# Small RNA profiles of HTLV-1 asymptomatic carriers with monoclonal and polyclonal rearrangement of the T-cell antigen receptor γ-chain using massively parallel sequencing: A pilot study

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**Abstract.** In the present pilot study, massively parallel sequencing (MPS) technology was used to investigate cellular small RNA (sRNA) levels in the peripheral blood mononuclear cells (PBMCs) of human T-lymphotropic virus type I (HTLV-I) infected asymptomatic carriers with monoclonal (ASM) and polyclonal (ASP) T cell receptor (TCR) γ gene. Blood samples from 15 HTLV-I asymptomatic carriers (seven ASM and eight ASP) were tested for the clonal TCR-γ gene and submitted for sRNA library construction together with blood samples of five healthy controls (HCs) using Illumina sequencing platform. The sRNA-sequencing reads were aligned, annotated and profiled using various bioinformatics tools. Based on these results, possible markers were validated in the study samples by performing reverse transcription-quantitative (RT-q)PCR analysis. A total of 76 known sRNAs and 52 putative novel sRNAs were identified. Among them, 44 known and 34 potential novel sRNAs were differentially expressed in the ASM and ASP libraries compared with HCs. In addition, 10 known sRNAs were exclusively dysregulated in the ASM group and one (transfer RNA 65) was significantly upregulated in the ASP group. *Homo sapiens* (hsa) microRNA (miRNA/mir)-23a-3p, -28-5p, hsa-let-7e-5p and hsa-mir-28-3p and -361-5p were the most abundantly upregulated mature miRNAs and hsa-mir-363-3p, -532-5p, -106a-5p, -25-3p and -30e-5p were significantly downregulated miRNAs (P<0.05) with a >2-fold difference between the ASM and ASP groups compared with HCs. Based on these results, hsa-mir-23a-3p and -363-3p were selected for additional validation. However, the quantification of these two miRNAs using RT-qPCR did not provide any significant differences. While the present study failed to identify predictive sRNA markers to distinguish between ASM and ASP, the MPS results revealed differential sRNA expression profiles in the PBMCs of HTLV-1 asymptomatic carriers (ASM and ASP) compared with HCs.

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## Introduction

Human T-lymphotropic virus type I (HTLV-I) is an oncogenic human retrovirus that was first isolated from the HUT102 T-cell line, which had been obtained from a patient with adult T-cell leukemia/lymphoma (ATLL) (1). Globally, there are an estimated 5-10 million individuals who carry HTLV-I, with the important caveat that the prevalence remains largely unknown in several areas of the world, such as India, China, Russia, Australia and several African countries (2). The disease burden is unevenly distributed, with a higher incidence of the disease in southwest Japan, the Caribbean islands, South America and parts of Central Africa (3). Infection with HTLV-I may result in a spectrum of clinical manifestations,

ranging from asymptomatic infection to several health conditions, most notably malignant ATLL and a type of chronic progressive neuromyelopathy, termed HTLV-I-associated myelopathy/tropical spastic paraparesis (4). The majority of patients with HTLV-I remain asymptomatic for life, while certain individuals progress to a pre-leukemic phase that is characterized by small numbers of circulating leukemic cells in the peripheral blood and skin lesions, but with a lack of involvement of other organ systems (5). Only 2.5-5% of the virus carriers eventually develop ATLL after a long asymptomatic period (6,7).

The reason why certain individuals develop the disease, whereas others remain asymptomatic, is likely dependent on host- and virus-associated factors (8). Available evidence from molecular studies indicates that impaired cellular functions mediated by viral proteins, such as immortalization and interleukin (IL)-2-independent proliferation of T cells induced by *TAX* protein (9) genetic and epigenetic changes, including DNA methylation, and the host's immune system may contribute to the leukemogenesis of ATLL (10-12). Despite the effective immortalization of the T cells, the markedly prolonged incubation period (>30 years) prior to the onset of ATLL suggests the additional acquisition of genetic changes besides the viral infection contributing to the pathogenesis (13).

Infections with HTLV-I are expected to produce an initial polyclonal T-cell proliferation followed by a monoclonal malignant transformation in asymptomatic HTLV-I carriers prior to the diagnosis of ATLL. HTLV-I carriers who have monoclonal integration of HTLV-I provirus DNA in their mononuclear cells are at high risk of developing ATLL, but the prognosis of these patients varies from being stable long-term carriers to developing ATLL (14-17). Carvalho and Da Fonseca Porto (18) also identified a correlation between monoclonal integration of provirus DNA and abnormal lymphocytes in peripheral blood, with a trend toward greater severity of the parasitic infection.

Certain studies have provided significant evidence to support transcription of several non-protein coding regions of the mammalian genome (19,20), yielding a complex network of transcripts that include tremendous numbers of non-coding RNAs (ncRNAs). These molecules have important roles in normal biological processes and in a variety of human diseases, including diabetes (21), aging heart (22) and cancer (23,24). Within these ncRNAs, small RNAs (sRNAs) have emerged as potential posttranscriptional regulators of gene expression in both eukaryotes and prokaryotes (25). sRNAs are highly complex in terms of structural diversity and function, and may be further divided into structural and regulatory ncRNAs (26). Structural ncRNAs include transfer RNA (tRNA) and ribosomal RNA, as well as other small but stable non-coding 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 (27,28). Regulatory ncRNAs include microRNAs (miRNAs/miRs), P-element induced wimpy testis-interacting RNAs (piRNAs) and long ncRNAs (29). miRNAs are perhaps the single most extensively investigated small ncRNAs. miRNAs are typically 18-25 nucleotides 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 (30), differentiation (31) and apoptosis (32). Mature miRNAs are assembled into ribonucleoprotein complexes called miRNA-induced silencing complexes, which inhibit gene expression by perfect complementary binding for mRNA degradation, or imperfect binding at the 3' untranslated region to inhibit translation (33). The rigid control of miRNA expression is important to maintain the normal physiological state of cells (34), while overexpression of miRNAs has been associated with the occurrence and development of various diseases including ATLL (35,36). t has been demonstrated that oncogenic miRNAs and tumor-suppressor miRNAs mediate important cell cycle components, thus resulting in either acceleration or deceleration of the cell cycle (37-39).

In theory, dysregulation of host cell miRNAs mediated by HTLV-I may influence the development of ATLL. Certain studies have demonstrated the role of cellular miRNA in the proliferation and survival of HTLV-I-infected T cells. Hybridization-based methodologies, including microarray and PCR-based assays, have been used thus far to identify and profile cellular miRNAs in HTLV-I-infected cell lines (40,41). Pichler *et al* (35), who first used quantitative (q)PCR to study the associations between HTLV-I and cellular miRNAs, confirmed that HTLV-I transforms host cells by inducing dysregulated expression of specific miRNAs, including miR-146a, which is upregulated by the Tax protein, an oncoprotein of HTLV-I. Subsequently, other studies have reported both up- and down-regulation of a number of miRNAs in HTLV-I/ATLL cell lines and primary ATLL cells (36,42). Previously, Ruggero *et al* (43) used massively parallel sequencing (MPS) to identify the miRNAs and tRNA fragments expressed in HTLV-I-infected cells compared with normal CD4^{+} T cells. Most of the aforementioned studies primarily focused on the expression of specific or diverse miRNAs in HTLV-I-infected cell lines, while few studies have focused on determining the expression profiles of sRNAs isolated from human blood samples. Thus, it would be worthwhile to investigate early gene regulatory mechanisms associated with cell transformation in HTLV-I asymptomatic carriers with monoclonal T cell receptor (TCR) γ gene rearrangement, designated here as ASM group.

In the present pilot study, Illumina MPS technology (Illumina, Inc.) was used to comprehensively characterize sRNA expression profiles in the peripheral blood mononuclear cells (PBMCs) of patients with ASM and HTLV-1 asymptomatic carriers with polyclonal TCR γ gene rearrangement, designated as ASP group, and to compare these to a healthy control (HC) group. The present results provide considerable insight to enhance the current understanding of the expression characteristics of sRNAs in ASM and ASP subjects.

## Materials and methods

*Patient enrolment and sample preparation.* Peripheral blood samples were initially collected from 367 HTLV-I asymptomatic carriers. These participants were receiving care at the HTLV-I outpatient clinic at the University of São Paulo and the Institute of Infectious Diseases Emilio Ribas (São Paulo, Brazil) between November 2012 and December 2014. All subjects were recruited for the present study after receiving adequate explanations of the enrollment procedure at the

collaborating institutions. Of the 367 HTLV-I asymptomatic carriers, seven had detectable monoclonal expansion of T-cells population, which resulted in the predominance of TCR γ gene rearrangement, classified as ASM group, were included in the present study. Furthermore, eight patients who were ASP were randomly selected from the 360 carriers to limit them to the ASM group. Furthermore, five HCs were selected from apparently healthy volunteers recruited from the laboratory staff who have no evidence of HTLV-1 infection. Thus, the final sample size was seven ASM and eight ASP cases, as well as five HCs. All eight participants in the ASP group were female and the median age was 55 years (range, 31-80 years). Furthermore, five females and two males were included in the ASM group and the median age of this group was 61 years (range, 28-74 years). The samples were collected after approval by the Ethics Committee for Review of Research Projects (CAPPesq; approval no. 1235/2017) and written informed consent was provided by all subjects. Isolation of PBMCs was performed using Ficoll-Hypaque (Amersham; Cyvita) density centrifugation for 20 min at 1,020 x g, followed by two washes with RPMI 1640 (R0883, Sigma-Aldrich; Merck KGaA) with 10% fetal calf serum (FCS, Gibco; Thermo Fisher Scientific, Inc.) and stored in liquid nitrogen (≤140°C) until use. A summary of the clinical characteristics of the patients is provided in Table I.

*Genomic DNA and RNA extraction.* Genomic DNA was extracted from PBMCs using a QIAamp blood kit (Qiagen GmbH). An miRNeasy Mini kit (Qiagen GmbH) in conjunction with TRIzol® (Thermo Fisher Scientific, Inc.) was used to extract total RNA and sRNA following the manufacturer's protocols. In brief, 700 µl TRIzol was added to 200 µl cryopreserved PBMCs, followed by incubation at room temperature for 5 min. After this, 200 µl chloroform (Sigma-Aldrich; Merck KGaA) was added and the samples were shaken during incubation at room temperature for 5 min, followed by centrifugation at 12,000 x g for 15 min at 4°C. The aqueous phase containing RNA was transferred to a new tube and 0.5 ml isopropanol (Thermo Fisher Scientific, Inc.) was added. The samples were incubated at room temperature for 10 min, followed by centrifugation at 12,000 x g for 15 min at 4°C. The pellet was washed with 75% ethanol (Sigma-Aldrich; Merck KGaG), air-dried and resuspended in 20 µl nuclease-free water (Ambion; Thermo Fisher Scientific, Inc.). For the addition of carriers, 2 µl glycogen (5 µg/µl) was added during the isopropanol precipitation step. Using the miRNeasy Mini kit (QIAgen, Hliden, Germany), the aqueous phase was added to ~525 µl 100% ethanol and the mixture was thoroughly blended by pipetting. The supernatants were then transferred to the miRNeasy Mini spin column and centrifuged at ≥8,000 x g. Thereafter, the column was washed with 700 µl Qiagen RWT buffer, 500 µl Qiagen RPE buffer and 500 µl 80% ethanol, and then centrifuged at 12,000 x g for 5 min at room temperature. Finally, the sRNAs on the membrane were eluted in 22 µl RNase-free water and stored at -80°C until further use. The concentration of DNA and RNA, including sRNA, was measured using fluorimetry, using a Qubit 2.0 fluorometer with a Qubit® DNA or an RNA HS Assay kit (Thermo Fisher Scientific, Inc.), respectively, according to manufacturer's instructions.

*HTLV-I proviral load determination.* Extracted DNA was used as a template to amplify a 97-base pair (bp) fluorimetry fragment from the HTLV-I *tax* region using previously described primers (44) and protocols (45). Amplification and analysis were performed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The standard curves for HTLV-I *tax* were generated from MT-2 cells (kindly provided by Dr Jorge Casseb, University of São Paulo, Brazil) of $\log_{10}$ dilutions (from $10^5$ to $10^0$ copies). The threshold cycle for each clinical sample was calculated by defining the point at which the fluorescence exceeded a threshold limit set at the mean plus 10 standard deviations above the baseline. Each sample was assayed in duplicate and the mean of the two values was considered the copy number of the sample. The HTLV-I proviral load was calculated as the copy number of HTLV-I (tax) per 1,000 cells=(copy number of HTLV-I tax)/(copy number of RNase P gene/2) x1,000 cells. The method was able to detect one copy per $10^3$ PBMCs.

*Analysis of TCR γ gene rearrangement.* DNA-based PCR of rearranged γTCR genes was performed according to a previously described protocol (46). For each of the patients, the PCR products were analyzed using 3130 ABI Prism capillary electrophoresis equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following was added to each well of a 96-well plate: 0.5 µl size GeneScan Rox (red) 500 size standard, 13 µl Highly deionized (Hi-Di) formamide and 1 µl template DNA sample. Data were analyzed using Genescan and Genotyper software version 2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). T-cell clonalities were blindly determined by visual examination of the electropherograms by two independent examiners and further confirmed by an expert hematology pathologist (coauthor JP).

*sRNA library construction and MPS.* For each sample in each group, sRNA libraries were prepared with the Small RNA v1.5 sample preparation kit, as per the manufacturer's instructions (Illumina, Inc.) and a previous protocol (47). In brief, 5 µl purified total RNA was ligated with 1 µl RNA 3' adapter and then with a 5' RNA adapter (both Illumina, Inc.). The 5' adapter also included the sequencing primer. After reverse transcription (RT-)qPCR amplification, the resulting products were analyzed using PAGE (6% Novex Tris-borate-EDTA PAGE; Invitrogen; Thermo Fisher Scientific, Inc.). After gel electrophoresis, sRNA bands sized 145-150 bp were excised and purified. Finally, every four libraries were pooled and up to 8-10 pM of the pooled libraries were loaded and sequenced using the MiSeq platform (Illumina, Inc.) with a 36-base single-end protocol, according to the manufacturer's protocol.

*sRNA data analysis and interpretation.* Base-calling, demultiplexing and trimmed FASTQ files were generated using MiSeq reporter 2.3 and 2.4.1.3, (Illumina, Inc.). Only high-quality reads with scores >30 on the Sanger scale were considered for further analysis. The reads were aligned against the whole genome build: Hg19 using Strand NGS version 3.1 (Strand Life Science). The default parameters and algorithms of this software package were also used for sequences annotations and deferential expression of known,

Table I. Demographic and clinical characteristics of HTLV-1 asymptomatic carriers and HCs subjected to small RNA analysis.

| Sample | Sex | Age, years | Clonality features | Proviral load (copies/1,000 PBMCs) | Total input reads | Total filtered reads, n (%) | Mapped reads |
|---|---|---|---|---|---|---|---|
| 131ASP | Female | 52 | Polyclonal | 17 | 4,029,773 | 766,480 (19.02) | 3,263,293 |
| 146ASP | Female | 59 | Polyclonal | 208 | 12,757,509 | 2,882,221 (22.59) | 9,875,288 |
| 151ASP | Female | 72 | Polyclonal | 1 | 11,791,086 | 1,821,389 (15.45) | 9,969,697 |
| 152ASP | Female | 34 | Polyclonal | 60 | 6,445,840 | 887,250 (13.76) | 5,558,590 |
| 167ASP | Female | 58 | Polyclonal | 32 | 5,942,373 | 941,689 (15.85) | 5,000,684 |
| 172ASP | Female | 31 | Polyclonal | 102 | 4,441,528 | 726,607 (16.36) | 3,714,921 |
| 182ASP | Female | 80 | Polyclonal | 22 | 7,313,049 | 1,410,187 (19.28) | 5,902,862 |
| 188ASP | Female | 49 | Polyclonal | 8 | 9,208,436 | 2,309,907 (25.08) | 6,898,529 |
| 054ASM | Female | 42 | Monoclonal | ND | 6,597,354 | 1,471,005 (22.30) | 5,126,349 |
| 075ASM | Female | 45 | Monoclonal | ND | 11,351,002 | 2,096,996 (18.47) | 9,254,006 |
| 124ASM | Male | 70 | Monoclonal | 327 | 8,270,885 | 1,327,603 (16.05) | 6,943,282 |
| 143ASM | Female | 65 | Monoclonal | 71 | 5,833,340 | 1,087,647 (18.65) | 4,745,693 |
| 154ASM | Female | 28 | Monoclonal | 163 | 11,211,867 | 1,715,983 (15.31) | 9,495,884 |
| 200ASM | Female | 74 | Monoclonal | ND | 8,077,749 | 1,649,537 (20.42) | 6,428,212 |
| 212ASM | Male | 61 | Monoclonal | 27 | 12,982,061 | 2,715,976 (20.92) | 10,266,085 |
| 002HC | Female | 38 | NA | NA | 3,707,168 | 432,213 (11.66) | 3,285,435 |
| 003HC | Male | 53 | NA | NA | 8,917,856 | 911,050 (10.22) | 8,051,395 |
| 004HC | Female | 37 | NA | NA | 5,078,632 | 847,574 (16.69) | 4,241,255 |
| 005HC | Male | 32 | NA | NA | 2,969,362 | 474,247 (15.97) | 2,504,144 |
| 006HC | Female | 32 | NA | NA | 6,665,538 | 868,400 (13.03) | 5,833,724 |

HTLV-1,Human T-lymphotropic virus type I; ASP, asymptomatic carriers with polyclonal T cell receptor γ gene rearrangement; ASM, asymptomatic carriers with monoclonal T cell receptor γ gene rearrangement ; HCs, healthy controls; ND, not determined; NA, not available; PBMCs, peripheral blood mononuclear cells.

novel sRNA, and mature miRNA. Novel sRNA was identified and classified using the decision tree method with 3-fold validation accuracy according to a previously described model by Langenberger *et al* (48). Distributions of the sRNA data in each clinical condition were conducted according to the quantile normalization algorithm (49), with a baseline transformation set to the median value for all samples. In addition, only sRNA sequences meeting the minimum read coverage criterion of >5 were considered as novel or known sRNA and were included in further analyses. For each sRNA within each group, the Shapiro-Wilk method was used to test for normality of the distribution of the clean data (Shapiro-Wilk P>0.95) and only data meeting this standard were considered for further analysis. sRNAs with fold-changes >2.0 were considered differentially expressed. All the sRNA raw data generated using MPS were deposited in the Zenodo repository (https://doi.org/10.5281/zenodo.1181925).

*Validation of miRNA using RT-qPCR.* A total of two miRNAs, namely Homo sapiens (hsa)-mir-23a-3p (MI0000079) and -363-3p (MIMAT0000707), demonstrating a tendency of strong dysregulation among patients with ASM compared with ASP and HCs according to MPS analysis, were selected and subjected to validation by RT-qPCR. A total of 5 $\mu$l of each enriched miRNA was converted into complementary DNA using a TaqMan™ MicroRNA Reverse Transcription kit (cat. no. 4366596; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was then performed using TaqMan™ Universal Master Mix II (cat. no. 4440040; Thermo Fisher Scientific, Inc.) using a 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). Each reaction, including no-template negative control, was assayed in triplicate. The relative expression levels of miRNA were normalized to the internal control of miR16 (MI0000070). The PCR conditions consisted of uracil-DNA glycosylase activation at 50˚C for 2 min, pre-denaturation and hot-start *Taq* activation at 95˚C for 20 sec, and then 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. Data were quantified using the $2^{-\Delta\Delta Cq}$ method (50). Comparison of the ASM and ASP groups was performed using paired t-tests. P<0.05 was considered to indicate a statistically significant difference.

*Statistical analysis.* Moderated t-test or one-way analysis of variance (ANOVA) followed by Benjamini-Hochberg correction for multiple comparisons of the false discovery rate (FDR) was used for statistical analysis with a *P*(Corr) cut-off <0.05 to indicate statistical significance. Hierarchical clustering was performed using Strand NGS version 3.1 (Strand Life Science).

## Results

*Patient characteristics.* The characteristics of the subjects in the three groups are presented in Table I. All eight participants in the ASP group were female and the median age was 55 years (range, 31-80 years). Five females and two males were assigned to the ASM group and the median age was 61 years (range, 28-74 years). The HC consisted of 5 participants (3 females and 2 males with a median age of 37 years and range, 32-53 years). HTLV-1 proviral load levels varied from one copy to 208 copies/10³ PBMCs in the ASP group and varied

from undetectable to 327 copies/10³ PBMCs in the ASM group (two-tailed P=0.7).

*Whole-genome sRNA sequencing data.* Illumina MPS revealed a total of 153,592,408 reads. After the removal of low-quality data and failed reads, 126,359,328 reads were retained from combined genes that included tRNAs, intronic genes, scRNA pseudogenes, snRNAs, snoRNAs, snoRNA pseudogenes, snRNA pseudogenes, piRNAs, scRNAs, miRNAs and tRNA pseudogenes. The sRNA reads filtered for quality metrics for each sample used in alignment with the human genome sequence dataset are presented in Table I. Illumina MPS yielded 128 sRNA molecules that passed the filter on normality with Shapiro-Wilk (P>0.95), of which 76 and 52 sequences were derived from known and novel sRNAs, respectively. Of the 76 known sRNAs, 41 were miRNAs, 4 scRNAs, 2 scRNA pseudogenes, 6 snoRNAs, 1 was an snRNA, 21 were tRNAs and one was a tRNA pseudogene (Table SI). Of the 52 novel genes, two were novel miRNAs, 6 snoRNAs, 2 tRNAs and 42 were unknown (Table SII). Considering-3p and -5p mature forms of miRNA, the present analysis revealed a total of 54 mature miRNAs that passed the filter on normality with Shapiro-Wilk (P>0.95; Table SIII).

*Analysis of differential sRNA expression in PBMCs between ASM and ASP HTLV-1 carriers and HCs.* To evaluate altered sRNA expression, replicative differential expression analysis was performed between the ASM and ASP groups and HCs subjects. This analysis revealed 28 upregulated and 25 downregulated sRNAs within the PBMCs of the ASM and ASP group compared with the HC group. These 53 molecules included 28 miRNAs, two scRNAs, one scRNA pseudogene, six snoRNAs, 15 tRNAs and one tRNA pseudogene (Table SIV). Furthermore, hsa-mir-155, -23a and -23b, SNORD17 and hsa-mir-21-5p were the top five upregulated sRNAs and hsa-mir-144, -550a-1, -550a-2, -186 and -486 were the five top downregulated sRNAs. The individual analysis of the ASM vs. HC group revealed 28 upregulated and 26 downregulated known genes, satisfying the corrected [FDR corrected p-value ≤0.05] in the ASM group. The annotations of these 54 sRNAs were 29 miRNAs, two scRNAs, one scRNA pseudogene, six snoRNAs, 15 tRNAs and one tRNA pseudogene (Table SV). The most notably upregulated five sRNAs were hsa-mir-23a, hsa-mir-23b, hsa-mir-155, SNORD119 and SNORD100. On the other hand, hsa-mir-550a-2, -550a-1, -144, -486 and -186 were the most notably downregulated sRNAs. The differential analysis of known sRNAs within the PBMCs of the ASP vs. HC group revealed 45 dysregulated sRNAs (20 upregulated and 25 downregulated), consisting of 26 miRNAs, two scRNAs, one scRNA pseudogene, six snoRNAs and 10 tRNAs (Table SVI). The most notably upregulated five sRNAs were hsa-mir-155, -23b, -23a, -21 and SNORD17, while hsa-mir-144, -486, -550a-2, -550a-1 and -186 were among the most significantly downregulated sRNAs (FDR corrected P≤0.05). The Venn diagram representation depicted in Fig. 1 displays the sRNAs differentially expressed in the ASM and ASP vs. HC groups. A total of 44 sRNAs were common differentially expressed sRNAs in the ASM and ASP groups, whereas 10 were exclusively dysregulated in the ASM group (hsa-mir-378a, 590, 99b, trna17, trna35, trna2, trna37, trna39,
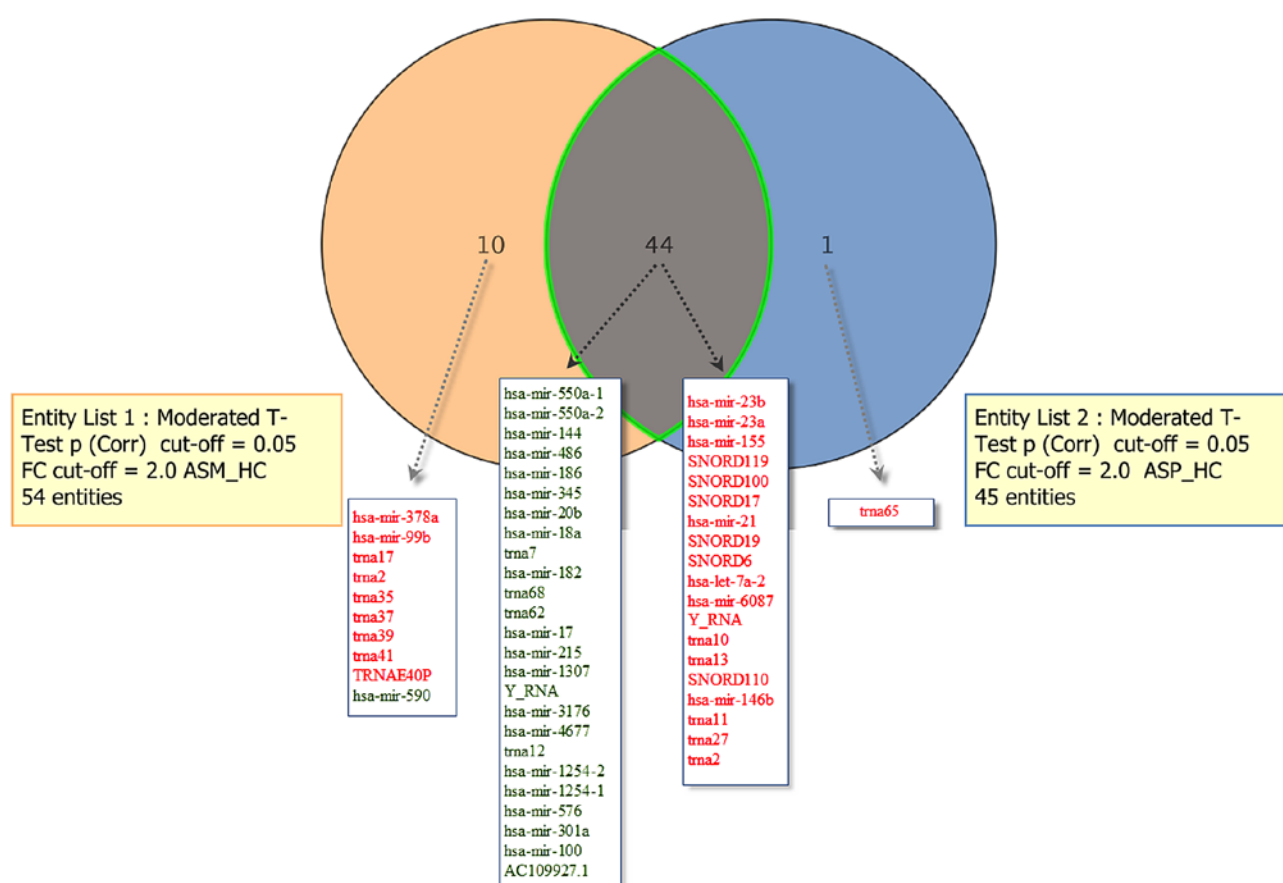
Figure 1. Comparisons of significantly dysregulated known sRNAs in peripheral blood mononuclear cells of patients with asymptomatic human T-lymphotropic virus type I. The Venn diagram indicates the number of sRNAs differentially expressed in entity list 1 (ASM vs. HC), entity list 2 (ASP vs. HC) and in both. For each entity, the sRNAs elevated (indicated in red) and downregulated (indicated in green) are indicated within the diagram. ASM, asymptomatic carriers with monoclonal T cell receptor γ gene rearrangement; ASP, asymptomatic carriers with polyclonal T cell receptor γ gene rearrangement; HC, healthy control; Corr, corrected; miR, microRNA; trna, transfer RNA; snoRNA, small nucleolar RNA;TRNAE40P, transfer RNA glutamic acid 40 (anticodon UUC) pseudogene; FC, fold-change; ASM_HC, ASM vs. HC; ASP_HC, ASP vs. HC; sRNA, small RNA; hsa, *Homo sapiens*.

trna41 and the transfer RNA glutamic acid 40 (anticodon UUC) pseudogene (TRNAE40P)) and one (trna65) was significantly upregulated in the ASP group.

Of the 52 novel genes, 37 reached an FDR significant value (FDR corrected P≤0.05] in the comparison of ASM and ASP vs. the HC group, of which 10 were upregulated and 27 down-regulated (Table SVII). A total of 28 of these 37 significantly dysregulated novel sRNAs (73.7%) have never been reported previously, to the best of our knowledge. The most significantly downregulated gene was annotated as NEWGENE156 [chromosome (chr)6, 8164298-8164349], followed by NEWGENE671 (chr20, 57414179-57414214). One miRNA (chr8, 136785981-136786100) followed by one snoRNA (chr1, 173836772-173836882) were significantly upregulated novel genes (both FDR corrected P≤0.05).

In the aforementioned results, the differential expression profiles of the sRNAs in the HTLV-I-infected groups (ASM and ASP) compared with the HC group were analyzed. It was then determined whether there is a significant change in the expression of known and novel sRNAs between the ASM and ASP groups. A pairwise comparison of the detected sRNAs between the two groups was performed using a moderated t-test with FDR corrected P≤0.05. The results revealed no significant difference in sRNA expression between the two groups (data not shown).

The extent to which the 53 and 37 known and novel sRNAs, respectively, were distinctly different in HTLV-I ASM and ASP carriers from HCs were explored via hierarchical clustering, which revealed a clear partition between these groups with all HC samples clustering within their group (Figs. 2and S1). The same cluster analysis of individual cases indicated no clearly distinct differences in RNA expression profiles between the ASM and ASP samples.

*Differential analysis of mature miRNA expression in PBMCs between HTLV-1 ASM and ASP carriers and HCs*. After correction for multiple testing using the Benjamini-Hochberg method, the replicative differential analysis of the 54 mature miRNAs was distributed normally following a Shapiro-Wilk test (P>0.95) between the HTLV-1 ASM and ASP carriers and HC group, and revealed 16 significantly upregulated and 14 significantly downregulated miRNAs [one-way ANOVA, FDR corrected P≤0.05; Table SVIII). The top five most upregulated mature miRNAs were hsa-mir-23a-3p and -28-5p, hsa-let-7e-5p, hsa-mir-28-3p and 361-5p, while the top five most downregulated miRNAs were hsa-miR-363-3p, -532-5p, -106a-5p, -25-3p and -30e-5p. The individual analysis of the mature miRNAs of the ASM vs. HC and ASP vs. HC groups yielded identical results to the replicative analysis.
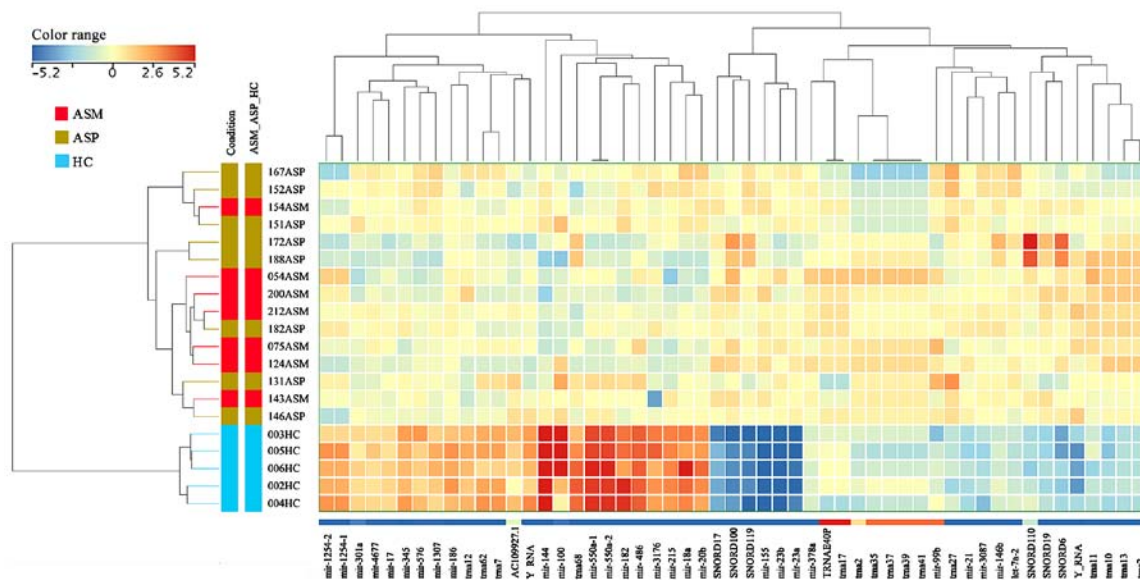
Figure 2. Unsupervised hierarchical clustering of sRNAs and samples from the Illumina MPS data. The heat map contains 53 unique and known sRNAs, which were differentially expressed in patients with ASM and ASP human T-lymphotropic virus type I. The sample clustering tree is displayed to the left and the sRNA clustering tree is above. The color scale at the top indicates the relative expression levels of sRNA across all samples. Red indicates that the expression levels are higher compared with the mean, whereas blue indicates that the expression levels are lower compared with the mean. Each column represents one known sRNA and each row represents one sample. ASM, asymptomatic carriers with monoclonal T cell receptor γ gene rearrangement; ASP, asymptomatic carriers with polyclonal T cell receptor γ gene rearrangement; HC, healthy control; miR, microRNA; trna, transfer RNA; snoRNA, small nucleolar RNA; TRNAE40P, transfer RNA glutamic acid 40 (anticodon UUC) pseudogene; ASM_ASP_HC, ASM vs. ASP vs. HC.

A total of 3 downregulated (hsa-miR-320b, -1468-5p and -4772-3p) and 2 upregulated (hsa-miR-339-5p and -625-3p) mature miRNAs were exclusively detected in the ASP group (Fig. 3). A Venn diagram representation of the number of significantly differentially expressed mature miRNAs overlapping between the ASM and ASP groups is provided in Fig. 3. The results also suggested that all of the 30 miRNAs significantly dysregulated in the ASM libraries were also significantly dysregulated in the ASP libraries. In other relaxed analysis, we used all mature miRNAs with 2-fold or greater change and a corrected p-value of <0.001 regardless of their normal distribution. The results revealed 144 highly dysregulated mature miRNA in PBMCs of ASM and ASP when compared to HC subjects as shown in Table SIX.

*miRNA validation using RT-qPCR.* To validate the expression levels of miRNAs among the three compared groups by RT-qPCR, two miRNAs (hsa-miR-23a-3p and -363-3p) were selected based on their expression profiles. However, the results revealed no significant differences in miRNAs expression among the three groups (P>0.05; data not shown).

## Discussion

In the present pilot study, Illumina high-throughput sequencing technology was used to analyze the global expression of the non-coding RNome in PBMCs of ASM, ASP, and HCs groups. Significant dysregulation of 76 known sRNAs and 52 putative novel sRNAs was detected. Of these, 44 and 34 sRNAs were known and potential novel sRNAs, respectively, and were commonly and differentially dysregulated in the ASM and ASP libraries when compared with HCs. In addition, 10 known sRNAs were exclusively dysregulated in the ASM group and

one (trna65) was exclusively and significantly upregulated in the ASP group. On the other hand, analysis of the 54 mature miRNAs between the ASM and ASP versus HC group revealed significantly 16 up-regulated and 14 down-regulated entities. Of these, hsa-mir-23a-3p and 363-3p, were selected for the validation among the three groups. However, subsequent RT-qPCR analyses of both miRNAs revealed no significant difference between the analyzed subgroups. As there were no differences between the samples used in the MPS experiment and the consequent validations, the discrepancies in miRNA quantification may have been caused by the small sample size available for validation using RT-qPCR or by the MiSeq platform. Other possible explanations for these differences include the enzymatic reactions and amplification step performed during sample preparation, as well as the RT-qPCR probe design (51-53). For the MPS assay, the integrity of RNA and precision of miRNA band excision from the gel during library preparation may impact the detection of miRNA and may have resulted in the detection of the precursor as mature miRNA (54). These factors could be minimized in future studies by optimizing PCR conditions and using high quality primer and probe, and by developing a robust methods for MPS library preparation.

As there is growing interest in personalized medicine and an individualized multimodality approach to treatment, it is important to identify early biomarkers to more accurately identify leukemic cells. Clonal changes have been detected in ~10% of cases progressing from asymptomatic HTLV-I infection to overt acute ATLL, and this may reflect the emergence of multiple premalignant clones in viral leukemogenesis as proposed in Epstein-Barr virus-associated lymphomagenesis (55,56). After infection, HTLV-I mediates the cellular transformation of primary human T cells through a multistep
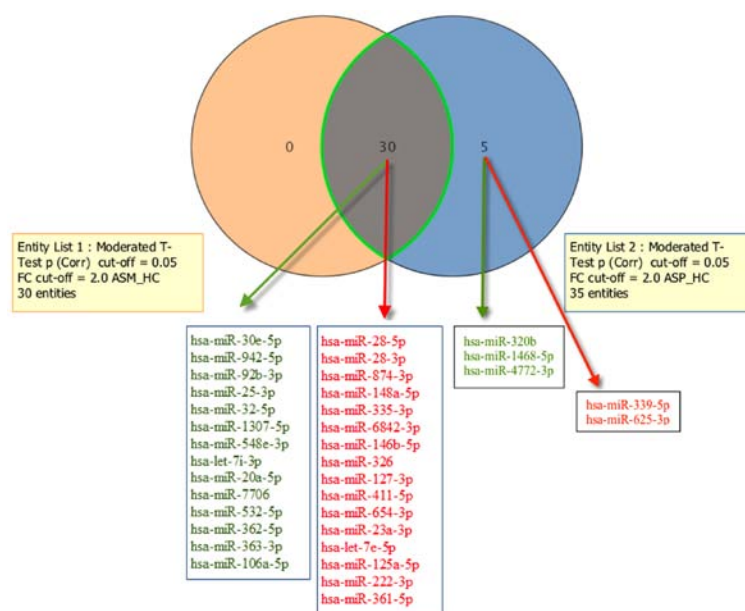
Figure 3. Comparisons of significantly dysregulated mature miRNA expression in peripheral blood mononuclear cells of patients with asymptomatic human T-lymphotropic virus type I. The Venn diagram indicates the number of mature miRNAs differentially expressed in entity list 1 (ASM vs. HC), entity list 2 (ASP vs. HC), and in both. For each entity, elevated miRNAs (indicated in red) and downregulated miRNAs (indicated in green) are indicated within the diagram. ASM, asymptomatic carriers with monoclonal T cell receptor γ gene rearrangement; ASP, asymptomatic carriers with polyclonal T cell receptor γ gene rearrangement; FC, fold-change; Corr, corrected; miR, microRNA; ASM_HC, ASM vs. HC; ASP_HC, ASP vs. HC.

process in which the virus induces genomic instability, thus ultimately promoting the accumulation of genetic mutations and enhancing the chronic proliferation of infected cells (57). It has been proposed that, during infection, the HTLV-I virus dysregulates the host cellular RNA interference pathway, including miRNAs, by different mechanisms, including suppression and degradation of Drosha through binding to the tax protein (58), or by inhibition of Dicer activation through direct interaction with the viral rex protein (59). Previous studies have identified multiple miRNAs that are significantly differentially expressed in ATLL cell lines and patients with ATLL infected with HTLV-I (35,36,42,60). However, studies concerning sRNA expression alterations in response to HTLV-I infection during chronic infection in asymptomatic carriers are lacking.

Using an MPS approach, the present study identified differential sRNA expression profiles between ASM and ASP versus HCs group. However, the present study failed to identify a significant predictive impact of these miRNAs using RT-qPCR analyses. The following significantly differentially expressed miRNAs were identified as possible predictive factors in asymptomatic HTLV-I carriers with monoclonal T cell receptor γ gene rearrangement: hsa-miR-26a-5p, -23a-3p, -144-3p, -146a, -28-5p, -23b-3p, -363-3p, -532-5p, -93-5p, -155-5p and -185-5p. The expression profiles of several of these miRNAs are consistent with those of previous studies and are involved in various cellular processes, including cell proliferation, differentiation and apoptosis. For instance, consistent with a previous study by Pichler *et al* (35), who examined the expression of miRNAs in HTLV-1-transformed cells, the present results obtained with clinical samples demonstrated upregulation of miR-21, miR-146a and miR-155 in PBMCs in HTLV-I-infected carriers regardless of their mode of T-cell γ gene rearrangements. Of note, the

same three miRNAs, together with miR-27a, reported in the present study (Table SIX), were upregulated in Epstein-Barr virus-infected B cells during latency III, the viral growth program that induces B-cell proliferation (61). The upregulation of miR-146a, miR-21 and miR-155 in uninfected CD4$^+$ T cells occurs after activation through the T-cell receptor (62). Tomita *et al* (63) altered the levels of miR-146a in HTLV-I-infected T-cell lines and observed that the inhibition of miR-146a by a specific antagomir in these cells elicited an inhibitory effect on cell proliferation, and the opposite effect was induced by forcing HTLV-I-infected T-cell lines to over-express miR-146a. In addition, the present study demonstrated a significant downregulation of miR-19a and suggested that miR-26a-5p was one of the most highly expressed miRNAs. Consistent with these results, Yeung *et al* (36) reported significant upregulation of miR-19a and downregulation of miR-26a in HTLV-I-transformed cell lines. As miR-26a is located in cancer-associated genomic regions, it may have a dual role in tumorigenesis and may act as a tumor suppressor (64-68) as well as oncogenic miRNA (69). It has been demonstrated that upregulation of miR-26a is able to phosphorylate mutant p53 and restore the wild-type function, thereby resulting in $G_0/G_1$ phase arrest and reducing the proliferation of tumor cells in pancreatic cancer (70). Furthermore, previous studies indicated the involvement of miR-26a in multiple biological pathways, including proliferation (71), invasion (65), differentiation (72,73), angiogenesis (74,75) and energy metabolism (75). The same patterns of miR-19a and miR-26a dysregulation observed in HTLV-I-transformed cell lines have been documented in B-cell chronic lymphocytic leukemia (76,77) and epithelial cancer types (78). Furthermore, miR-19a has been demonstrated to target a tumor suppressor gene, phosphatase and tensin homolog (PTEN), in CD5$^+$ and chronic lymphocytic

leukemia cells (76,79). Of note, it has been indicated that PTEN is downregulated by HTLV-I tax through the NF-κB signaling pathway (80).

In the present study, miR-144-3p was the most notably downregulated miRNA, consistent with previous studies reporting its low expression in chronic myeloid leukemia (81), acute myeloid leukemia (82) and in the PBMCs of patients with HTLV-I as well as ATLL cell lines (36). In addition, miR-28-5p was another highly expressed miRNA in the present study. This miR has been reported to inhibit HTLV-I virus expression and its replication, except ATK1 strains, by targeting a specific site within the genomic gag/pol viral mRNA (83). Taking into consideration the aforementioned data, it was hypothesized that the dysregulated miRs detected in the present study have important functions in the pathology of HTLV-I infection.

Previous studies have demonstrated that snoRNAs are important in the regulation of cell fate and tumorigenesis (84,85). The present study identified six significantly upregulated snoRNAs (snoRD17, snoRD119, snoRD100, snoRD19, snoRD6 and snoRD110). At present, the understanding of the biological and clinical implications of these results is limited; however, an improved understanding of these molecules may highlight their value as diagnostic markers or therapeutic targets. Validation using large clinical samples is required to confirm the biological function of the dysregulated snoRNAs identified in the present study.

Finally, 37 significantly dysregulated novel sRNAs were identified, including two molecules that met the current miRNA criteria, 1 tRNA, 6 snoRNAs, and 28 unknown sRNAs. Validation and future analysis will be required to address the regulatory mechanisms and biological effects of these small molecules.

The present pilot study has several limitations, particularly regarding its retrospective design with a small number of patients. Thus, future studies with larger sample sizes are required to validate these results. Despite these caveats, these data provide various known and novel subsets of dysregulated sRNAs, including a signature in the PBMCs of the ASM group. sRNAs with aberrant expression may be of value as diagnostic and prognostic biomarkers and/or therapeutic targets in early HTLV-I pathogenesis and it is therefore important to investigate the molecular mechanisms underlying their function.

In conclusion, the present pilot study revealed no significant changes in expression for the selected miRNAs using separate RT-qPCR analysis; however, the MPS results yielded differential sRNA expression profiles. Therefore, further investigation with a larger number of patients is required to elucidate the exact pathogenic impact of these ncRNA molecules in HTLV-I infection.

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## Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the Zenodo repository (https://doi.org/10.5281/zenodo.1181925).

## Authors' contributions

SSS conceived the study and designed the experiments. DRVS, RP, AN, PBC and SSS performed the experiments. DRVS, RP, PBC and SSS analyzed the data. DRVS, RP, YN, JP, ACPO, AJSD, JC, PBC, and SSS analyzed the data and involved in drafting the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The collection and the use of all samples was approved by the Ethics Committee for Review of Research Projects (CAPPesq; approval no. 1235/2017) All patients provided informed written consent.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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