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Research Report

Plasma-Based Circulating MicroRNA Biomarkers for Parkinson's Disease

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Abstract.
Background: The current "gold-standard" for Parkinson's disease (PD) diagnosis is based primarily on subjective clinical rating scales related with motor features. Molecular biomarkers that are objective and quantifiable remain attractive as clinical tools to detect PD prior to its motor onsets.
Objective: Here, we aimed to identify, develop, and validate plasma-based circulating microRNA (miRNAs) as biomarkers for PD.
Methods: Global miRNA expressions were acquired from a discovery set of 32 PD/32 controls using microarrays. k-Top Scoring Pairs (k-TSP) algorithm and significance analysis of microarrays (SAM) were applied to obtain comprehensive panels of PD-predictive biomarkers. TaqMan miRNA-specific real-time PCR assays were performed to validate the microarray data and to evaluate the biomarker performance using a new replication set of 42 PD/30 controls. Data was analyzed in a paired PD-control fashion. The validation set was composed of 30 PD, 5 progressive supranuclear palsy, and 4 multiple system atrophy samples from a new clinical site.
Results: We identified 9 pairs of PD-predictive classifiers using k-TSP analysis and 13 most differentially-expressed miRNAs by SAM. A combination of both data sets produced a panel of PD-predictive biomarkers: k-TSP1 (miR-1826/miR-450b-3p), miR-626, and miR-505, and achieved the highest predictive power of 91% sensitivity, 100% specificity, 100% positive predicted value, and 88% negative predicted value in the replication set. However, low predictive values were shown in the validation set.

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Conclusions: This proof-of-concept study demonstrates the feasibility of using plasma-based circulating miRNAs as biomarkers for neurodegenerative disorders such as PD and shows the challenges of molecular biomarker research using samples from multiple clinical sites.

Keywords: Circulating microRNAs, plasma, biomarkers, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States, affecting approximately 1 million Americans and 5 million people worldwide [1]. Its prevalence is projected to double by 2030 [2]. It is a complex and heterogeneous neurodegenerative disease that results in deficits in movement and cognitive competency, and additional peripheral symptoms such as GI dysfunction. Currently, differential diagnosis of PD is based primarily on clinical rating scales associated with motor functions. The scales are subjective and can only be employed when motor features are present. Due to the need for such observable symptoms, by the time of diagnosis, 60–70% of a patient's dopaminergic neurons are already lost [3]. Since there is no reliable quantitative diagnostic test for PD, molecular biomarkers that are objective and measurable can be potential clinical tools to facilitate PD diagnosis especially during its early stage.

Current PD candidate biomarkers are heavily based on individual proteins related to the pathogenesis of PD in cerebral spinal fluid (CSF) and brain tissue (eg. DJ1 for mitochondrial dysfunction; α-synuclein for protein aggregation and Lewy body formation; BDNF for inflammation and glial activation) [4, 5]. To use these biomarkers on PD patients, sample collection is either invasive (CSF) or quite impossible (brain tissue). Moreover, conflicting results among studied CSF proteins have been reported due to assay differences and/or blood contamination [6]. In contrast, blood is an ideal source for biomarkers due to its propensity to contain biological and chemical signals from relevant sources such as cells associated with a disease [7]. Moreover, sampling from blood is quick, simple, inexpensive and minimally-invasive. Global proteomic studies using blood serum or plasma as a source of biomarker discoveries have been reported in Alzheimer's disease [8–10] and, most recently, in PD with cognitive impairment [11]. Hence, the development of blood-based biomarkers for PD has great potential but surprisingly is still in its infancy.

MicroRNAs (miRNAs) are small, conserved RNAs (18 to 22 nucleotides) that interact with target sequences on messenger RNAs (mRNAs) resulting in decreased mRNA stability and/or translation. miRNAs are derived from primary transcripts through processing by the Drosha ribonuclease and the Dicer enzyme. miRNAs have been proven to regulate the expression of many genes and play important roles in normal cell development, differentiation, proliferation, and apoptosis. In neuroscience, genetically-inactivated Dicer enzyme in miRNAs can contribute to neurodegeneration in animal models and cultured neurons [12–14]. MiR-133b has been reported to be specifically expressed in normal dopaminergic neurons and yet is deficient in midbrain tissue of PD patients. Most recently, a miRNA that regulates the learning processes and may play a central role in Alzheimer's disease has been identified [15]. miRNAs are known to be abundant, tissue-specific, highly stable, non-post process modified, and quantifiable in plasma. Its unique characteristics have made it feasible to develop as plasma-based biomarkers for diseases such as Huntington's disease, myelodysplastic syndrome, myocardial infarction, and various cancers [16–23]. In this study, we presented a proof-of-concept study to demonstrate the feasibility of utilizing circulating miRNAs in the plasma for PD biomarker discoveries.

MATERIALS AND METHODS

Subject and plasma sampling

Patients diagnosed with idiopathic PD based on UK Brain Bank criteria [24] were recruited between January 2006 and November 2009 from the Saint Mary's Health Care Hauenstein Parkinson's Center (SMHCPC). Normal healthy controls were also recruited at the same center. These samples were used as our discovery and replication sets. In addition, PD, progressive supranuclear palsy (PSP), multiple system atrophy (MSA) patients and healthy controls recruited from the Department of Neurology at Umeå University Hospital (UUH) were used as a new, independent validation set. All subjects provided their informed consents and this study was approved by the institutional review boards of Saint Mary's Health Care, Umeå University Hospital, and the Van Andel Institute. Peripheral blood samples were obtained using 10 ml EDTA tubes, placed on ice immediately and

centrifuged at 4°C, 1,000 g for 15 minutes. Plasma supernatant was aliquotted into 500 μl aliquots and stored immediately at −80°C until analysis.

RNA isolation and miRNA expression microarrays

Total RNA that included miRNAs was isolated from plasma using the TRI reagent RT-blood protocol (Molecular Research Center, Cincinnati, OH) with slight modification. Polyacryl carrier was added to improve RNA recovery. The Agilent whole human genome miRNA microarray v.3 (Agilent, Santa Clara, CA) containing 866 human miRNAs based on Sanger miRBase release 12.0 were used to obtain the global miRNA expression profiles. RNA samples were processed, labeled, and hybridized onto the microarrays according to the manufacturer's protocols. Microarray slides were then scanned using the G3 High Resolution Scanner and microarray data extracted by Feature Extraction software v.10.7.3.1 (Agilent).

Statistical analyses

The k-Top Scoring Pairs (k-TSP) algorithm [25] was used to identify highly discriminative classifiers that can distinguish PD patients from normal controls. This algorithm directly addresses the tradeoff between sample size and model complexity in machine learning algorithms as well as between "bias-variance" by incorporating simplifying assumptions. In brief, this comparison-based method seeks to discriminate the disease group from appropriate controls by finding pairs of miRNAs whose expression levels typically invert from one group to another. In addition, we identified the differentially expressed miRNAs between PD and control group by applying the significance analysis of microarrays (SAM) procedure [26]. The Newton's Gamma-Gamma Bernoulli correction [27] was applied to both analyses to provide more precise estimates of differentially-expressed miRNAs and more accurate assessments of significant changes. TargetScanHuman (www.targetscan.org) which predicts biological targets of miRNAs by searching for the presence of conserved 7mer and 8mer sites that match the seed region of each miRNA was used to search for predicted miRNA targets of the classifiers.

Quantitative real-time PCR (qRT-PCR) validation and biomarkers evaluation

The miRNA microarray expression of all candidate miRNA biomarkers were validated with qRT-PCR in triplicate. In brief, 40 ng of total RNA was reverse transcribed to cDNA in a PCR thermal cycler using the Taqman miRNA-specific assay and qRT-PCR was performed using the StepOnePlus real time PCR system (Applied Biosystems, Foster City, CA) to detect each miRNA expression. Human miR-16 and non-human ath-miR-156a were used as an endogenous and negative control, respectively. To calculate the expression of each target miRNA relative to each endogenous control, the comparative C_T ($\Delta\Delta C_T$) method was used [28]. Each candidate miRNA biomarker whose qRT-PCR expression was in concordance with its microarray expression was then performance evaluated using a new set of PD patients and normal controls. We used 95% normalized C_T values of individual miRNAs as thresholds for classifying PD and normal controls. A multiple thresholds approach was applied to overcome the potential inter-center variability [29].

RESULTS

Study subjects

Using samples from SMHCPC, 32 treated PD patients were assigned as the discovery set (microarray) and 42 patients as the replication set (qRT-PCR). Sixty-two normal controls were also included. Normal control spouses of PD patients were used to minimize the environmental component as a confounding factor of this study. Data was analyzed in paired PD-control spouse fashion. In addition, 20 treated PD, 10 newly diagnosed untreated PD, 5 PSP, 4 MSA, and 8 healthy controls from UUH were used as a new, independent validation set (qRT-PCR). The clinical characteristics were similar among discovery, replication, and validation sets (Table 1).

Identification of plasma-based circulating miRNA biomarkers for PD

Global miRNA expression profiles were acquired from circulating miRNAs in plasma of PD patients and normal controls using microarray technology. We then identified 9 pairs of PD-predictive miRNA classifiers using the k-TSP algorithm. The paired k-TSP biomarkers were miR-1307/miR-632, miR-647/miR-99a*, miR-1225-5p/miR-891b, miR-1826/miR-450b-3p, miR-579/miR-708*, miR-506/miR-1253, miR-200a/miR-455-3p, miR-192*/miR-485-5p, and miR-488/miR-518c* (Fig. 1A). The interpretation for the first miR-1307/miR-632 is: if the expression of miR-1307 is higher than miR-632, then the patient

Table 1
Characteristics of PD and normal control samples

Characteristics	Parkinson's disease		Normal control	
Discovery set (miRNA microarray)				
Sex	Male	Female	Male	Female
N	16 (50%)	16 (50%)	15 (47%)	17 (53%)
Age, year				
Median	65	69	67	68
Mean	66 ± 11	67 ± 11	65 ± 10	62 ± 17
Hoehn and Yahr	1.75 ± 2.25	1.50 ± 2.39		
Replication set (qRT-PCR)				
Sex	Male	Female	Male	Female
N	20 (48%)	22 (52%)	10 (33%)	20 (67%)
Age, year				
Median	69	73	65	63
Mean	68 ± 6	72 ± 8	64 ± 15	59 ± 14
Hoehn and Yahr	1.75 ± 1.83	1.63 ± 1.51		
Validation set (qRT-PCR)				
Sex	Male	Female	Male	Female
N	16 (53%)	14 (47%)	3 (37.5%)	5 (62.5%)
Age, year				
Median	66	73	71	73
Mean	68 ± 10	71 ± 7	71 ± 3	73 ± 4
Hoehn and Yahr	2.06 ± 0.66	2.38 ± 0.63		

is predicted as PD, else it is predicted as normal control. Similar interpretations are also applied to other miRNA k-TSP pairs. As each individual miRNA pair can be used as a classifier, the final prediction is based on the majority voting of the 9 miRNA pairs. In addition, SAM analysis with 500 permutations identified 13 most differentially-expressed miRNAs: miR-1307, miR-647, miR-548b-3p, miR-192*, miR-505, miR-506, miR-626, miR-1826, miR-572, miR-671-5p, miR-222, miR-9*, and miR-1225-5p (Fig. 1B), with 6 miRNAs (miR-1307, miR-647, miR-192*, miR-506, miR-1826, and miR-1225-5p) mutually inclusive with k-TSP biomarkers.

Plasma miRNA expression can be validated using qRT-PCR

We validated the miRNA expression of all 9 pairs of PD-predictive biomarkers as well as 3 of the most-differentially expressed miRNAs: miR-626, miR-222, and miR-505, using qRT-PCR (Supplementary Table 1). miR-626, miR-222, and miR-505 were selected as miR-626 is a possible target for LRRK2 and PARK2, while miR-222 and miR-505 are possible targets for PARK2 by TargetScan Human. We observed very high concordance (>70%) between miRNA expression measured in the microarrays vs. qRT-PCR. However, 4 k-TSP pairs were not detectable by qRT-PCR even after optimizing with various cycles and RNA quantities. Thus, only 5 k-TSP pairs of biomarkers (miR-1826/miR-450b-3p – k-TSP1, miR-506/miR-1253 – k-TSP2, miR-200a/miR-455-3p – k-TSP3, miR-192*/miR-485-5p – k-TSP4, and miR-488/miR-518c* – k-TSP5) and 3 differentially expressed miRNAs (miR-626, miR-222, and miR-505) were evaluated further for biomarker performance using two new sets of PD patients and controls (replication and validation sets).

k-TSP PD-predictive miRNA biomarkers show high sensitivity

Sensitivity, specificity, positive and negative predicted values of each k-TSP pair as well as various combinations of the k-TSP pairs were evaluated in the replication set. The results are summarized in Table 2. k-TSP1 or miR-1826/miR-450b-3p pair showed the highest predictive power with 100% sensitivity, 56% specificity, and 92 and 100% positive and negative predicted value, respectively. A combination of k-TSP1, k-TSP3, and k-TSP4 improved specificity to 64% and maintained its high sensitivity (96%). Interestingly, the miRNA expression detection rate of k-TSP pairs is higher in PD than controls (eg. k-TSP1: 9/30 in controls, 45/46 in PD; k-TSP4: 28/30 in controls, 43/46 in PD).

Differentially expressed miRNAs identified by SAM show high specificity

RNA from the same replication set (42 PDs and 30 normal controls) were used to evaluate the performance of miR-626, miR-505, and miR-222. MiR-626 showed the highest performance: 83% sensitivity, 100% specificity, 100% positive predicted value, and 78% negative predicted value (Table 2). Scatter plots of normalized C_T values, thresholds (95% cut-off), and *P* values of all 3 miRNAs are shown in Fig. 2A to C.

High predictive biomarker performance from panel of k-TSP1 (miR-1826/miR-450b-3p), miR-626, and miR-505 in replication set

Since k-TSP classifiers showed high sensitivity while miR-626, miR-505, and miR-222 from SAM showed high specificity, we integrated both sets of data to evaluate the combined predictive power. We found

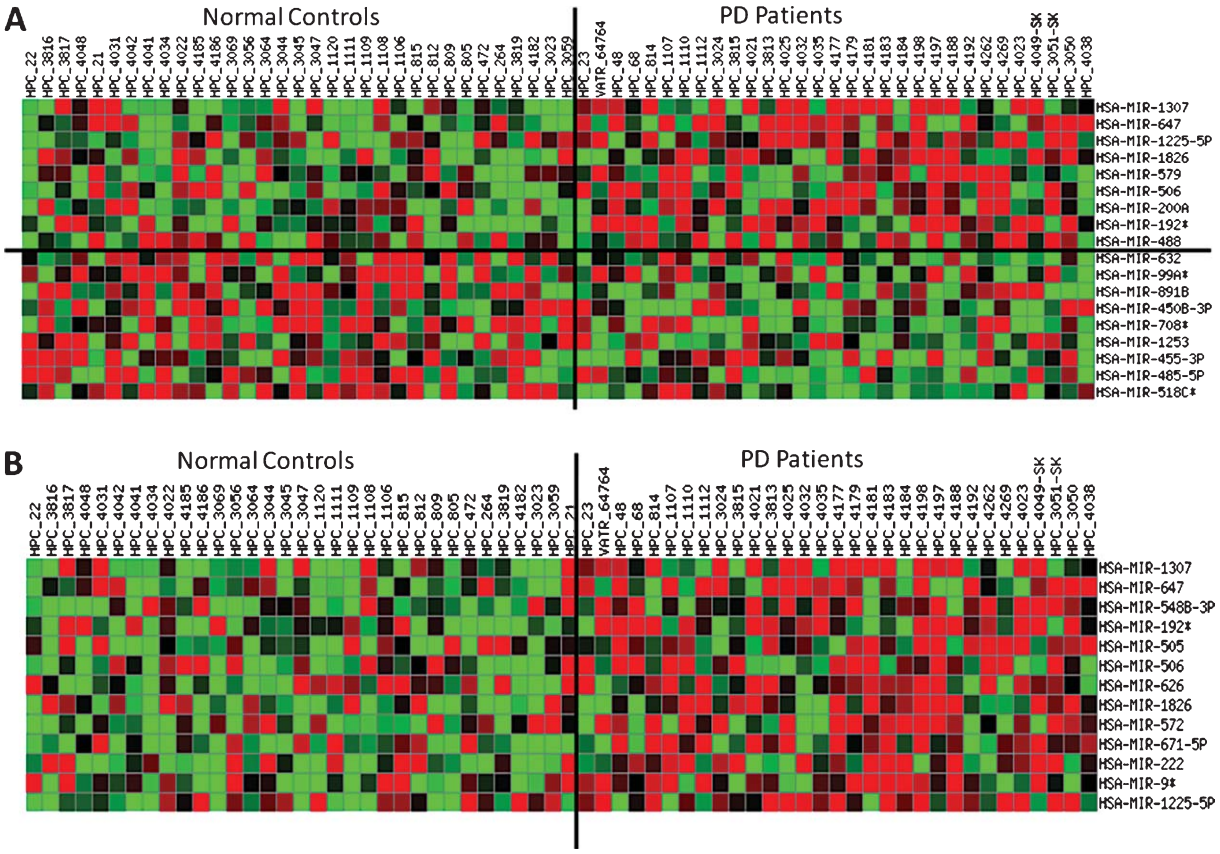


Fig. 1. Heatmaps represent the expressions of circulating miRNAs in plasma of controls and PD patients. A. The expressions of nine pairs of highest ranked k-TSP PD-predictive circulating miRNAs. B. The expressions of thirteen most differentially-expressed circulating miRNAs by SAM analysis. The expression levels are exhibited in shades of green and red. Lightest green, lowest expression; black, neutral; lightest red, highest expression. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPD-012144)

the combination of k-TSP1 (miR-1826/miR-450b-3p), miR-626, and miR-505 achieved the highest predictive performance: 91% sensitivity, 100% specificity, 100% positive predicted value, and 88% negative predicted value in the replication set (Table 3). The performance of this panel of miRNA biomarker candidates was then evaluated in a new, independent validation set. The expression of miR-450b-3p from k-TSP1 could not be detected and hence, unfortunately, the overall performance for this specific panel of biomarkers could not be evaluated in this data set. However, interestingly, SAM's miR-626 showed high specificity (100%; all normal controls, PSP, and MSA were predicted as controls) but poor sensitivity (0%; all PD were predicted as controls) while miR-505 showed the reverse: low specificity (18%) and average sensitivity (76%; 2 of 4 MSA and 5 of 5 PSP predicted as PD) (Supplementary Fig. 1). We also examined the associations between age, gender, and treatment of these biomarkers but there were no significant statistical differences between these groups.

DISCUSSION

In this comprehensive proof-of-concept study, we obtained the global miRNA expressions in plasma of an initial discovery set of 32 PD patients and 32 normal controls. We identified 9 pairs of PD-predictive classifiers and 13 most-differentially expressed miRNAs as potential biomarkers to discriminate PD patients from normal controls. We then used qRT-PCR to validate and evaluate the performance of these biomarkers in a new replication set of 42 PD patients and 30 controls from the same clinical site. We found that the combination of k-TSP1 (miR-1826/miR-450b-3p), miR-626, and miR-505 achieved the highest predictive biomarker performance. We then performed a follow-

Table 2
Performance evaluation of k-TSP PD-predictive miRNA biomarkers show high sensitivity and miRNA biomarkers by SAM show high specificity

miRNA	Sensitivity %	Specificity %	+Predicted Value %	– Predicted Value %
k-TSP1	100	56	92	100
k-TSP2	32	0	93	0
k-TSP3	96	9	66	50
k-TSP4	0	100	0	31
k-TSP5	0	20	0	3
k-TSP1–5	96	27	94	60
k-TSP1–4	96	55	90	75
k-TSP1–3	96	55	90	75
k-TSP1,3,4	96	64	91	78
miR-626	83	100	100	78
miR-505	72	97	97	69
miR-222	78	73	79	58

Note: +, positive; –, negative.

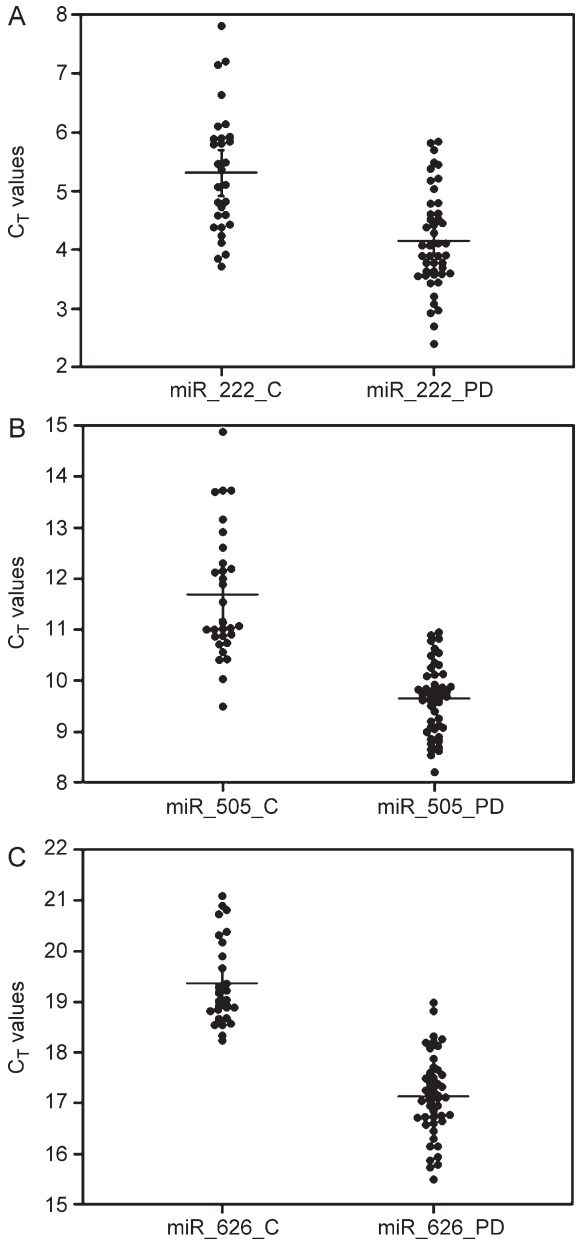


Fig. 2. Scatter plots of differentially miRNA expression of 3 PD biomarkers (from SAM) in healthy controls vs PD patients by qRT-PCR assay. A. The threshold of C_T and P values for miR-222 are 4.5 and 0.0004, respectively. B. The threshold of C_T and P values for miR-505 are 10 and 0.0001, respectively. C. The threshold of C_T and P values for miR-626 are 18 and 0.0001, respectively. The horizontal bar represents the median cycle threshold (C_T) value of each subject in each specific group. C, control group; PD, PD group.

up study, applying this panel of biomarkers to a new, independent validation set of samples (30 PD) from a different clinical site, which showed lower biomarker performance.

Standard clinical diagnosis based on comprehensive neurological examination and neuro-imaging assessment is subjective and the sensitivity of a final ante-mortem diagnosis of PD was 91% compared to autopsy findings [3]. Hence, there is an urgent need to develop objective, measureable biomarkers to improve PD diagnostics and define its subtypes. The ideal biomarker should be minimally-invasive, cost efficient, quantifiable, reproducible, specific, and sensitive. Biofluids such as plasma could provide an ideal resource for development of such desirable biomarkers. However, clinical diagnostic tests based on biochemical analysis of biofluids from PD patients have yet to be established. Here, we reported the first plasma-based circulating miRNA biomarkers for PD. The miRNAs we identified fit the criteria of high-quality biomarkers: minimally-invasive protocol (collection of blood plasma sample), simple and quantifiable detection method (qRT-PCR), stable, non-post process modification and sequence conservation of biochemical (miRNA characteristics), specific and sensitive in performance. Moreover, using a plasma-based diagnostic method as in this study allows monitoring of a PD patient over time with fewer difficulties, while biopsy is almost impossible and neuro-imaging every 3 months is financially burdensome.

It is known that miRNAs detected in various cells and tissues can also be found in biofluids such as blood plasma and serum [30]. Our preliminary study using miRNA microarrays showed that approximately 4% (35/866) of miRNAs from healthy brain tissues could also be detected in the plasma of healthy controls. The expression and composition of plasma

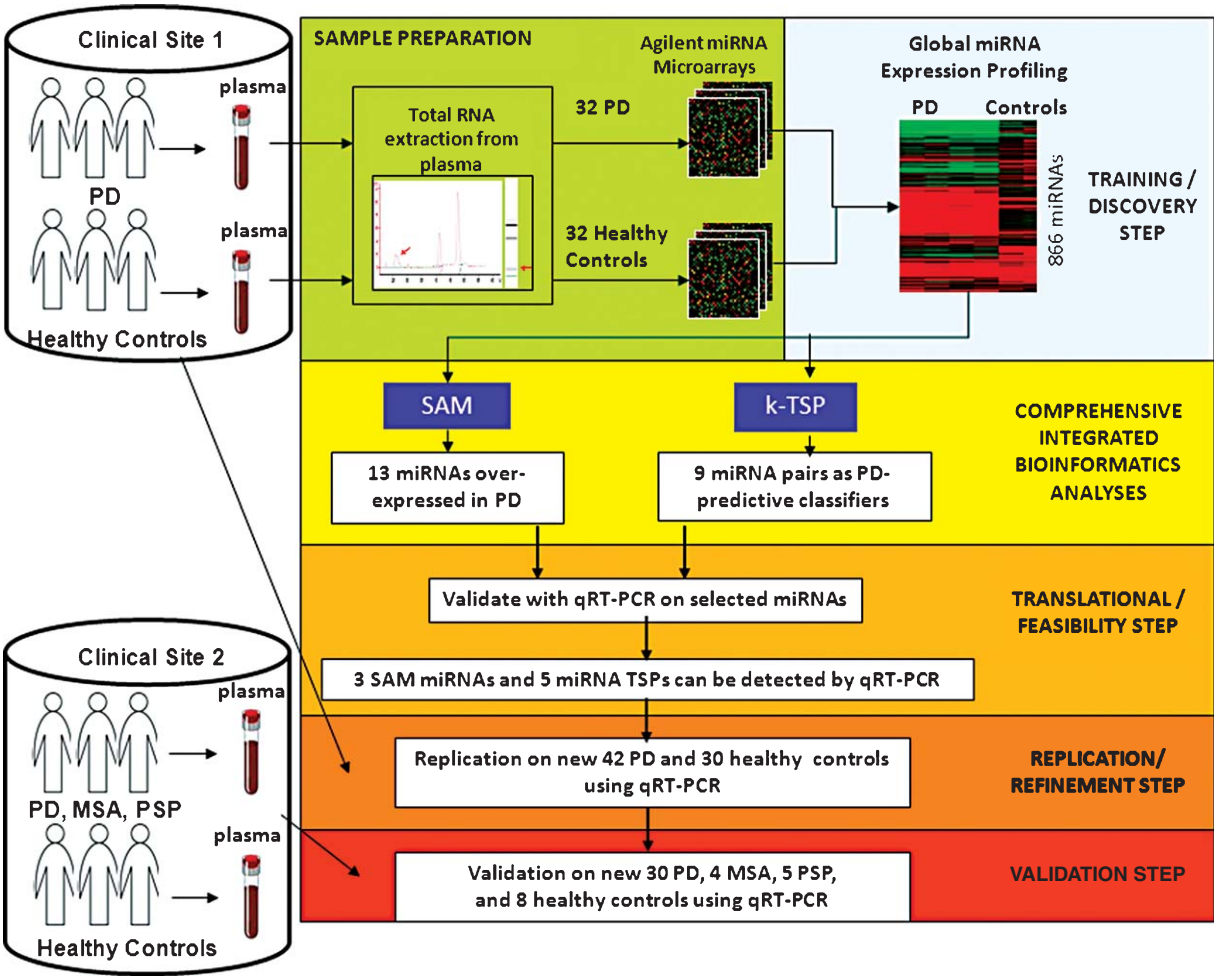


Fig. 3. Our proof-of-concept study demonstrates the feasibility of obtaining PD biomarker candidates from plasma circulating miRNAs.

Table 3
Identification of plasma-based PD biomarker panels with highest predictive performance

miRNA	Sensitivity %	Specificity %	+Predicted Value %	– Predicted Value %
k-TSP1+miR626	98	83	90	96
k-TSP1+miR505	100	87	92	95
k-TSP1+miR-222	98	63	80	95
k-TSP1+miR626 +miR505	91	100	100	88

Note: +, positive; –, negative.

circulating miRNAs is known to correlate with disease manifestation, including neurodegenerative disorders such as Huntington's disease [17]. In this study, we hypothesized that specific miRNAs can be detected in plasma to discriminate PD patients from healthy controls. Higher expression of k-TSP pairs in PD patients that lead to a higher detection rate in general compared to controls may indicate incremental changes of miRNA expression as PD progresses and are worthy of further investigation. We also identified a panel of biomarker candidates for PD: k-TSP1 (miR-1826/miR-450b-3p), miR-626, and miR-505. Interestingly, miR-1826 has also been recently found to be up-regulated in plasma of multiple sclerosis patients [31]. miR-505 is reported to be modulated by LRF (leukemia/lymphoma-related factor) in mouse embryonic fibroblasts, targeting alternative splicing factor/splicing factor 2 (ASF/SF2), a serine/arginine protein essential for cell proliferation and survival [32]. On the other hand, currently, there is no report on miR-626 or miR-450b-3p that correlates with any diseases or cell functions. Whether these miRNA biomarkers

play direct or indirect roles in the pathogenesis of PD are pertinent questions to examine in the future.

Some limitations should be considered when interpreting our results. Firstly, there are factors that influence miRNA expression, such as medication and medical co-morbidities. Xenobiotic drugs have been known to alter miRNA expression in human cancer cell lines [33]. In peripheral blood, differentially-expressed miRNAs were found in levodopa treated vs. untreated patients [34]. Although we did not perform global miRNA profiling on our treated/untreated PD patients, from our qRT-PCR results, we found no statistical significance in the plasma miRNA expression of our 10 non-medicated vs. 20 medicated PD patients (biomarkers miR-626 and miR-505, $P = 0.70$ and $P = 0.12$, respectively), suggesting that variable miRNA expression may be due to disease severity or medical co-morbidity. Plasma alpha-synuclein levels are also known to be similar between treated and untreated PD patients [35]. In patients with complex regional pain syndrome, a chronic pain condition resulting from dysfunction in the central nervous system, miRNAs in blood have been associated with co-morbidities such as headache and use of antiepileptic drugs [36]. Here, the association between miRNA expression and multiple co-morbidities of PD was not studied due to a small sample size. That brings us to the second limitation: that our studies can be strengthened with a larger number of patients in the discovery phase to increase the power of statistical analyses and obtain statistically significant results in various subtypes of PD.

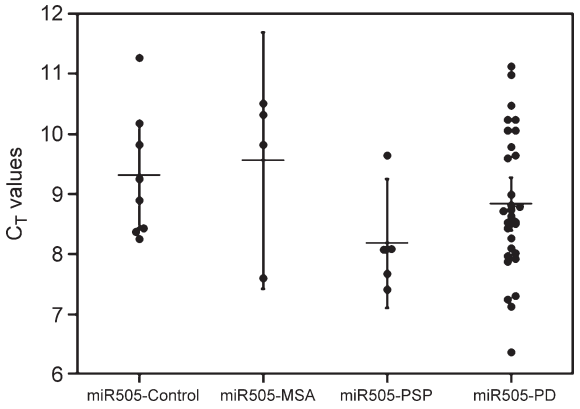
The obstacles of validating biomarker candidates due to clinical and sample variability are widely known [1, 37]. Here, our discovery and replication sets were recruited with the same clinical criteria and sampling manner for the same center. Thus, like most single-center samples, coefficients of variation are reduced significantly [38], resulting in high validity of our biomarker candidates in the replication set. However, biomarkers' performance was reduced in our validation set from another center, despite applying a multiple thresholds approach. Here, we have three things to consider: 1) unlike the discovery and replication sets, the validation set also included non-PD controls (MSA and PSP), besides PD and normal controls. It is commonly known that a single miRNA can target multiple genes and vice versa [39] and therefore, a specific miRNA may be modulated and expressed in multiple disease syndromes. The lower sensitivity and specificity in the validation set that include MSA/PSP as non-PD controls (Supplementary Fig. 1) may indicate that these movement disorders share similar pathogenic mechanisms with PD, making the miRNA candidates targeting movement disorder-related neurodegenerative disease in general instead of PD-specific, but this needs further confirmation with a larger number of MSA and PSP samples, 2) besides considerable variation among all studied human subjects, the commonly reported inter-center variability resulting from "noisiness" in the clinical data and samples from different centers [11] existed in our studies. Lack of standardization in clinical and biological procedures is one of the most common pitfalls that deters cross-validation findings. Hence, ideally, recruitment using standardized clinical and biological protocols from multiple centers such as the Parkinson's Progression Markers Initiative (PPMI) is recommended for PD biomarker discovery studies. However, to eventually test biomarker candidates in practicing clinical settings, non-standardization of clinical and biological procedures is probably unavoidable and may remain a challenge, and 3) TaqMan qRT-PCR may not be the most sensitive technology to validate the expression of all miRNA biomarkers as there were examples of miRNA non-expression using this platform. Direct measurement of miRNA expression without enzymatic reactions or bias using the NanoString technology [40] may be an alternate approach to validate the biomarkers.

In summary, this is a proof-of-concept study to demonstrate the feasibility of using plasma-based circulating miRNAs for PD biomarker discovery (Fig. 3). The hypothesis that miRNA expression changes are associated with the neurodegenerative disease process, either directly or as part of positive feedback loops, is emerging rapidly [41, 42]. Hence, this pilot study opens new opportunities to the exploration of circulating miRNAs for diagnostic, prognostic, and therapeutic interventions for PD and possibly other neurodegenerative diseases. Our future plans include cross-platform re-discovery and re-validation using next-generation sequencing of miRNAs (whole-genome miRNA sequencing) and miRNA microarrays in larger and independent multi-center standardized samples such as from PPMI. In order to achieve the ultimate goal of developing a quantifiable clinical diagnostic test that can better define PD risk, an integrated and multi-disciplinary approach such as coupling plasma-based biomarkers with imaging technology may be necessary to develop a powerful next-generation diagnostic tool for PD.

SUPPLEMENTARY MATERIAL

Supplementary Table 1
miRNA assays and sequences for validation of miRNA gene expression using TaqMan real time PCR on PD and normal control samples

miRNA assay	Sequence
hsa-miR-1307	ACUCGGCGUGGCGUCGGUCGUG
hsa-miR-632	GUGUCUGCUUCCUGUGGGA
hsa-miR-647	GUGGCUGCACUCACUUCCUUC
hsa-miR-99a*	CAAGCUCGCUUCUAUGGGUCUG
hsa-miR1225-5p	GUGGGUACGGCCCAGUGGGGGG
hsa-miR-891b	UGCAACUUACCUGAGUCAUUGA
hsa-miR-1826	AUUGAUCAUCGACACUUCGAACGCAAU
hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA
hsa-miR-579	UUCAUUUGGUAUAAACCGCGAUU
hsa-miR-708*	CAACUAGACUGUGAGCUUCUAG
hsa-miR-506	UAAGGCACCCUUCUGAGUAGA
hsa-miR-1253	AGAGAAGAAGAUCAGCCUGCA
hsa-miR-200a	UAACACUGUCUGGUAACGAUGU
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC
hsa-miR-192*	CUGCCAAUUCCAUAGGUCACAG
hsa-miR-485-5p	AGAGGCUGGCCGUGAUGAAUUC
hsa-miR-488	UUGAAAGGCUAUUUCUUGGUC
hsa-miR-518c*	UCUCUGGAGGGAAGCACUUUCUG
hsa-miR-626	AGCUGUCUGAAAAUGUCUU
hsa-miR-222	AGCUACAUCUGGCUACUGGGU
hsa-miR-505	CGUCAACACUUGCUGGUUUCCU



Supplementary Fig. 1. miR-505 showed lower specificity and sensitivity when non-PD controls (MSA and PSP) were included to evaluate the biomarker performance in the validation set.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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