3p hsa-miR-22-3p
hsa-miR-378a-3p hsa-miR-324-5p
hsa-miR-339-3p hsa-miR-423-5p
hsa-miR-196b-5p hsa-miR-454-5p
hsa-miR-93-3p hsa-miR-21-3p
hsa-miR-106b-3p hsa-miR-181c-5p
hsa-miR-598 hsa-let-7c
hsa-miR-320a hsa-miR-130b-3p
hsa-miR-486-3p hsa-let-7b-5p
hsa-miR-151a-3p hsa-miR-532-3p
hsa-miR-181a-5p hsa-miR-500a-3p
hsa-miR-199b-5p hsa-miR-1468
hsa-miR-421

51

33

3

44

38

16

11

hsa-miR-30e-3p hsa-miR-182-5p hsa-miR-152
hsa-miR-197-3p hsa-miR-183-5p hsa-miR-10a-5p
hsa-miR-181b-5p hsa-miR-335-5p hsa-miR-21-5p
hsa-miR-181a-3p hsa-miR-29a-3p hsa-miR-338-3p
hsa-miR-194-5p hsa-miR-27b-3p hsa-miR-769-5p
hsa-miR-664-3p hsa-miR-32-5p hsa-miR-150-5p
hsa-miR-128 hsa-miR-181b-5p hsa-miR-125a-5p
hsa-miR-10b-5p hsa-miR-192-5p hsa-miR-185-5p
hsa-miR-26b-5p hsa-miR-194-5p hsa-miR-221-5p
hsa-miR-26b-3p hsa-miR-26a-5p hsa-miR-532-5p
hsa-miR-128 hsa-miR-19a-3p hsa-miR-223-5p
hsa-miR-26a-5p hsa-miR-19b-3p hsa-miR-223-3p
hsa-miR-28-5p hsa-miR-625-3p hsa-miR-361-3p
hsa-miR-874 hsa-miR-342-3p hsa-miR-363-3p
hsa-miR-484 hsa-miR-19b-3p

Entity List 3 : ASP vs HAM
109 entities

hsa-miR-29c-3p hsa-miR-146b-5p
hsa-miR-29b-3p hsa-miR-548k
hsa-miR-4677-3p hsa-let-7a-5p
hsa-let-7g-5p hsa-miR-141-3p
hsa-miR-15b-3p hsa-let-7i-3p
hsa-miR-582-3p hsa-miR-92a-3p
hsa-miR-143-3p hsa-miR-140-5p
hsa-miR-103a-3p hsa-miR-22-5p
hsa-miR-340-5p hsa-miR-454-3p
hsa-miR-589-5p hsa-miR-301a-3p
hsa-miR-148a-3p hsa-miR-24-3p
hsa-miR-548o-3p hsa-miR-27a-3p
hsa-miR-29b-3p hsa-miR-103a-3p
hsa-miR-151a-5p hsa-miR-548o-3p
hsa-let-7a-5p hsa-miR-99a-5p
hsa-let-7f-5p hsa-let-7a-5p
hsa-miR-24-3p hsa-miR-98
hsa-miR-126-5p hsa-let-7f-5p
hsa-miR-126-3p hsa-miR-92a-3p

hsa-miR-92b-3p
hsa-miR-576-5p
hsa-miR-590-3p
hsa-miR-107
hsa-miR-378c
hsa-miR-125b-5p
hsa-miR-142-3p
hsa-miR-142-5p
hsa-miR-125b-5p
hsa-miR-362-5p
hsa-miR-502-3p

hsa-miR-101-3p
hsa-miR-16-5p
hsa-miR-25-3p
hsa-miR-106b-5p
hsa-miR-101-3p
hsa-miR-1307-5p
hsa-miR-16-5p
hsa-miR-15a-5p
hsa-miR-17-3p
hsa-miR-18a-5p
hsa-miR-20a-5p
hsa-miR-144-5p
hsa-miR-374a-3p
hsa-miR-374a-5p
hsa-miR-20b-5p
hsa-miR-106a-5p

Figure 9. A Venn diagram showing miRnas whose expression was either upregulated or downregulated (>2- fold) in peripheral blood mononuclear cells of entity list 1 (ASP vs HC), entity list 2 (HAM vs HC), and entity list 3 (ASP vs HAM). Up-regulated miRNA are shown in red and down-regulated miRNA are shown in blue. MiRNA written in green were downregulated in one entity list while upregulated in another entity list.

Validation of the deregulated miRnas by Qrt-PCR

The two most deregulated miRNAs between the HAM and ASP groups in our sequencing data (hsa-miR-29b-3p and hsa-let-7f-5p) were selected for validation by qRT-PCR in 9 samples from this study and 14 other independent samples from HAM/TSP patients. Quantification of hsa-miR-29b-3p showed significant correlation with sequencing data (Figure 10). Statistical analysis also revealed a nonsignificant trend showing that hsa-let-7f-5p was downregulated in HAM patients compared with ASP.
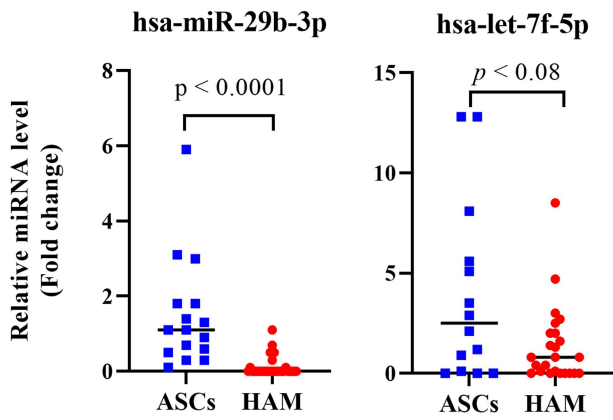
Figure 10. Comparison of miRNA expression levels (hsa-miR -29b-3p and hsa-let-7f-5p) in peripheral blood mononuclear cells from HTLV-1-associated HAM patients compared with HTLV-I-infected asymptomatic carriers with polyclonal T-cell receptor γ gene. The expression levels of selected miRnas were analysed by Qrt-PCR.

Target genes, KEGG pathway, and GO enrichment analysis

Data from the seven most downregulated nonredundant miRNAs between the HAM and ASP groups were used to predict miRNA target genes. This analysis by miRWalk v.3 resulted in 61 genes, as shown in **Table S6**. The results showed that hsa-miR-342-3p has two target genes (INO80D and SYNPO2L), whereas hsa-miR-192-5p has three (RB1, DBT, and ZBTB34), hsa-miR-141-3p has five (YAP1, PRELID2, IGF1R, GATA6, and ATXN7L1), hsa-miR-140-5p has five (GALNT16, KLK10, VEZF1, RALA, and TSC22D2), hsa-miR-32-5p has eight target genes (CPEB2, SPOCK2, FAM126B, CPEB4, CNEP1R1, DUS2, TENT4B, and MIA3), hsa-miR-29b-3p has 17 target genes (including ENPP2, NREP, and HMGCR) and hsa-let-7f-5p has 23 miRNAs (including MDM4, NF200, and BZW)
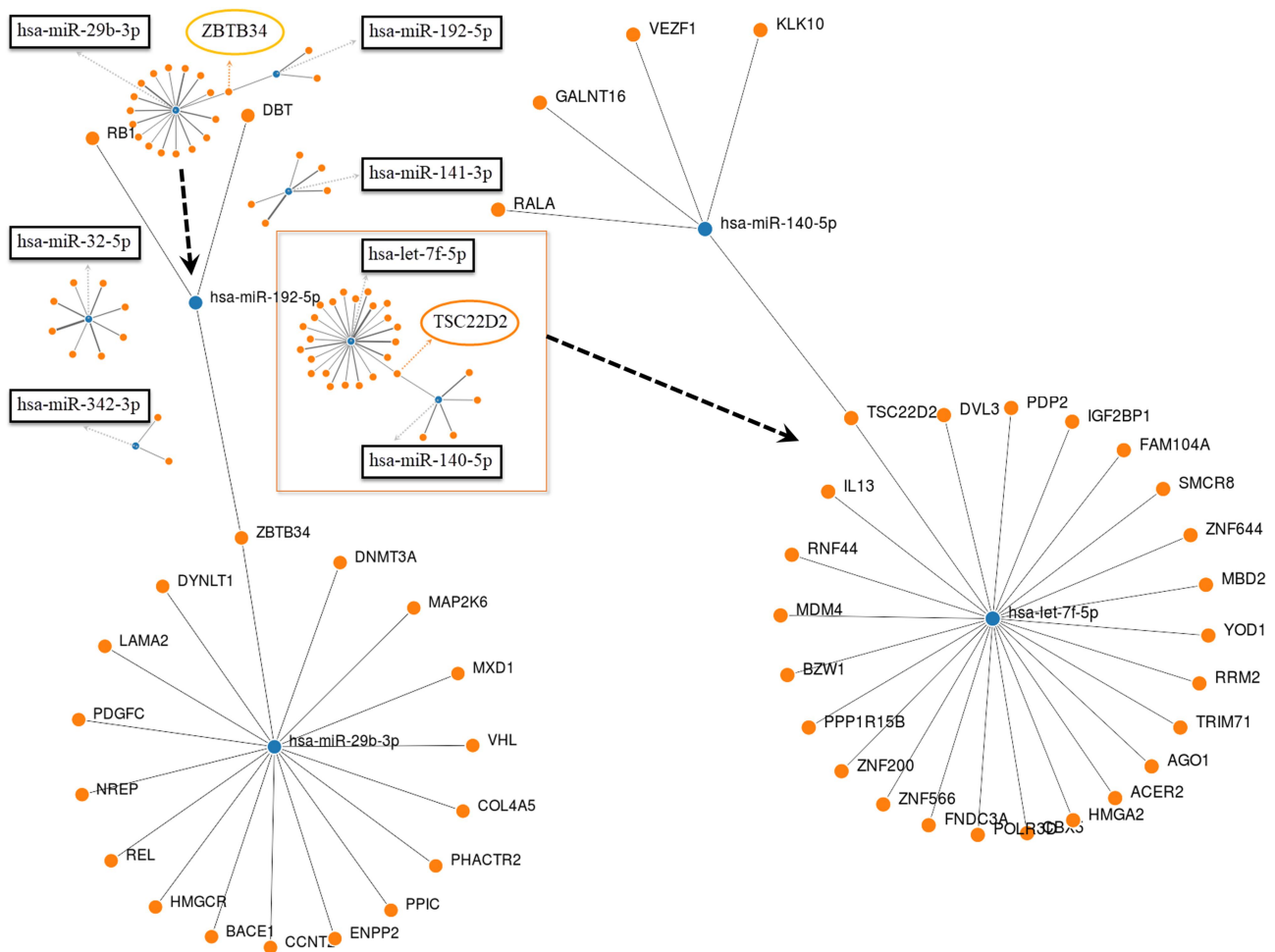
Figure 11. Interaction network between the seven most deregulated mature miRnas and their target genes created with MirWalk v3. Blue circles represent miRnas, orange circles represent mRnas. The more connections there are between miRnas and genes, the more connections there are in the network.
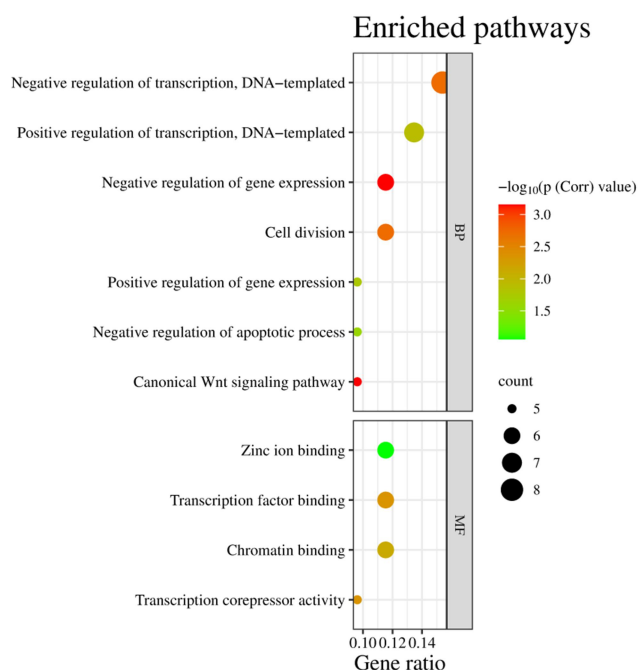
Enriched pathways

Figure 12. Bubble plot of the significantly enriched GO enriched pathways.

(Figure 11). The 61 target genes were then used in a pathway enrichment analysis to search for molecular pathways related to HAM/TSP. As shown in **Table S7**, 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 were associated with biological processes and molecular functions, respectively (Figure 12).

Functional in silico investigation by IPA revealed that 8 of 97 experimentally validated and putative predicted target genes were directly associated with collagenase response in HAM/TSP patients, which consists treatment approved for progressive Dupuytren contracture (PDCD) [35] (**Figure S2**).

Discussion

Although there is no ideal cure for HAM/TSP, early detection allows for therapeutic measures that can slow or even halt the progression of the disease. HAM/TSP is diagnosed by recognizing a pattern of clinical symptoms and signs, detecting HTLV-I antibodies in the CSF, and ruling out other diseases with similar symptom [36]. Methods based on quantitative PCR testing of the provirus have been developed, but they have not been able to diagnose HAM/TSP with acceptable sensitivity because of the broad spectrum of disease symptoms [37]. Thus, there is a clear need to search 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 pathogenicity mechanism and identify relevant signalling pathways and potential targets for therapy development. It is difficult to find a single biomarker that can perform all of these tasks; indeed, each of these biomarkers may require different properties. MicroRNAs and other small RNAs have been identified as potential biomarkers for a wide range of human diseases [38]. Circulating miRNAs have also been thoroughly explored for their potential to serve as diagnostic or prognostic early warning biomarkers for a broad spectrum of infection-causing pathogens [39,40]. Evidence suggests that host miRNAs can bind to a broad spectrum of RNA viruses, impairing replication, limiting response to antiviral therapies, inhibiting apoptosis, and promoting cell growth [41,42].

The interaction between HTLV-1 and host cells is critically dependent on miRNAs. For example, Pichler and colleagues [20] reported that HTLV-1-infected cell lines had increased levels of miR-21, miR-24, miR-146a, and miR-155 but lower levels of miR-223 compared with control cells. Tax protein [43], Rex [44], and HBZ [45] are viral proteins that have been shown to interact with the synthesis of mature miRNAs by promoting the degradation of Drosha [43]. Rex and HBZ proteins have been shown to inhibit the formation of mature miRNAs by inhibiting Dicer activity [44] and expression [45], respectively. Although miRNAs are being investigated as prognostic molecular markers in ATLL, their role as important biomarkers for HAM/TSP diagnosis and prognosis remains unexplored [20–22,46,47]. Using PBMC samples and Illumina small RNA high-throughput sequencing technology, we have identified for the first time unique small RNA profiles that can distinguish patients with HAM/TSP from ASP and healthy individuals. 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 most downregulated miRNAs in PBMCs from 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 these genes showed significant dysregulation between patients and controls, our small sample size did not allow us to investigate the association between their expression and the severity of HAM/TSP. In view of our results, further studies are needed to determine whether there is an association between the expression of the above miRNAs and the severity of HAM/TSP and, if so, how strong this association is.

Deregulation of miR-29, including its family member miR-29b-3p, contributes to the pathogenesis of tumours, autoimmune diseases, and fibrotic diseases [48,49]. Significant suppression of miR-29b has been reported in the literature in patients with multiple sclerosis (MS) [50], an autoimmune disease that is clinically closely related to HAM/TSP in certain forms, suggesting that miR-29 expression may be deregulated in both diseases and contribute to their pathogenesis. In contrast, hsa-let-7f-5p and 25 other miRNAs were found to be significantly downregulated in PBMCs from patients with all subtypes of MS, including primary progressive, secondary progressive, and relapsing-remitting disease [51]. Low expression of let-7 family miRNAs in HIV-1 infection has been associated with increased production of IL −10 by CD4+ T cells, which confers an important survival advantage to the virus by modulating the host immune response [52]. A recent study by Kumar et al [53] showed 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 [32]. Consistent with our sequencing results, we found a nearly 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 be partially responsible for the discrepancies in miR-let-7f quantification. Deregulation of hsa-miR-32-5p efficiently reduces primate foamy virus type 1 (PFV-1) retrovirus accumulation in human cells [54]. As far as we know, the biological role of hsa-miR-32-5p in HAM/TSP has not yet been investigated. With regard to miR-192-5p, there is ample evidence for the possible role of transforming growth factor-β (TGF-β) in increasing the expression of miR-192-5p in epithelial cells of the human, mouse, rat, and mouse proximal tubule [55–58]. Since TGF-β signalling is downregulated in HAM/TSP patients, suppression of this pathway could lead to low expression of miR-192-5p in these patients [59].

We searched the MirWalk database for target genes for the seven most downregulated miRNAs and found a relevant Reactome pathway, "R-HSA-2559583 Cellular Senescence," to uncover signalling pathways relevant to HAM/TSP. This signalling pathway was observed by the discovery of target genes for hsa-miR-192-5p, 29b-3p, and hsa-let-7f-5p. Consequently, cellular senescence, an irreversible state of cell cycle arrest, is a molecular signalling pathway possibly associated with HAM/TSP. Merling

and colleagues [60] had previously proposed that Tax protein triggers cellular senescence by a mechanism other than IKK/NF-B activation. In another study, hyperactivation of NF-B signalling by Tax was found to lead to cellular senescence, contradicting the general view that NF-B activity promotes growth of HTLV-1-infected cells [61]. The data presented above suggest that HTLV-1-infected cells must counteract this senescence phenotype to proliferate. Previous studies have shown that these miRNAs play a role in regulating cellular senescence [62,63]. For example, previous studies have shown that elevated levels of IL −17 directly inhibit miR-192 and that IL −17 increases NF-κB [64] and MAPK [65] activation. NF-κB is a trigger of proinflammatory cytokines and thereby promotes senescence. Our results showed that hsa-miR-192-5p was downregulated in HAM/TSP patients. A conceivable, albeit speculative, result is that downregulation of hsa-miR-192-5p would lead to upregulation of the NF-κB pathway because NF-κB is a direct target of miR-192, which in turn would promote senescence. The exact mechanism of selective modulation of inflammation in HAM/TSP disease by hsa-miR-192-5p, hsa-let-7f-5p, and hsa-miR-29b-3 needs further investigation.

Collagenase, an enzyme involved in the degradation and remodelling of the extracellular matrix, which includes collagen and other components, was identified by the IPA as the top listed drug that directly interacts with eight target genes. To our knowledge, the use of collagenase as a therapeutic approach in HAM/TSP patients has not yet been described. Therefore, these results warrant further investigation to determine whether collagenase is a therapeutic candidate for the disease HAM/TSP.

Currently, no specific therapies have been developed to prevent or reduce the risk of developing HAM/TSP complications in asymptomatic HTLV-1 carriers, and it has proven difficult to predict who will develop the disease [66]. Predictive biomarkers of disease activity have been previously identified in PBMCs using PvL, which correlates positively with severity of motor disability and is associated with long-term prognosis [67]. Increased cell counts, anti-HTLV-1 antibody titres, protein levels, C-X-C motif chemokine 10, and neopterin in CSF significantly correlated with the rate of progression of HAM/TSP [68–71]. Despite the small sample size, our results suggest that the differentially expressed miRNAs are potential biomarkers for HAM/TSP. Based on our results, further research is needed to develop an integrative approach to derive the candidate miRNA biomarkers described here by linking miRNA and gene expression data in a large sample of HAM/TSP patients.

The novelty of our study was its main strength. To our knowledge, this is the first study to investigate the role of small RNAs, particularly miRNAs, in HAM/TSP patients infected with HTLV-1. Moreover, among the seven most down-regulated miRNAs, we identified two important common REACTOMES pathways, namely, "R-HSA-74160 gene expression" and "R-HSA-2559583 cellular senescence."

Although our study is the first to identify multiple PBMC miRNAs associated with HAM/TSP disorder in HTLV-1-infected patients, there are several limitations that should be noted. The most important limitation of our study is the small number of patients and controls. This limitation might reduce the power of the presented results regarding the role of the presented miRNAs in HAM/TSP patients and their prognostic value as potential markers in this disease. In the future, large prospective studies are needed to confirm and investigate the true role of these miRNAs in HAM/TSP disease. Another limitation of this study is that we only investigated miRNAs. The information on other small RNAs discovered in this study could be used in future research to develop and improve analytical methods.

Conclusion

In summary, our results reveal a variety of miRNAs that have the potential to predict the development of HAM/TSP. These miRNAs and their targets are involved in a known pathogenesis pathway for the disease. Our results pave the way for the discovery of novel molecular miRNA signatures that can predict not only the diagnosis of HAM/TSP but also virus carriers that will develop the disease. To our knowledge, this is the first study to investigate differential expression of miRNAs in PBMCs from HAM/TSP patients to determine if they can be used as early prognostic markers.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All raw sRNA data generated by MPS have been deposited in the Zenodo repository https://doi.org/10.5281/zenodo.6528176 and https://doi.org/10.5281/zenodo.1181925.

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