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DEFINING PLASMA MICRORNAS ASSOCIATED WITH COGNITIVE IMPAIRMENT IN HIV-INFECTED PATIENTS†

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Abstract

Human Immunodeficiency Virus (HIV)-infected individuals are at increased risk for developing neurocognitive disorders and depression. These conditions collectively affect more than 50% of people living with HIV/AIDS and adversely impact adherence to HIV therapy. Thus, identification of early markers of neurocognitive impairment could lead to interventions that improve psychosocial functioning and slow or reverse disease progression through improved treatment adherence. Evidence has accumulated for the role and function of microRNAs in normal and pathological conditions. We have optimized a protocol to profile microRNAs in body fluids. Using this methodology, we have profiled plasma microRNA expression for 30 age-matched, HIV-infected (HIV⁺) patients and identified highly sensitive and specific microRNA signatures distinguishing HIV⁺ patients with cognitive impairment from those without cognitive impairment. These results justify follow-on studies to determine whether plasma microRNA signatures can be used as a screening or prognostic tool for HIV⁺ patients with neurocognitive impairment.

Keywords

microRNA; HIV; neurological disorders

Introduction

Neurocognitive impairments affect more than 50% of HIV⁺ individuals, with negative consequences for antiretroviral therapy (ART) adherence and high-risk behaviors, including drug and alcohol abuse (Hinkin et al., 2007; Tozzi et al., 2003). The introduction of ART

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greatly improved the lifespan of people with HIV/AIDS. However, for many patients immune fitness is not fully restored, and persistent viral replication increases the risk for chronic cognitive impairment (Deeks et al., 2013). Since diagnosis of cognitive disorders of the central nervous system (CNS) often cannot be achieved through direct assessment (tissue biopsies or spinal fluid analyses), they are determined through evaluation of symptoms which are often evident only at later stages of disease. Thus, the discovery of circulating (plasma or cell-based) biomarkers for early diagnosis is a top priority for CNS disorders, including HIV-associated neurocognitive disorders (HAND). Critical obstacles in identifying biomarkers for those slowly progressing/chronic conditions are the adaptive responses that take place to re-balance acutely activated factors and networks. Nevertheless, in the past few years the discovery of cell-free microRNAs, together with the development of new technologies for their detection in body fluids, has permitted formulation of new hypotheses for biomarker discovery. For example, microRNA signatures related to cancer have been proposed for identifying patients at risk for disease progression (Erkan et al., 2011; Gibcus et al., 2009; O'Hara et al., 2008; Pedranzini et al., 2010; Robertus et al., 2010; Shah et al., 2010), and diagnostic and prognostic utility has been identified for microRNA quantification within body fluids for both cancers and neurocognitive disorders (Alevizos and Illei, 2010; Cho, 2010; De Smaele et al., 2010; Ferracin et al., 2010; Heneghan et al., 2010; Ju, 2010; Kosaka et al., 2010; Shafi et al., 2010; Taft et al., 2009; Wittmann and Jack, 2010; Xie et al., 2010). In addition, existing data strongly implicate brain-enriched microRNAs in pathogenesis for HIV⁺ individuals with encephalopathy (HIVE) (Eletto et al., 2008; Noorbakhsh et al., 2010; Rom et al., 2010; Tatro et al., 2010; Yelamanchili et al., 2010), and two independent studies enrolling HIV-negative patients have identified plasma microRNAs which are enriched in brain tissue and may identify patients with mild cognitive impairment (Sheinerman et al., 2013a; Sheinerman et al., 2012). These latter data and others indicate that because of their high stability and resistance to RNases, plasma microRNAs may be good candidate biomarkers for CNS diseases (De Felice et al., 2012; Gandhi et al., 2013; Jin et al., 2013; Rao et al., 2013; Sheinerman and Umansky, 2013a; Witwer et al., 2011). We have recently identified two microRNAs, miR-23a-3p and miR-23b-3p, which are consistently expressed at high levels within plasma from HIV⁺ patients and, therefore, can serve as reference genes for normalization and indicators of RNA quality. Taking advantage of this advance, we sought to identify microRNA-pairs that can distinguish, with high sensitivity and specificity, HIV⁺ individuals with and without cognitive impairment.

Materials and Methods

Whole blood collection

Following informed consent, 30 ml whole blood was collected from HIV-infected patients at the Louisiana State University Health Sciences Center (LSUHSC) HIV Outpatient (HOP) Clinic in New Orleans, Louisiana in the context of routine health assessment visits to this clinic. Samples were de-identified using an alphanumeric coding system, and Whole blood was transported twice daily (within two hours of collection) to the HIV Specimen Biorepository housed within the Stanley S. Scott Cancer Center (SSSCC) where plasma was immediately separated from cell fractions and stored at -80°C . Patients with opportunistic infections involving the CNS, existing CNS tumors, history of significant head injury,

multiple sclerosis, and other dementing disorders (Alzheimer's disease) were excluded from the study. All studies were performed with approval from the LSUHSC Institutional Review Board and in conjunction with national guidelines for protection of patient confidentiality and safety.

Cognitive testing

Cognitive functioning was assessed using a modified version of the Multicenter AIDS Cohort Study (MACS) screening battery, which measures memory, attention, processing speed, language, and motor skill. Participants completed six measures of cognitive functioning: WAIS-IV Digit Span, Controlled Oral Word Association Test, Rey Auditory Verbal Learning Test, Trail Making Test, Symbol Digit Modalities Test, and Grooved Pegboard Test. Raw scores on these measures were converted to t-scores using demographically-adjusted normative data. T-scores were converted to deficit scores and averaged to develop Global Deficit Scores (GDS) using methods previously reported by Heaton and Carey (Carey et al., 2004; Heaton et al., 1995). GDS scores range from 0 to 5 with higher scores indicative of greater cognitive impairment. A GDS of 0.5 or higher is considered a positive predictive value in establishing HIV-associated cognitive impairment (Carey et al., 2004). Accordingly, patients were categorized as either cognitively impaired (CI; GDS $\geq$ 0.5) or unimpaired (nonCI; GDS < 0.5) based on their performance across these six measures. The categories of cognitively impaired and unimpaired were used to determine between-group differences in expression of microRNAs.

RNA extraction, quality control, and miRNA profiling

RNA extraction and microRNA profiling were performed as previously reported (Pacifi et al., 2014). RNA was obtained from 200 μ l of plasma using the miRCURY RNA extraction kit (Exiqon, Woburn, MA). To increase the RNA recovery, 1 μ g of MS2 carrier RNA were added to each plasma sample. 8 μ l of total RNA was subjected to retro-transcription using the Universal cDNA synthesis kit (Exiqon, Woburn, MA), followed by qRT-PCR using microRNA ready-to-use PCR human Panels I and II V3.0 (Exiqon, Woburn, MA). qRT-PCR was carried out on a Roche LightCycler 480 Real-Time PCR System according to the Exiqon recommended protocol. Cycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. Fluorescent data were converted into cycle threshold (Ct) measurements by the Roche LightCycler system software (Version 1.5; Roche). Quantification using 2nd derivative maximum were further calculated with Roche Lightcycler 480 software. qPCR data were analyzed in GenEx Professional 5 software (MultiD Analyses AB, Goteborg, Sweden). Degree of hemolysis was determined as the difference in Ct of miR-23a-3p (a microRNA not affected by hemolysis) and miR-451a (an indicator of hemolysis); this calculation was performed in GenEx. All samples had a Ct < 5, which indicated no hemolysis. After inter-plate calibration, the amount of target microRNAs was normalized relative to the amount of reference genes, determined using the GeNorm algorithm, an application of GenEx software.

Statistical Analyses

Statistical calculations were performed in GenEx Professional software. Mann-Whitney tests were two-sided and set at 5% level and 95% confidence intervals (CIs). Bonferroni

correction was applied to determine statistically significant microRNA pairs. The potential of microRNA pairs for use in diagnosis of neurocognitive impairment was assessed by estimating sensitivity and specificity based on an ROC analysis using GraphPad Prism 5. Pearson's correlations were performed in GraphPad Prism 5.

Results

We profiled plasma microRNAs from 30 individual patients recruited from an existing cohort of >400 individuals participating in the HOP Clinic Cancer Prevention Study (HOP CPS Cohort). Participants enrolled in the HOP CPS Cohort exhibit characteristics similar to those receiving care at other academic centers: 94% are on ART with a mean CD4⁺ T-cell count of 458 cells/mm³ (range 12 to 1773); 14% exhibit undetectable HIV viral loads (< 20 copies/ml), and 62% have viral loads < 400 copies/ml; African Americans comprise 88% of the cohort. Patients in the subsample underwent neurocognitive evaluations and were categorized as either cognitively impaired (CI, *n*=17) (Global Deficit Score, GDS = 0.5) or unimpaired (nonCI, *n*=13, GDS < 0.5) based on their performance across six measures, as detailed in Methods. The mean age for the two groups was 50. Demographics, neurocognitive status and other relevant parameters are shown in Table I.

Use of MiR-23a-3p and MiR-23b-3p for Normalization and for RNA Quality

A total of 752 unique microRNAs were profiled for each sample. After interplate calibration, a cycle threshold (Ct) cutoff of 39 was applied, and 230 microRNAs were discarded. The pre-processing also included a 60% validation of the data, in which the microRNAs that were not detected in at least 60% of the samples and were discarded. This accounted for the additional removal of 281 microRNAs. The remaining 241 microRNAs were considered for further analysis. First, we determined the frequency of missing data (valid Ct values). 57 microRNAs were detected in all (100%) plasma samples; a similar number (61 microRNAs) was detected in 60 to 70% of samples. The remaining microRNAs (a total of 110) were almost equally represented among 70 to 99% of the samples. Next, the 57 microRNAs detected in all samples were tested for their possible use as reference genes, using geNorm algorithm (incorporated in the GenEx software, see Materials and Methods). MiR-23a-3p and miR-23b-3p had the best M-value (0.3), which indicates the variation of a microRNA compared to all other candidate microRNAs (Vandesompele et al., 2002). The range of detection (Ct) of those microRNAs across the samples was between 20.7 and 32.1 for miR-23a-3p and between 24.5 and 30.4 for miR-23b-3p, with a standard deviation of 2.74 and 2.90, respectively (Fig. 1A, left two plots). We also recognized that samples which had the highest Ct values for miR-23a-3p and miR-23b-3p were also those with the highest number of missing values in the arrays. Indeed, the Ct values of both microRNAs correlated very well with the percentage of missing data (Fig. 1C–D). Therefore for additional validation, we repeated RNA extraction from plasma samples with missing data to specifically reassess expression levels of miR-23a-3p and miR-23b-3p, and found improved Ct values for both microRNAs in the newly prepared RNA samples (Fig. 1A, right two plots). The relationship between the expression levels of miR-23a-3p and miR-23b-3p across our samples was further assessed using Pearson's correlation, which indicated a strong positive correlation ($R^2 = 0.985$, Fig. 1B). Overall, these results indicate that miR-23a-3p and miR-23b-3p can be

utilized to assess the quality of RNA/cDNA in plasma samples and can serve as reference genes.

MicroRNA Pairs Associate with CI in HIV⁺ Patients

To identify microRNAs that could distinguish CI from nonCI in HIV⁺ patients, we performed microRNA pair-wise analyses (Hennessey et al., 2012; Sheinerman et al., 2012), as described in detail in Materials and Methods. After unequal variance t-test analysis, we subjected microRNA-pairs to the Receiver-Operating Characteristic (ROC) curve analyses to evaluate the discriminating value of microRNAs as biomarkers of cognitive impairment. This method resulted in over 30 microRNA pairs with an AUC > 0.8. Table II shows the microRNA pairs with a *p* value < 0.03. Interestingly, the most heavily represented microRNA among pairs with a low *p* value was miR-495-3p. It is important to note that although the miR-495-3p/miR-19b-5p pairing exhibited a perfect AUC, one or both microRNAs were not detected in 5 and 6 samples within CI and nonCI groups, respectively. In contrast, miR-376a-3p/miR-16-5p and miR-495-3p/miR-29a-5p were detected in almost every sample from each group, and therefore despite lower AUC, rationalized these pairings as stronger candidates for further validation. Comparisons are depicted in Figure 2, showing the relative expression of the three microRNA pairs including miR-495-3p.

Validation of microRNA pairs with individual qRT-PCRs

Next, we performed qRT-PCR for individual microRNAs and their respective pairs for further validation. Selected microRNAs were assayed from each of the 30 plasma samples, and miR-23a-3p and miR-23b-3p used to normalize Ct values prior to determining the microRNA pairs that best distinguished CI and nonCI groups (Table III). Ten microRNA pairs were confirmed differentially expressed, and the pair miR-495-3p/let-7b-5p actually exhibited improved sensitivity using individual qRT-PCR relative to the array approach (compare Tables II and III). Four microRNA pairs best distinguished CI from nonCI groups (miR-495-3p/let-7b-5p, miR-495-3p/miR-151-5p, miR-495-3p/miR-744-5p and miR-376a-3p/miR-16-5p), and there was no association between expression of these microRNA-pairs and any of the clinical and demographic variables listed in Table I. Finally, combinations of microRNA-pairs were tested for their ability to further distinguish CI from nonCI groups. ROC analyses revealed two sets of microRNA-pairs exhibiting improved sensitivity and specificity for distinguishing CI from nonCI groups: miR-376a-3p/miR-532-3p combined with either miR-495-3p/miR-744-5p (Fig. 4A) or miR-495-3p/let-7b-5p (Fig. 4B).

Discussion

A minimally invasive test for the early detection and monitoring of CI in HIV⁺ patients is not currently available. MicroRNAs regulate gene expression, and changes in their concentration may reflect changes in cellular function. MicroRNAs are also secreted and remarkably stable in the extracellular environment, making them attractive molecules for biomarker discovery. The current view of microRNAs as biomarkers relates to their increased expression and secretion as a result of cellular/organ injury, resulting in their detection in plasma. Although advances in microRNA technology have allowed increased

detection of minute amounts of circulating microRNAs, their application as predictive or prognostic tools for neurodegenerative diseases remains underdeveloped. A growing need exists to identify chronic neurodegeneration during its early phases, and only a small number of reports show correlation between dysregulated plasma microRNAs and neuropsychological conditions [reviewed in (Jin et al., 2013; Sheinerman and Umansky, 2013a)]. To the best of our knowledge, of the putative role of microRNAs as biomarkers for HIV-associated neurocognitive impairment has not been reported.

In this study, we utilized our published protocol for isolating microRNAs from body fluids (Pacifi ci et al., 2013; Pacifi ci et al., 2014) and optimized this protocol for profiling plasma microRNAs from HIV⁺ individuals. As methodologies are often overlooked in the literature (Moldovan et al., 2014), we have curated critical procedural aspects, such as blood collection and processing, that can affect stability of microRNAs. To avoid introducing technical variations we used a standardized procedure for sampling in which EDTA-anticoagulated blood was collected from patients and processed for plasma separation within two hours of collection.

While new technologies have greatly improved the recovery and measurement of circulating microRNAs, less attention has been given to standardization procedures for data analysis that would allow comparison across different studies (Kroh et al., 2010; Mitchell et al., 2008; Moldovan et al., 2014). We have identified two microRNAs, miR-23a-3p and miR-23b-3p, that were consistently expressed across all samples from HIV⁺ patients and that could be used as reference genes. We also determined that these microRNAs may serve as indicators of RNA/cDNA quality. In fact, high threshold values for miR-23a-3p and miR-23b-3p correlated with poor performance of samples using the array approach, resulting in a high rate of missing cycle threshold values (Fig. 1C-D). Importantly, the identification of reference genes for HIV⁺ samples could pave the way to standardized protocols that would enable investigators to compare profiles across different cohorts of patients.

For data analysis we used a microRNA pairing approach (Hennessey et al., 2012; Sheinerman et al., 2012) based on empirical search of microRNA pairs that differentiate cognitively impaired from unimpaired control patients with the best sensitivity and specificity. The miRNA-pairing approach is becoming the preferred method for circulating microRNA biomarker discovery (Hennessey et al., 2012; Hsu et al., 2014; Sheinerman et al., 2012; Sheinerman and Umansky, 2013b). A modification of this approach, in which only organ-enriched microRNAs are screened in plasma, has been successfully applied for the discovery of microRNA-pairs that discriminate mild cognitive impairments from controls (Sheinerman et al., 2013a; Sheinerman et al., 2012; Sheinerman and Umansky, 2013a; Sheinerman and Umansky, 2013b) or other pathologies like pulmonary and gastrointestinal cancers (Hennessey et al., 2012; Sheinerman et al., 2013b). In this study, we applied the microRNA pair-wise approach to array data, resulting in discovery of more than 30 microRNA-pairs potentially distinguishing CI from nonCI patients with good sensitivity and specificity (Table II). After validation through individual qRT-PCR, a total of 10 microRNA pairs were confirmed to distinguish CI from nonCI (Table III). We observed a loss in the sensitivity for some microRNA-pairs using the latter approach, due likely to the high

number of undetected microRNAs in the arrays, while individual qRT-PCR, and in some instances isolation of fresh RNA, eliminated this problem. The microRNA-pairs best distinguishing CI from nonCI in our HIV⁺ cohort were: miR-495-3p in combination with let-7b-5p, miR-151a-5p or miR-744-5p, and the pair miR-376a-3p/miR-16-5p (Figure 3). Improved sensitivity (0.88) was achieved with the combination of two microRNA pairs, miR-495-3p/miR-744-5p and miR-376a-3p/miR-532-3p. Finally, we wanted to assess whether other patient's characteristics might account for observed differences in miRNAs expression in CI/nonCI groups. Thus, we compared expression of the four microRNA pairs, miR-495-3p/let-7b-5p, miR-495-3p/miR-151-5p, miR-495-3p/miR-744-5p and miR-376a-3p/miR-16-5p, with other clinical and demographic variables, as shown in Table I. However, this type of analysis did not return any significant association.

The microRNAs identified as potentially distinguishing CI from nonCI in this study are either brain-enriched, or have validated neuronal functions (Im and Kenny, 2012; Landgraf et al., 2007; Liang et al., 2007). For example, miR-495, miR-16, and miR-30 target brain-derived neurotrophic factor (BDNF) (Mellios et al., 2008), a factor downregulated in several neurodegenerative disorders (Durany et al., 2000; Ferrer et al., 2000; Zuccato et al., 2001), including HAND (Bachis et al., 2012), and known not only to support neuronal survival but also promote synaptic transmission, growth and plasticity [reviewed by (Lu et al., 2013)]. Expression of miR-495 appears to be restricted to layer II of the pre-frontal cortex, suggesting its critical role in neurogenesis and synaptic regulation during adulthood in this layer (Mellios et al., 2008). Notably, six distinguishing microRNAs in the array (miR-495, miR-376a, miR-376b, miR-376c, miR-127, and miR-379; Table II) and four from individual qRT-PCR (miR-495, miR-376a, miR-431, and miR-377; Table III) are clustered in the chromosome 14q32 region. This region contains paternally imprinted/maternally expressed *DLK1-DIO3* domain and two large microRNA clusters that encode a population of brain enriched microRNAs (Benetatos et al., 2013; Gardiner et al., 2012). Much of the 14q32-derived microRNAs are involved in the maintenance of the white matter where they are implicated in cell adhesion, differentiation, neuronal myelination, and oligodendrocyte maturation (Manzardo et al., 2013). Therefore, detection of this cluster in the periphery may reflect white matter pathology observed in HIV⁺ patients by MRI imaging (Pomara et al., 2001), although MRIs were not available for patients in our cohort. Mir-19b-5p, which demonstrated distinguishing capacities in combination with other microRNAs (Tables II and III), associates with Argonaute 2 (Ago2) in the amigdala following chronic stress and modulates behavioral responses (Volk et al., 2014), and is upregulated in ischemic models of stroke (Dhiraj et al., 2013). Furthermore, miR-532 is an axon-enriched microRNA which localizes in granules at distal axons and growth cones (Sasaki et al., 2014).

In summary, using novel approaches validated using plasma samples from HIV⁺ patients we have identified potential microRNA-pairs that may distinguish CI from nonCI in HIV⁺ patients with good sensitivity and specificity. While further validation studies are needed in other cohorts of HIV⁺ patients, these data establish a framework for longitudinal testing to determine whether plasma microRNA profiling can be used to predict the onset and/or progression of neurocognitive impairment among HIV⁺ individuals.

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Figure 1. Use of miR-23a-3p and miR-23b-3p as reference gene and for sample quality
 (A) Box plots represent cycle threshold (Ct) for miR-23a-3p and miR-23b-3p in the arrays (left two plots) and in single qRT-PCRs (right two plots). The mean value is indicated in each box plot. (B) Pearson's correlation of miR-23a-3p and miR-23b-3p as determined by individual qRT-PCRs. (C-D). Scattered plots show the analysis of correlation between the frequency of missing data in arrays and Ct values for miR-23a-3p (C) or miR-23b-3p (D). Pearson's rank correlation coefficient r^2 and p values are indicated.

Figure 2. Differentially regulated plasma microRNA pairs in HIV-patients with neurocognitive impairment (CI) compared to non-cognitively impaired patients (nonCI)

For each microRNA-pair, relative expression in the two groups is represented on the left graphs as $2^{-\Delta Ct}$. Actual p -values are reported for each experiment. Receiver-operator Characteristic (ROC) curves (right panels) were constructed and the area under the curves was calculated to evaluate sensitivity and specificity of the indicated biomarker sets (see also Table II).

Figure 3. Validated microRNA pairs that distinguish CI from nonCI

Left panels show expression levels of the indicated microRNA pairs as determined by individual qRT-PCR assays in the HIV⁺ samples. Exact p -values are shown. Right panels show the results from ROC analysis. Area under the curve (AUC), specificity and sensitivity are indicated in each graph.

Figure 4. Diagnostic value of combined microRNA pairs

Plots showing ROC curve analysis resulting from the combination of the pair miR-495-3p/miR-744-5p with miR-376a-3p/miR-532-3p (A), and miR-495-3p/let-7b-5p with miR-376a-3p/miR-532-3p (B). Actual p-values, AUC, specificity and sensitivity are shown.

Table IDemographic and medical parameters of HIV⁺ patients.

		CI (n=17)	nonCI (n=13)
Age	Avg	50.64	49.38
	Min-Max	41–59	42–57
Education	8 grade	5	1
	> 8 grade	12	12
Gender	Males	9	10
	Females	8	4
Ethnicity	African-American	10	12
	Caucasian	6	1
	Multiracial	1	0
GDS		0.5	< 0.5
HCV		1	1
VL	Avg	978	610
	Min-Max	20–7083	20–3925
	N/A	2	1
	<400	10 (58.8%)	8 (61.5%)
	<20	1	2
CD4	Avg	548	453
	Min-Max	194–1034	202–806
	N/A	1	1

n= number of patients; GDS= Global Deficit Score; VL= viral load/ml; CD4= CD4 count/ml; N/A= number of samples in which the parameter is not available

Table II

List of microRNA pairs that discriminate cognitively impaired from non-impaired HIV⁺ patients obtained with qRT-PCR arrays.

miRNA pairs	AUC	Sensitivity	Specificity	P-Value	CI (n=17)	nonCI (n=13)
miR-495-3p/miR-151a-5p	0.97	1.00	0.82	1.37E-06	15	9
miR-495-3p/miR-194-5p	0.98	0.93	0.91	3.16E-06	15	10
miR-495-3p/miR-30d-3p	0.99	1.00	0.89	0.000167	12	8
miR-495-3p/miR-130b-5p	0.96	0.92	0.90	0.00020	13	9
miR-495-3p/miR-942	0.98	1.00	0.89	0.000222	12	8
miR-495-3p/miR-19b-1-5p	1.00	1.00	0.86	0.000389	12	7
miR-221-5p/miR-19b-1-5p	1.00	1.00	0.86	0.000389	12	7
miR-495-3p/miR-135a-5p	0.97	1.00	0.88	0.000391	13	7
miR-495-3p/miR-16-5p	0.99	1.00	0.89	0.000491	9	8
miR-495-3p/miR-92b-3p	0.95	0.92	0.89	0.000501	12	8
miR-495-3p/miR-139-3p	0.95	0.80	0.90	0.000675	10	9
miR-495-3p/miR-1271-5p	0.98	0.92	0.86	0.000729	12	6
miR-32-3p/miR-19b-1-5p	0.99	1.00	0.86	0.000913	10	6
hsa-miR-376a-3p/miR-16-5p	0.89	0.67	0.90	0.001381	12	11
miR-376b-3p/miR-19b-1-5p	0.95	0.82	0.86	0.001791	11	6
miR-376c-3p/miR-19b-1-5p	1.00	1.00	0.83	0.001957	8	6
miR-411-5p/miR-19b-1-5p	0.92	0.83	0.86	0.00271	12	6
miR-127-3p/miR-19b-1-5p	1.00	1.00	0.83	0.002713	7	6
miR-495-3p/miR-29a-5p	0.92	0.92	0.86	0.003111	12	6
miR-495-3p/miR-744-5p	0.88	0.90	0.70	0.003115	11	10
miR-22-5p/miR-19b-1-5p	0.86	0.89	0.86	0.01727	9	7
miR-495-3p/let-7b-5p	0.80	0.67	0.89	0.02095	12	9
miR-379-3p/miR-19b-1-5p	0.83	0.90	0.87	0.02096	11	7

The area under the curve (AUC) is indicated together with specificity, sensitivity and *p*-values. The last two columns indicate the number of samples in which the two microRNAs were detected.

Table III

List of microRNA pairs that discriminate cognitively impaired from non-impaired HIV⁺ patients confirmed with individual qRT-PCRs.

microRNA pairs	AUC	Sensitivity	Specificity	P-value
miR-495-3p/let-7b-5p	0.89	0.71	0.92	0.000322
miR-532-3p/miR-377-3p	0.89	0.63	0.92	0.000326
miR-495-3p/miR-744-5p	0.89	0.76	0.92	0.000349
miR-495-3p/miR-194-5p	0.89	0.82	0.92	0.000400
miR-495-3p/miR-151a-5p	0.89	0.76	0.92	0.000473
miR-495-3p/miR-16-5p	0.87	0.53	0.92	0.000653
miR-495-3p/miR-106a-5p	0.87	0.76	0.92	0.000653
miR-376a-3p/miR-16-5p	0.87	0.76	0.92	0.000704
miR-376a-3p/miR-532-3p	0.86	0.59	0.92	0.000884
miR-431-5p/miR-16-5p	0.85	0.76	0.93	0.000992