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Research article

Parkinson's Disease-related Circulating microRNA Biomarkers — a Validation Study

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Abstract: Parkinson's disease (PD) is the second most common neurodegenerative disease. One of the major challenges in studying this progressive neurological disorder is to identify and develop biomarkers for early detection. Recently, several blood-based microRNA (miRNA) biomarkers for PD have been reported. However, follow-up studies with new, independent cohorts have been rare. Previously, we identified a panel of four circulating miRNA biomarkers for PD (miR-1826, miR-450b-3p, miR-505, and miR-626) with biomarker performance of 91% sensitivity and 100% specificity. However, the expression of miR-450b-3p could not be detected in a new, independent validation set. In our current study, we improved the detection power by including a non-biased pre-amplification step in quantitative real-time PCR (qRT-PCR) and reevaluated the biomarker performance. We found the panel of four PD-related miRNAs achieved the predictive power of 83% sensitivity and 75% specificity in our validation set. This is the first biomarker validation study of PD which showed reproducibility and robustness of plasma-based circulating miRNAs as molecular biomarkers and qRT-PCR as potential diagnostic assay.

Keywords: Parkinson's disease; circulating microRNAs; plasma; biomarkers; validation; quantitative real-time PCR

1. Introduction

Parkinson's disease (PD) is a complex and heterogeneous neurological disorder involving both progressive loss of dopaminergic neurons and intraneuronal aggregation of the protein alpha-synuclein. Its current diagnosis is based primarily on subjective clinical rating scales such as the Unified Parkinson's disease rating scale, applicable only in the presence of its motor symptoms. However, it is well known that by the time of diagnosis, neurodegeneration has already reached a fairly advanced stage. Hence, there is an urgent need to develop biomarkers that are objective and measurable for early detection of PD.

MicroRNAs (miRNAs) are small, conserved RNAs that mediate post-translational gene regulations. They are involved in many essential biological processes such as cell development, differentiation, proliferation, and apoptosis. The unique characteristics of miRNAs include tissue-specificity, high stability, quantifiability, and ease of isolation, making them ideal candidates for biomarker development.

miRNAs that are associated with PD have been reported in the peripheral blood [1,2]. We demonstrated in a proof-of-concept study that plasma-based circulating miRNAs can distinguish PD patients from healthy controls and are potential molecular biomarkers for PD [3]. It was then followed by several other PD-related biomarker studies in serum, plasma, and cerebral spinal fluid [4–8]. None of these studies have reported a follow-up replication and validation study except ours. However, one of the biomarkers (from a panel of four) was not detectable in our previous validation study, making the biomarker performance unavailable.

Validation of candidate biomarkers is one of the biggest challenges in biomarker research [9]. A validation study of potential biomarker candidates can provide a "gauge" for a go or no- go decision to move forward and develop these biomarkers into clinical assays, in our case, a diagnostic test for PD. In this study, we improved the detection power of miRNA biomarkers using a non-biased pre-amplification step and reassessed the performance of potential PD biomarkers in a validation set. This is the first reported validation study for plasma-based biomarkers in PD. We showed reproducibility and comparable sensitivity and specificity using circulating miRNAs as potential molecular biomarkers for PD.

2. Materials and Method

2.1. Study subjects

PD patients and healthy controls were recruited at the Department of Neurology at Umeå University Hospital in Sweden. In addition, progressive supranuclear palsy (PSP) and multiple system atrophy (MSA) patients were also recruited as non-PD controls. All subjects agreed to participate by providing their informed consent forms and this study was approved by the institutional review board. The validation set in this study was composed of 30 PD (10 early-diagnosed — PD-E and 20 advanced PD — PD-L), 7 healthy controls, 5 PSP, and 4 MSA (Table 1).

Table 1.   Characteristics of study subjects for PD biomarker validation set

	PD	Healthy Controls	PSP	MSA
Sample size	16 M 14 F	3 M 4 F	2 M 3 F	2 M 2 F
Age	69.4 ± 9.0	72.3 ± 3.4	75.2 ± 8.8	72.5 ± 17.8
Hoehn & Yahr	2.2 ± 0.7		4 ± 1.2	4.5 ± 1.0

PD = Parkinson's disease; PSP = progressive supranuclear palsy;

MSA = multiple system atrophy; M = Male; F = Female

2.2. Sample collection and RNA isolation

Peripheral blood samples were obtained in 10 ml EDTA blood collection tubes and centrifuged at 4 °C at 1,000g for 15 min. Plasma was collected from the supernatant and store in 500 µl aliquots at −80 °C until further analysis. Total RNA including miRNAs was isolated from plasma using a TRI reagent RT-blood kit (Molecular Research Center). The quality and quantity of RNA were evaluated by RNA 6000 pico kits using a BioAnalyzer (Agilent).

2.3. Quantitative real-time PCR (qRT-PCR) analysis

miRNA expression of miR-1826, miR-450b-3p, miR-505, and miR-626 was evaluated using Taqman miRNA-specific assays (Life Technologies) [3]. An additional pre-amplification of reverse transcription product prior qRT-PCR step was executed according to Kroh et al. [10] with slight modification to improve detection sensitivity. qRT-PCR was performed in a MX3000P QPCR system (Agilent). MiR-15b was used as an endogenous control. The expression of each target miRNA is calculated relative to the endogenous control as ΔCT (delta cycle threshold). Higher value of ΔC_T means lower miRNA expression and vice versa. Comparative C_T ($\Delta\Delta C_T$) method was used to determine the differential miRNA expression [11]. Student t-test was performed and $P < 0.05$ was considered as statistically significant.

2.4. Biomarker performance analysis

Receiver operating characteristics (ROC) curve analysis was performed to summarize the accuracy of biomarker predictions, with an AUC (area under the curve) value close to 1 indicating an excellent diagnostic test [12]. This analysis was performed to confirm the ability of our candidate biomarkers to classify PD vs. controls correctly regardless of their specific thresholds. We then used 95% normalized ΔC_T values of individual miRNAs as optimal thresholds for classifying PD and healthy controls.

3.   Results

3.1. Validation study of circulating miRNA biomarkers by qRT-PCR

The expression of all circulating miRNA biomarkers (miR-1826, miR-450b-3p, miR-505, and miR-626) could be detected using qRT-PCR. ΔC_T values for each biomarker were calculated and

compared among the experimental groups (Figures 1–4). ΔCT of miR-450b-3p in PD was statistically significant ($P < 0.032$) when compared with healthy controls. On the other hand, ΔC_T for miR-1826 in PSP was statistically significant ($P < 0.045$) when compared with healthy controls. We also separated PD into PD-E (early-diagnosed, non-medicated) and PD-L (advanced PD, medicated) for further analysis. Interestingly, we found ΔC_T of miR-1826 and miR-450b-3p statistically significant in PD-L when compared with healthy controls ($P < 0.036$ and $P < 0.039$, respectively) but not in PD-E vs. controls. ΔC_T of miR-626 was found significant in PD-L and all PD (PD-E + PD-L) ($P < 0.026$ and $P < 0.027$, respectively) only when MSA and PSP were combined as a single group of non-PD. $\Delta\Delta C_T$ values of each miRNA for all PD vs. healthy controls, MSA, or PSP were presented in Table 2.
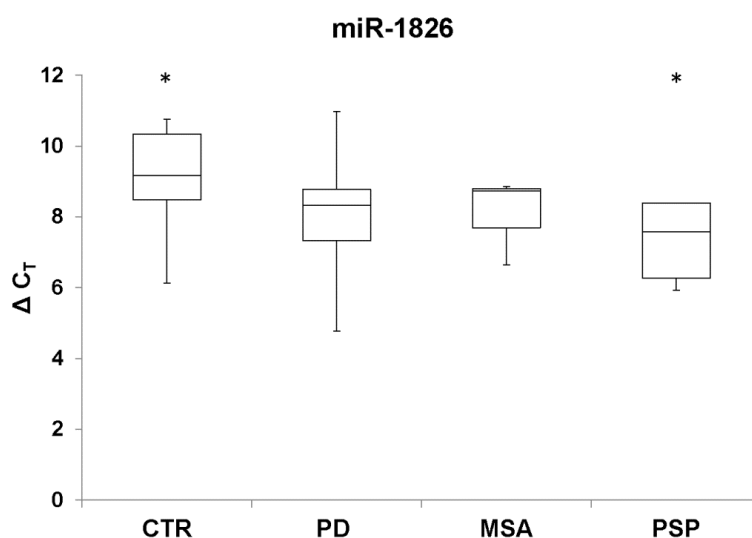

Figure 1. miRNA expression levels of circulating miR-1826 in healthy controls (CTR), Parkinson's disease (PD), multiple system atrophy (MSA), and progressive supranuclear palsy (PSP) patients. * Statistically significance between groups ($P < 0.05$).
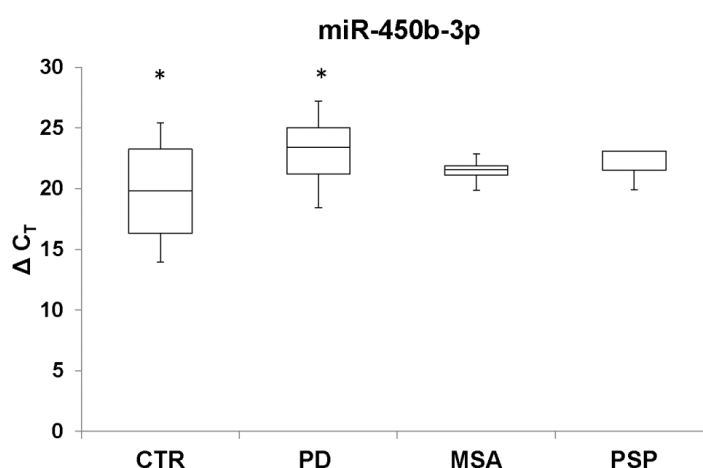

Figure 2. miRNA expression levels of circulating miR-450b-3p in healthy controls (CTR), Parkinson's disease (PD), multiple system atrophy (MSA), and progressive supranuclear palsy (PSP) patients. * Statistically significance between groups ($P < 0.05$).
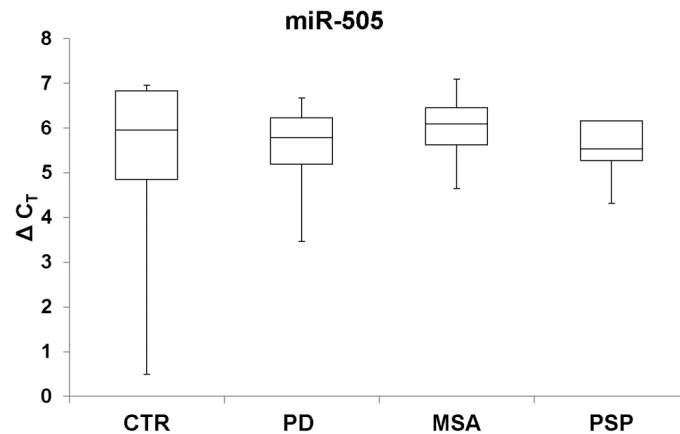
Figure 3. miRNA expression levels of circulating miR-505 in healthy controls (CTR), Parkinson's disease (PD), multiple system atrophy (MSA), and progressive supranuclear palsy (PSP) patients.
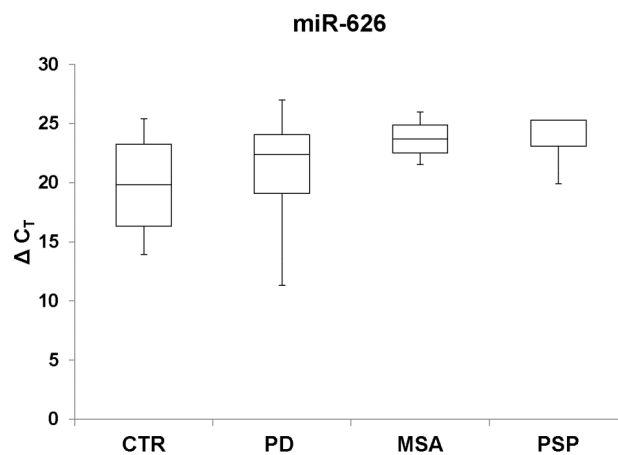

Figure 4. miRNA expression levels of circulating miR-626 in healthy controls (CTR), Parkinson's disease (PD), multiple system atrophy (MSA), and progressive supranuclear palsy (PSP) patients.

3.2. Circulating miRNA biomarker performance

ROC analysis confirmed that the panel of four PD-related biomarkers has the ability to correctly predict PD and healthy controls. In this validation study, the optimal ΔC_T threshold for each individual miRNAs: miR-1826, miR-450b-3p, miR-505, and miR-626, are 10, 18, 4, and 18, respectively. Since miR-1826 and miR-450b-3p were identified by the k-TSP (k-Top Scoring Pairs) algorithm [13], they always worked as a pair and only when the expression of miR-1826 > miR-450b-3p (ΔC_T of miR-1826 < miR-450b-3p), PD is predicted. The combined predictive power of all four biomarkers for PD is 83% sensitivity, 75% specificity, 94% positive predicted value, and 60% negative predicted value.

Table 2. Average $\Delta\Delta C_T$ in log$_2$ fold change for miRNA-1826, miR-450b-3p, miR-505, and miR-626

		CTR	MSA	PSP
PD	miR-1826	2.046	1.055	−1.613
	miR-450b-3p	−9.355	−2.857	−1.920
	miR-505	−1.260	1.316	−1.067
	miR-626	0.326	5.125	3.918
MSA	miR-1826	1.939		−1.702
	miR-450b-3p	−3.274		1.488
	miR-505	−1.659		−1.405
	miR-626	−15.698		−1.308
PSP	miR-1826	3.300		
	miR-450b-3	−4.874		
	miR-505	−1.181		
	miR-626	−12.000		

PD = Parkinson's disease; CTR = healthy controls; MSA = multiple system atrophy;
PSP =progressive supranuclear palsy

4. Discussion

Molecular biomarker discoveries for PD have been emerging rapidly for the last few years. However, insufficient follow-up validation studies, coupled with non-standardized molecular assays, have resulted in a stagnancy of biomarker development for PD. In addition, biomarker validation is known as a "tar pit" [14] as the majority of biomarker candidates were not reproducible and hence failed to move forward to preclinical or clinical settings. Therefore, the validation study presented here is the first step to reaffirm the potential use of circulating miRNAs as molecular biomarkers for PD.

In this study, we reevaluated the performance of a panel of four circulating miRNAs as molecular biomarkers for PD in a previously tested validation set. Previously, the overall biomarker performance could not be obtained due to lack of detection in one of the biomarkers. Here, we improved the sensitivity of detection using a known, non-biased pre-amplification step which enabled all circulating miRNA biomarkers to be validated by qRT-PCR. We found 1) miRNA expression of individual biomarkers such as miR-450b-3p and miR-1826 could significantly differentiate PD and PSP patients from healthy controls, 2) combination of 4 biomarkers brought the predictive power to 83% sensitivity and 75% specificity in the validation set, which is lower when compared with the replication set, 3) no statistical significance could be found in the miRNA expression of all investigated biomarkers between PD vs. PSP and PD vs. MSA, and 4) miR-1826 expression was significantly higher in PD-L than healthy controls and vice versa for miR-450b-3p, but not in PD-E vs. healthy controls.

Although the biomarker predictive power was not as high as the discovery and replication sets, the performance was reproducible in the validation set. Further, this validation study was performed

in a different laboratory setting with different qRT-PCR equipment, further demonstrating potential high reproducibility for this assay using a variety of qRT-PCR instrumentation. Hence, our validation study confirmed the reproducibility and robustness of circulating miRNAs as molecular biomarkers as well as using the qRT-PCR assay as a potential molecular diagnostic tool. Future plans include further evaluation with digital PCR for cross-platform validation studies.

We included MSA and PSP as non-PD controls (MSA and PAP) in the validation study and found that the panel of 4 PD-related biomarkers could not significantly differentiate PD from MSA or PSP (except when MSA and PSP were combined into one group as non-PD, then expression of miR-626 was significantly higher in PD-L and PD-E + PD-L than non-PD). Larger sample size for non-PD controls is required to confirm whether these biomarkers can be used to differentiate non-PD from PD or are more appropriate to target movement disorders-related neurodegenerative disease in general. For the same reason (small sample size), it is also uncertain whether the difference in miRNA expression between early diagnosed, naïve PD patients vs. advanced, medicated PD patients are due to their disease stages and/or medication or merely lack of statistical power.

As noted, small sample size is a major limitation in this validation study. Even so, our findings are promising as we confirmed the validity of the biomarkers as well as the diagnostic assay. It would be reasonable to consider performing a much larger validation study from the same and/or a new, independent collection site to further test the performance and robustness of these biomarkers. It is also worth considering the assembly of PD-related circulating miRNAs from different reports to build a more comprehensive biomarker panel that may further improve prediction sensitivity and specificity.

5. Conclusion

We reported the first validation study of PD-related plasma-based circulating miRNA biomarkers using qRT-PCR and confirmed the validity of the biomarkers and the molecular assay. Implementing vigorous validation studies will ensure development of reliable and robust disease biomarkers before translation into clinical applications.

Acknowledgments

Conflict of Interest

All authors declare no conflict of interest.

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