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Research paper

Circulating miR-29c, miR-30c, miR-193a-5p and miR-885-5p: Novel potential biomarkers for HTLV-1 infection diagnosis

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) is an oncoretrovirus that infects 5–10 million people worldwide. Currently, different methods are used to test HTLV-1 infection. However, a biomarker that could enable an early and accurate diagnosis of HTLV-1 infection is still lacking. Here, we compared the serum miRNA expression profile in HTLV-1 infected patients versus healthy individuals to identify a potential biomarker for diagnosis of HTLV-1 infection.TaqMan miRNA microarray (TLDA) was carried out to compare the miRNA expression profile in infected versus healthy individuals. Quantitative real-time RT-PCR (qRT-PCR) was applied to validate TLDA results. Receiver-operator characteristic (ROC) curve analysis was performed to determine the diagnostic accuracy of the most highly and significantly identified deregulated miRNA(s) as potential biomarker(s). We identified deregulated expression for ten miRNAs with miR-127, miR-136, miR-142-3p, miR-221, and miR-423-5p being down-regulated whilst let-7b, miR-29c, miR-30c, miR-193a-5p, and miR-885-5p being up-regulated in infected individuals. ROC curve analyses showed an AUC (Areas Under the ROC Curve) of 0.875 (95% CI: 0.7819–0.9581; $P = .0021$), 0.861 (95% CI: 0.7596–0.9754; $P = .003$), 0.856 (95% CI: 0.689–0.895; $P = .011$), and 0.849 (95% CI: 0.678–0.855; $P = .017$) for miR-29c, miR-30c, miR-193a-5p, and miR-885-5p respectively. Combined ROC analyses using these 4 miRNAs showed a greater AUC of 0.907 (95% CI: 0.809–1; $P = .000001$) indicating a robust diagnostic value of these 4 miRNAs. Our findings highlight serum miR-29c, miR-30c, miR-193a-5p and miR-885-5p as novel potential biomarkers important for HTLV-1 diagnosis.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1), an oncogenic retrovirus, was first idetified, almost 40 years ago, in United states and Japan and is estimated to infect about 20 million people worldwide (Gessain and Cassar, 2012). HTLV-1 can trigger malignant transformation of CD4+ T cells leading to severe leukemia/lymphoma designated Adult T-cell Leukemia/Lymphoma (ATL) disease (Poiesz et al., 1980). HTLV-1 genome consists of long terminal repeats (LTRs), *gag*, *pol*, *env* genes, encoding structural proteins, and extra ORFs including those encoding the transcriptional transactivator (Tax) and post- transcriptional regulatory protein (Rex) (Kashanchi and Brady, 2005). HTLV-1 infection can occur due to a sexual intercourse, vertical transmission, blood transfusion and intravenous drug injection (Gonçalves et al., 2010). While the majority of HTLV-1 infected individuals remain asymptomatic throughout their life, a significant percentage of HTLV-1 infected patients develop symptoms of distinct diseases, mainly HTLV-1 associated myelopathy (HAM/TSP) and ATL (Gonçalves et al., 2010). High proviral load, lymphocyte activation and exaggerated inflammatory response are all markers of HTLV-1-associated diseases (Starling et al., 2015). So far, no gold standard test exists for HTLV infection diagnosis. The most common used screening strategies are enzyme-linked immunoassay (EIA), Western blot (WB), immunofluorescence assay (IFA), and particle agglutination (PA). However, these screening tests might give indeterminate results in certain conditions such as the window period, virus variants, and non-specific reaction of the patient's antibodies to viral antigens (Thorstensson et al., 2002). Polymerase Chain Reaction (PCR) test which detect proviral DNA can be used as a confirmatory test (Proietti et al., 2005). Biomarkers for diagnosis of microbial infection have been widely described (Kapasi et al., 2016; Yin et al., 2018; Yusa et al., 2017). However, till date, no specific biomarker for HTLV-1 infection has been reported. MicroRNAs (miRNAs) are short

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(19–22-nt) single-stranded non-coding RNAs capable of regulating a wide range of developmental and physiological processes upon directly binding to their target sites in the 3′ untranslated region (3'UTRs) of certain target mRNAs, leading to mRNA degradation or translation inhibition (Bartel, 2004). Dysregulation of miRNA expression has been associated with the emergence of different diseases including cancer (Tüfekci et al., 2014). Circulating miRNAs and exosomal miRNAs have emerged as useful diagnostics biomarkers for diseases and viral infections (Correia et al., 2017; Drury et al., 2017; Wang et al., 2016).

In this study, we aimed at identifying putative miRNAs with a potential to be used in the diagnosis of HTLV-1 infection. Following assessement of circulating miRNAs profile in the serum of healthy and HTLV-1 infected individuals, our results revealed several dysregulated miRNAs among which miR-29c, miR-30c, miR-193a-5p, and miR-885-5p that were upregulated in infected individuals, emerged as potential biomarkers for HTLV-1 infection at diagnosis stage based on ROC curve analysis.

2. Materials and methods

2.1. Patients and healthy controls

Blood samples were obtained from six HTLV-1 infected patients at the time of diagnosis. Healthy individuals were collected as negative controls where none of these controls had previously been diagnosed for HTLV-1 infection. This study was conducted in accordance with the Declaration of Helsinki (1964) and approved by the local ethics committee of the "Lebanese University". All donors gave written informed consent.

2.2. Serum sampling and RNA extraction

Total RNA was isolated from the serum using a mirVana PARIS isolation kit (Ambion, Austin, Texas) according to the manufacturer's instructions for serum samples. Briefly, 400 µl of human serum was used to extract total RNA. Each sample was eluted in 100 µL of RNAse-free water and was concentrated to a final volume of 20 µl by using Eppendorf Concentrator Plus 5301 (Eppendorf, Germany). RNA sample concentration was quantified by NanoDrop ND-1000 (Nanodrop, USA). All RNA samples were analyzed for miR-16 expression, a stable endogenous reference miRNA, to assess an approximate yield of RNA extraction and to ensure that comparable amounts of starting material were used in each reverse transcription reaction.

2.3. MiRNA expression profile

The method was previosuly described by Fayyad-Kazan et al. (2013). A three-step procedure was carried out to profile the miRNAs in the serum samples. First, for cDNA synthesis from the miRNAs, 30 ng of total RNA was subjected to RT (reverse transcription) using a TaqMan® microRNA Reverse Transcription Kit (#4366596; Applied Biosystems) and Megaplex RT primers (Human Pool A, #4399966; Applied Biosystems) following the manufacturer's protocol, allowing simultaneous reverse transcription of 380 mature human miRNAs to generate a miRNA cDNA library corresponding to each serum sample. RT was performed on a Mastercycler Epgradient thermocycler (Eppendorf) with the following cycling conditions: 40 cycles at 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s followed by a final step of 80 °C for 5 min to inactivate reverse transcriptase. Thereafter, to generate enough miRNA cDNA template for the following real-time PCR, the cDNA libraries were pre-amplified using Megaplex PreAmp primer (Humam Pool A, #4399233; Applied Biosystems) and PreAmp Master Mix (#4384266; AppliedBiosystems) following the manufacturer's instructions. The PreAmp primer pool used here consisted of forward primers specific for each of the 380 human miRNAs and a universal reverse primer. The pre-amplification cycling conditions were as follows: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min followed by 12 cycles at 95 °C for 30 s and 60 °C for 4 min; the samples were then held at 99.9 °C for 10 min. After the preamplification step, the products were diluted with RNase-free water, combined with TaqMan gene expression Master Mix and then loaded into TaqMan Human MicroRNA Array A (#4398965; Applied Biosystems), which is a 384-well formatted plate and real-time PCR-based microfluidic card with embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs; MiR-16 transcript was used as a normalization signal.

Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the following cycling conditions: 50 °C for 2 min, 94.5 °C for 10 min followed by 40 cycles at 95 °C for 30 s and 59.7 °C for 1 min. The Ct (cycle threshold) was automatically given by SDS 2.3 software (Applied Biosystems) and is defined as the fractional cycle number at which the fluorescence passes the fixed threshold of 0.2. MiR-16 embedded in the TaqMan Human MicroRNA Arrays was used as an endogenous control. The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method as described previously (Schmittgen and Livak, 2008). The fold changes in miRNAs were calculated by the eq. $2^{-\Delta\Delta Ct}$.

Table 1
Differentially expressed plasma miRNAs in infected versus Healthy objectes as identified by TLDA technique.

MicroRNA	HTLV/Healthy donor ratio	*P* value
hsa-miR-337	70.55	0.012
hsa-miR-135b	55.2	0.035
hsa-miR-193a-5p	50	0.005
hsa-miR-224	42.5	0.0225
hsa-miR-29c	40.2	0.000136
hsa-miR-143	37.44	0.015
hsa-miR-30c	35.7	0.00017
hsa-miR-885-5p	35.2	0.00435
hsa-miR-155	31.5	0.025
hsa-miR-132	26.5	0.044
hsa-let-7b	25.5	0.041
hsa-miR-186	20.5	0.011
hsa-miR-215	17.2	0.031
hsa-miR-199a	15.35	0.035
hsa-miR-340	0.0461	0.026
hsa-miR-423	0.0067	0.0239
hsa-miR-19a	0.0045	0.025
hsa-miR-191	0.0031	0.0155
hsa-miR-127	0.0018	0.0182
hsa-miR-205	0.0012	0.0143
hsa-miR-146b	0.0007	0.031
hsa-miR-142-3p	0.0007	0.0153
hsa-miR-136	0.00005	0.022
hsa-miR-221	0.00001	0.028

2.4. Taqman miRNA assay for individual miRNAs

The method was previosuly reported by Fayyad-Kazan et al. (2013). Gene-specific reverse transcription was performed for each miR using 10 ng of purified total RNA, 100 mM dNTPs, 50 U MultiScribe reverse transcriptase, 20 U RNase inhibitor, and 50 nM of gene-specific RT primer samples using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium). 15 µl reactions were incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C to inactivate the reverse transcriptase. Real time RT-PCR reactions (5 µl of RT product, 10 µl TaqMan 2× Universal PCR master Mix, (Applied Biosystems, Gent, Belgium), and 1 µl TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes) were carried out on ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Gent, Belgium) at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The qRT-PCR reactions were performed in triplicate, and the signal was collected at the end of every cycle. Due to a lack of generally accepted standards, all qRT-PCR data on single miRNA expression were

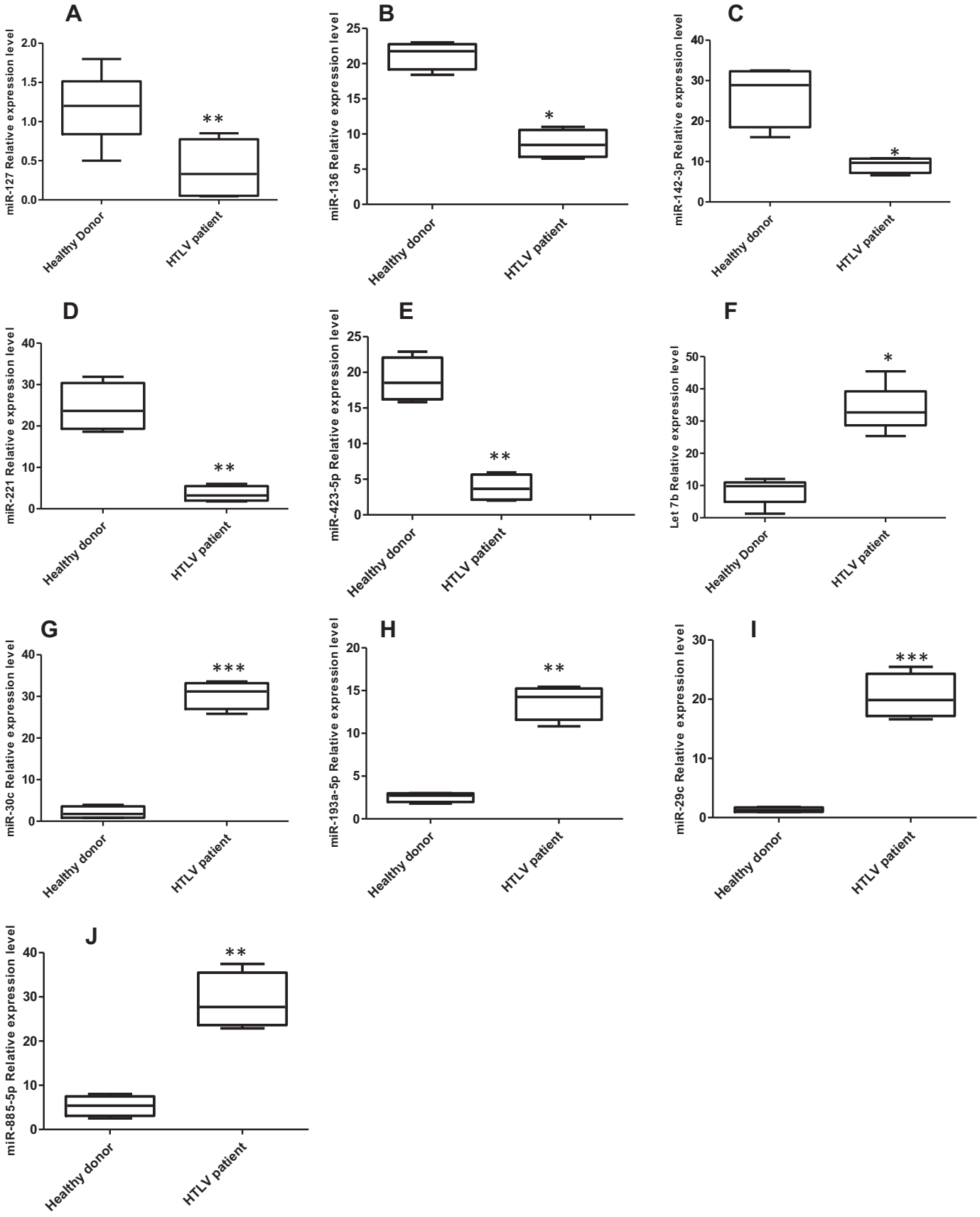

Fig. 1. Relative expression levels of differentially expressed miRNAs in serum of infected versus healthy individuals. Serum was derived from 12 individuals (6 HTLV-1 infected pateints and 6 helathy donors). The relative expression of miRNAs was determined by quantitative RT-PCR and plotted. Boxes correspond to standard error (SE). Error bars represent standard deviation (SD); $*p < .05$, $**p < .01$ infected patient versus Healthy individuals (Student's *t*-test).

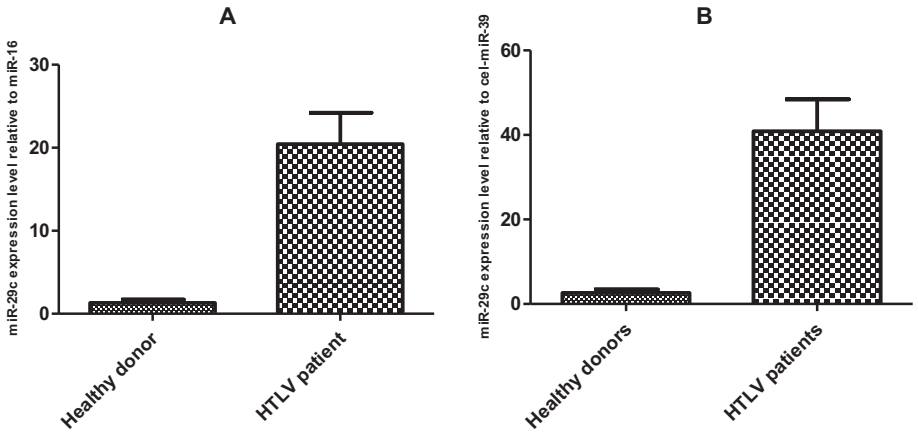

Fig. 2. miR-16 expression level is stable in both healthy controls and HTLV patients. MiR-16 expression levels were assessed by qRT-PCR and normalized by cel-miR-39. Shown are the relative levels (mean ± S.D.) of five independent experiments performed on all participants, each done in triplicate.

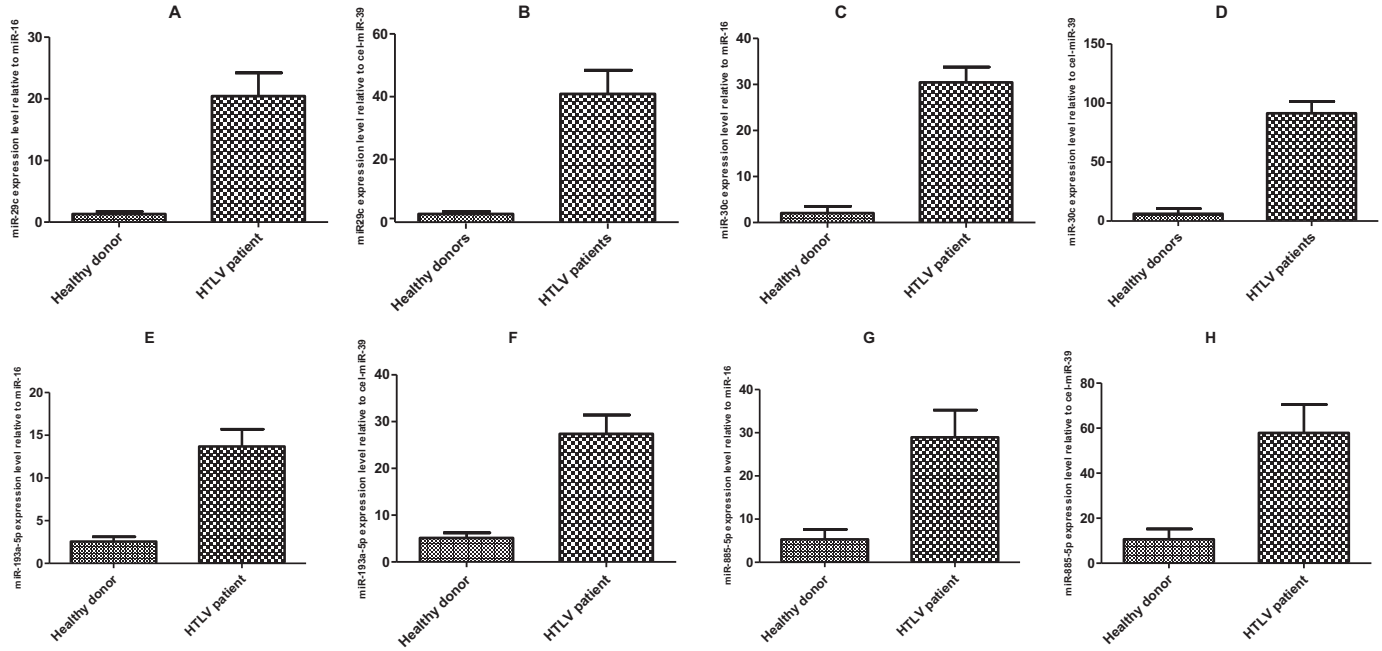

Fig. 3. Relative serum miR-29c, miR-30c, miR-193a-5p, and miR-885-5p expression levels normalized by cel-miR-39 and hsa-miR-16. MiR-29c (A, B), miR-30c (C, D), miR-193a-5p (E, F), and miR-885-5p (G, H) expression levels were assessed by qRT-PCR and normalized by cel-miR-39 and hsa-miR-16, respectively in healthy controls and HTLV patients. Shown are the relative levels (mean ± S.D.) of five independent experiments performed on all participants, each done in triplicate. Statistical significance was determined by Student's *t*-test and is denoted as follows: ** $p < .01$ versus healthy donors.

analyzed as unadjusted Ct values and standardized to miR-16. The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method as described previously (Schmittgen and Livak, 2008). The fold changes in miRNAs were calculated by the eq. $2^{-\Delta\Delta Ct}$.

2.5. Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk normality tests showed that our data is characterized by a normal distribution and was expressed as the mean and standard deviation. Widely presented using the $2^{-\Delta\Delta Ct}$ method, the relative gene expression involves the gene of interest data (Ct gene of interest) relative to an internal control gene (Ct internal control gene), named delta Ct. The calculated delta Ct ± SD for the patients was compared with the delta Ct ± SD (SD stands for the standard deviation of the average delta Ct of the group) for the healthy control group and tested for statistical significance. Sensitivity, specificity, and the area under the curve (AUC) for serum miRNAs were determined using Receiver Operator Characteristic (ROC) analysis. Data was analyzed by unpaired Student's *t*-test. *P*-values < .05 (*), < 0.01 (**), and < 0.001(***) were considered statistically significant.

3. Results

3.1. Circulating miRNA expression profiling in the serum of HTLV-1 infected patients

In a first step, twelve individuals were recruited (six HTLV-1 infected patients and six healthy controls). TaqMan low-density array (TLDA) was carried out in order to identify miRNAs differentially expressed in the serum of infected patients versus healthy individuals. Our TLDA analysis revealed that 24 miRNAs were significantly differentially expressed between infected patients and healthy controls (Table 1).

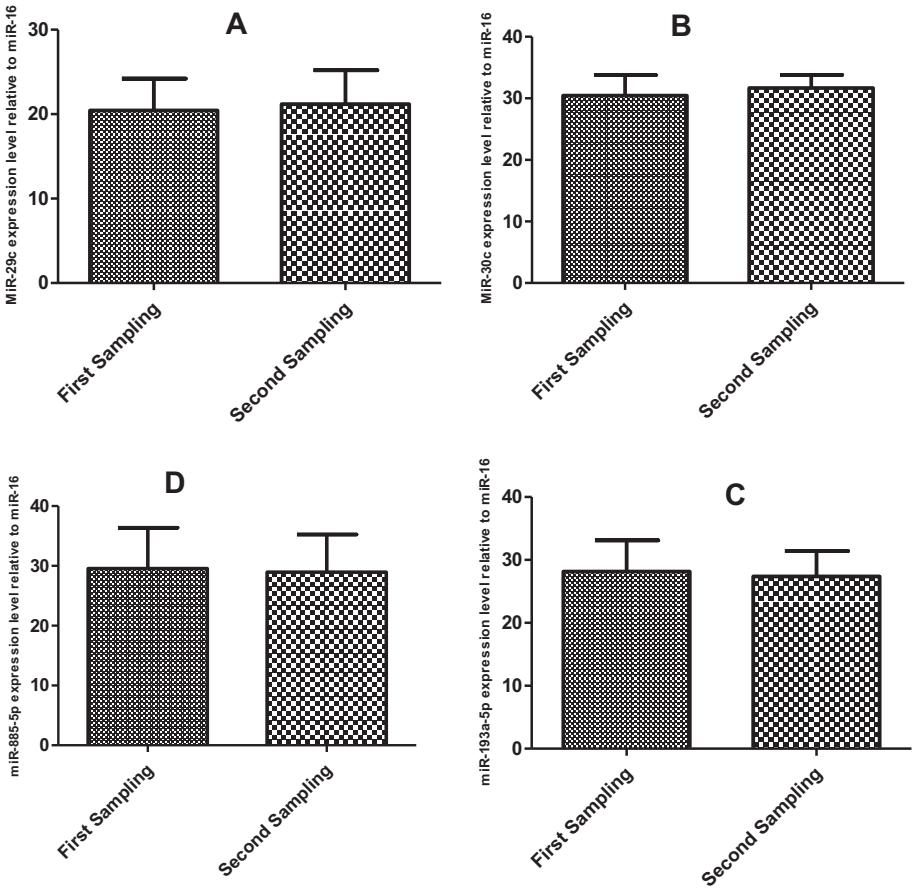

Fig. 4. miR-29c, miR-30c, miR-193a-5p, and miR-885-5p expression levels do not vary between the first and second sampling. Serum miR-29c, miR-30c, miR-193a-5p, and miR-885-5p expression levels in the first sampling and second sampling were assessed by qRT-PCR. The expression level of these four microRNAs was normalized to miR-16. Shown are the relative levels (mean ± S.D.) of five independent experiments performed on all participants, each done in triplicate.

3.2. Validation of differential miRNA expression in infected patients

These identified deregulated miRNAs were checked further, using quantitative Real Time PCR (qRT-PCR), to validate their differential expression in all samples. Fig. 1 represents the change in expression pattern for the candidate miRNAs in infected patients versus the healthy donors. Noteworthy that the expression level of miR-16, a commonly used endogenous reference miRNA that was also confirmed to be unchanged in our experiments (TLDA cards), was used to normalize our data. Moreover, miR-16 stability was comparable to that of cel-miR-39 (Fig. 2). Further, and upon comparing the difference in the expression level of the above confirmed differentially expressed miRNAs, especially miR-29c, miR-30c, miR-193a-5p and miR-885-5p, on which our study was focused due to their highly significant deregulation, between infected patients and healthy controls, we obtained a similar output regardless of whether data was normalized against cel-miR-39 or miR-16 (Fig. 3), which further corroborate the stability of miR-16 reference in this study. Infected patients-derived serum was characterized by downregulated expression of miR-127, miR-136, miR-142-3p, miR-221, and miR-423-5p but upregulation of let-7b, miR-29c, miR-30c, miR-193a-5p, and miR-885-5p in comparison to healthy controls (Fig. 1). To confirm further that our assay is reproducible, we analyzed miRNA expression patterns in serum (second sampling) obtained 1 h after the first sampling of the serum derived from the six infected patients. Interestingly, the expression levels of the above mentioned miRNAs showed no significant difference between the first and second sampling, indicating that the assay was reproducible (Fig. 4).

3.3. Diagnostic accuracy of serum miR-29c and miR-30c in HTLV-1 infected patients

We used the ROC curve analysis to assess the diagnostic accuracy of the most statistically significant and differentially expressed serum miRNAs, (miR-29c, miR-30c, miR-193a-5p, and miR-885-5p). This analysis revealed that miR-29c, miR-30c, miR-193a-5p, and miR-885-5p could serve as valuable biomarkers for differentiating HTLV-1 infected patients from healthy individuals with an AUC (the areas under the ROC curve) of 0.875 (95% CI: 0.7819–0.9581; $P = .0021$) (Fig. 5, Panel A), 0.861 (95% CI: 0.7596–0.9754; $P = .003$) (Fig. 5, Panel B), 0.856 (95% CI: 0.689–0.895; $P = .011$) (Fig. 5, Panel C), and 0.849 (95% CI: 0.678–0.855; $P = .017$) respectively (Fig. 5, Panel D).

At the cut-off value > 40,962.72 for miR-29c, the sensitivity and the specificity were 82.9% and 89%, respectively. At the cut-off value > 23,480.59 for miR-30c, the sensitivity and the specificity were 82% and 87%, respectively. At the cut-off value > 0.155 for miR-193a-5p, the sensitivity and the specificity were 83.3% and 87%, respectively. At the cut-off value > 0.405 for miR-885-5p, the sensitivity and the specificity were 83% and 87.9%, respectively.

Combination ROC analyses resulted in an increased AUC of 0.907 (95% CI: 0.809–1; $P = .000001$) with 89% sensitivity and 88% specificity at a cut-off value > 0.299 indicating the additive effect in the diagnostic value of these 4 miRNAs (Fig. 5E).

4. Discussion

Early, rapid and accurate diagnosis of HTLV-1 infection is highly importat to guide therapeutic decisions to control viral replication and prevent disease development. Currently, HTLV-1 diagnosis is mainly based on detection of specific antibodies against the virus in the blood or cerebrospinal fluid. In certain cases, techniques that detect HTLV-I

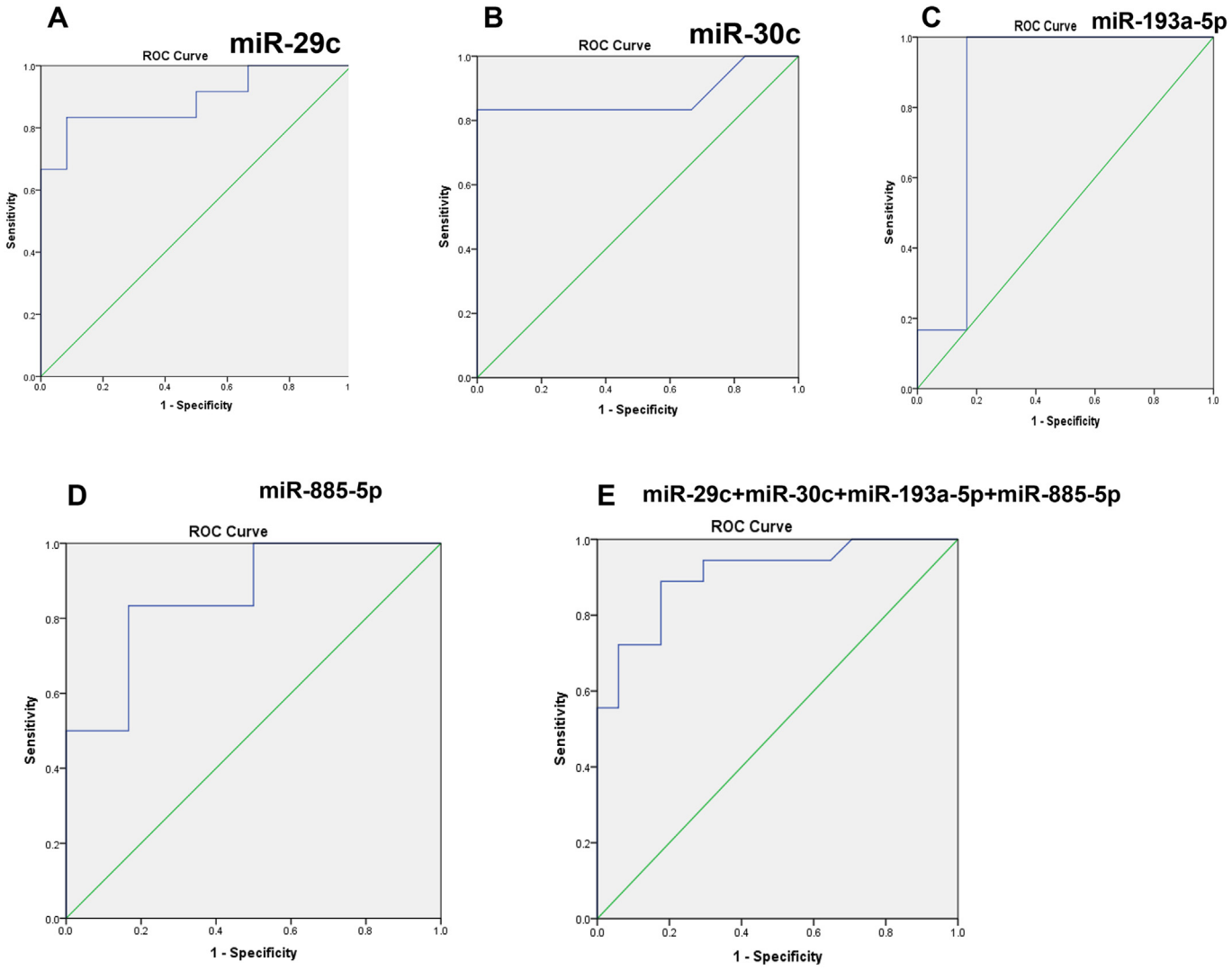

Fig. 5. Receiver operating characteristics (ROC) curve analysis using miR-29c, miR-30c, miR-193a-5p, and miR-885-5p for discriminating HTLV-1 infected patients. Serum miR-29c yielded an AUC of 0.875 (95% CI: 0.7819–0.9581; $P = .0021$) with 82.9% sensitivity and 89% specificity in discriminating infected patients (Panel A). Serum miR-30c yielded AUC of 0.861 (95% CI: 0.7596–0.9754; $P = .003$) with 82% sensitivity and 87% specificity in discriminating infected patients (Panel B). Serum miR-193a-5p yielded an AUC of 0.856 (95% CI: 0.689–0.895; $P = .011$) with 83.3% sensitivity and 87% specificity in discriminating infected patients (Panel C). Serum miR-885-5p yielded and AUC of 0.849 (95% CI: 0.678–0.855; $P = .017$) with 83% sensitivity and 87.9% specificity in discriminating infected patients (Panel D).
Combined ROC analysis revealed a greater AUC of 0.907 (95% CI: 0.809–1; $P = .000001$) with 89% sensitivity and 88% specificity in discriminating infected patients (Panel E).

DNA in infected cells may be indespensable. Identification of biomarkers that are associated with HTLV-1 infection would therefore allow better diagnosis of infected patients. In the present study, we identified serum miR-29c, miR-30c, miR-193a-5p, and miR-885-5p as potential new biomarkers for diagnosis of HTLV-1 infection.

As cells can actively secrete miRNAs, and as these non-coding RNAs are stable and can be simply detected and quantified in different biological fluids using distinct molecular techniques such as qRT-PCR, miRNAs has been extensively used as biomarkers to diagnose a wide variety of human disorders including cancer, cardiovascular diseases, diabetes and metaboloc diseases (Correia et al., 2017; Wang et al., 2016). During systemic infection, the presence of an infectious agent often triggers a significant alteration in the expression profiles not only of cellular miRNAs but also of circulating ones which in turn facilitates their use as biomarkers to track disease development and progression. In fact, miRNAs are tightly associated with viral infection especially in the case of HTLV, EBV (Epstein Barr virus), HBV (hepatitis B virus), HCV (hepatitis C virus) and HIV (human immunodeficiency virus) where those miRNAs can regulate different mechanisms including viral infection, replication and host transcription programs (Wang et al., 2016). Intriguingly, circulating miRNAs have been used as biomarkers during viral infection. For instance, circulating miR-122, miR-22, and miR-34a have been described to be correlated with the etiology of liver injury in HIV-infected patients (Anadol et al., 2015). Moreover, circulating miRNA signature has been employed to assess the risk of develoîng Hepatocellular Carcinoma ((HCC) in cirrhotic patients (Huang et al., 2017). In this study, we showed deregulated expression of several miRNAs, among which miR-29c, miR-30c, miR-193a-5p, and miR-885-5p that were most significantly upregulated in the serum of infected patients appeared as potential biomarkers for HTLV-1 infection diagnosis.

It is note worthy that serum miR-29 levels have previously been reported as biomakers for disease progression in patients with chronic hepatitis B virus infection (Huang et al., 2014). Moreover, serum miR-30c levels have been described to be correlated with disease progression in Xinjiang Uygur patients with chronic hepatitis B and thus

highlighted as a marker for risk stratification of HBV infection (Zhang et al., 2017). Further, miR-885-5p has emerged as a potential marker for detecting liver pathologies (Gui et al., 2011). In our study, the combination of the four deregulated miRNAs (miR-29c, miR-30c, miR-193a-5p, and miR-885-5p), improves the specificty of these suggested biomarkers for HTLV-1 infection diagnosis.

In our study, and to ensure accurate quantification of miRNA levels with qRT-PCR, normalization was carried out using miR-16 that showed similar levels in serum of normal controls and patients. Besides, different studies have validated the use of miR-16 for plasma miRNA quantification (Resnick et al., 2009; Zhu et al., 2009). Further, the control group examined in this study was confirmed to be free of HTLV-1 infection. On the other hand, it would be helpful to enlarge the sample size in order to eliminate potential sampling errors. To our knowledge, this is the first study to correlate circulating miRNA signature with diagnosis of HTLV-1 infection.

5. Conclusion

In conclusion, we identified, in this study, 4 miRNAs with a good potential for diagnosing HTLV-1 infection. This would be enable more accurate diagnosis and better therapeutic decisions.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the local ethics committee of the “Lebanese University”and with the 1964 Helsinki declaration and its later amendments.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Declaration of Competing Interest

None.

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