

Combinatory microRNA serum signatures as classifiers of Parkinson's disease

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Abstract

Introduction

As current clinical diagnostic protocols for Parkinson's disease (PD) may be prone to inaccuracies there is a need to identify and validate molecular biomarkers, such as circulating microRNAs, which will complement current practices and increase diagnostic accuracy. This study identifies, verifies and validates combinatory serum microRNA signatures as diagnostic classifiers of PD across different patient cohorts.

Methods

370 PD (drug naïve) and control serum samples from the Norwegian ParkWest study were used for identification and verification of differential microRNA levels in PD which were validated in a blind study using 64 NY Parkinsonism in UMeå (NYPUM) study serum samples and tested for specificity in 48 Dementia Study of Western Norway (DemWest) study Alzheimer's disease (AD) serum samples using miRNA-microarrays, and quantitative (q) RT-PCR. Proteomic approaches identified potential molecular targets for these microRNAs.

Results

Using Affymetrix GeneChip® miRNA 4.0 arrays and qRT-PCR we comprehensively analyzed serum microRNA levels and found that the microRNA (PARKmiR)-combinations, hsa-miR-335-5p/hsa-miR-3613-3p (95% CI, 0.87-0.94), hsa-miR-335-5p/hsa-miR-6865-3p (95% CI, 0.87-0.93), and miR-335-5p/miR-3613-3p/miR-6865-3p (95% CI, 0.87-0.94) show a high degree of discriminatory accuracy (AUC 0.9-1.0). The PARKmiR signatures were validated in an independent PD cohort (AUC \leq 0.71) and analysis in AD serum samples showed PARKmiR signature specificity to PD. Proteomic

analyses showed that the PARKmiRs regulate key PD-associated proteins, including alpha-synuclein and Leucine Rich Repeat Kinase 2.

Conclusions

Our study has identified and validated unique miRNA serum signatures that represent PD classifiers, which may complement and increase the accuracy of current diagnostic protocols.

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by neuronal cell death and Lewy bodies formation[1]. No molecular PD diagnostic tests exist and current diagnostic protocols, relying on disease presentation, treatment responses, and functional neuroimaging, are prone to diagnostic inaccuracies[2].

MicroRNAs (miRNAs), have been associated with many neuronal processes and some show inappropriate regulation in a PD-specific manner including, miR-548d, miR-224, miR-373, miR-198[3-6]. Circulating miRNAs may reflect cellular miRNA status where miRNA secretion and/or release into serum and plasma may involve active transport or release from apoptotic cells[7]. Studies have identified a number of miRNAs that show differential levels in PD including, miR-16 (blood/serum), miR-19a/b (CSF/blood), miR-29 members (blood), and miR-30 members (blood/serum)[6, 8, 9]. Recently, differential expression of circulating miRNAs (mir-103a, mir-30b, mir-29a) in L-dopa-treated PD patients was reported[10]. Although promising, the miRNAs were identified in relatively small PD cohorts, lacking validation, and by screening only a select number of miRNAs[3, 6, 8, 9].

In this study we comprehensively screened 4603 human mature/pre-miRNAs and 1996 human snoRNA/scaRNAs in order to identify miRNAs that show differential expression in PD patients. Utilizing a large longitudinal PD cohort of drug naïve patients with newly-diagnosed PD (n=180) and matched control subjects (n=190) from the Norwegian ParkWest study we verified a set of combinatory serum miRNA (PARKmiR) signatures that can act as robust classifiers of disease[11]. We further validated the PARKmiR signatures in an independent NY (new) Parkinsonism in UMeå (NYPUM)

study longitudinal cohort and tested for specificity in an Alzheimer's Disease (AD) longitudinal cohort from the Dementia Study of Western Norway (DemWest study)[12, 13]. We also performed proteome analysis in PARKmiR overexpressing SH-SY5Y neuroblastoma cells to identify potential protein targets of the PARKmiRs.

Methods

Ethical parameters

The investigation was conducted in accordance with the ethical standards of the Declaration of Helsinki, and national and international guidelines on research with human subjects. Protocols were approved by the Western Norway Regional Committee for Medical and Health Research Ethics, the Regional Swedish Medical Ethical Review Board and the St. John's University Institutional Review Board.

Patients and controls from the Norwegian ParkWest study, the Swedish NYPUM study and the Norwegian DemWest study

The Norwegian ParkWest and Swedish NYPUM studies are ongoing prospective population-based longitudinal PD cohort studies[11]. Patients with newly-diagnosed PD were recruited from Western and Southern Norway between November 1, 2004, and August 31, 2006, and from the Southern part of Västerbotten county in Northern Sweden between January 1, 2004 and April 30, 2009, respectively, to establish population-representative incident PD cohorts[11, 12]. All 370 ParkWest and 64 NYPUM serum samples were obtained during baseline examination before dopaminergic treatment. Patients and controls are under ongoing follow-up and patients were included if they provided serum at baseline and fulfilled the National Institute of Neurological Disorders and Stroke and UK Brain Bank diagnostic criteria of PD at their final follow-up prior to this study.

The Norwegian DemWest study recruited patients with mild dementia at time of diagnosis in Western Norway during 2005-2007 for a longitudinal cohort study[13].

Patients were diagnosed as AD according to consensus criteria by The National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association. 48 AD patients, age and gender matched with the ParkWest cohort, were selected.

All serum samples from all three cohorts were collected the same day as the clinical examinations and then stored at -70°C until transported on dry ice to New York.

RNA Isolation and cDNA synthesis

24 (16 PD and eight control) serum samples were centrifuged, the supernatant used for miRNA isolation, and the isolated RNA quantified on a Nanodrop 2000 (Thermo Scientific). cel-miR-39-3p (spike-in control) (Qiagen) was added to the RNA lysis buffer.

miRNA microarray

The isolated RNA from 16 patient and eight control serum samples (ParkWest study) were quantified and subjected to Affymetrix GeneChip® miRNA 4.0 Arrays containing 4,603 human mature and pre-miRNAs and 1,996 human snoRNA/scaRNAs at the Yale Center for Genome Analysis (<http://medicine.yale.edu/keck/ycga/index.aspx>)

Data analysis of miRNA microarray

The normalized Affymetrix Expression Console software .CEL files were imported into Partek Genomics Suite version 6.6 Copyright © 2012 (Partek, MO) for analysis. [The 24 Affymetrix Expression Console array files were analyzed using The Partek Genomics](#)

[Suite – microRNA Expression Workflow. Using ANOVA \(a fold change of \$\geq 1.4\$ and a \$p\$ -value cutoff of \$<0.05\$ \) we determined differentially expressed miRNAs.](#)

Quantitative Reverse Transcriptase Polymerase Chain Reaction

cDNA was synthesized following the manufacturer's protocol and subsequent qRT-PCRs were performed using miRNA specific primers (Supplemental Table 3) and PerfeCTa® Universal PCR primer (Quanta Biosciences). scaRNA17 was used as a reference small RNAs for normalizing qRT-PCR Cq values and cel-miR-39-3p (Qiagen) was used as a spike-in control. Standard curve for cel-miR-39-3p was analyzed in MS Excel with $R^2 = 0.98$ and PCR efficiency 92.96%.

Cell culture and transient cell transfection

SH-SY5Y cells (CRL-2266; ATCC) were cultured in a base medium mixture (Invitrogen) supplemented with 10% v/v fetal bovine serum (Atlanta biologics) and 2 mM GlutaMAX (Invitrogen). Cells were transfected with scrambled control mimic, miR-335-5p mimic, miR-3613-3p mimic, and miR-6865-3p mimic all mirVana™ (Life Technologies) in triplicate as previously described[14]. AntagomiR transfections were carried out in similar manner. Cells were harvested after 24 hours for qRT-PCR analysis and after 48 hours for Western blotting and LC-MS analysis.

RNA isolation, RT-PCR and quantitative PCR for neuroblastoma cells

RNA was isolated 24 hours post-transfection followed by cDNA synthesis and qRT-PCR.

Western blotting

Whole cell lysates were prepared, using RIPA buffer 48 hours post-transfection, and used for Western blot analysis following published protocols[15]. Antibodies used were rabbit polyclonal anti-a-syn (Abcam, Cambridge, MA), rabbit polyclonal anti-LRRK2 (Abcam, Cambridge, MA), mouse monoclonal anti-b-Actin (Sigma, St. Louis, MO) and rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology) along with the appropriate HRP-conjugated secondary antibody (Jackson Immuno-research, West Grove, PA).

Sample preparation for liquid chromatography and mass spectrometry analysis

One million frozen cells were lysed and sonicated. The cleared lysate was trypsinized followed by drying and reconstitution in formic acid according to previous protocols[16].

Liquid chromatography and mass spectrometry

Two μL of each sample (1 μg protein) were loaded onto a ReproSil-Pur C18-AQ beads self-packed column (Dr. Maisch GmbH, Germany). Peptides were eluted using a Thermo Scientific EASY-nLC 1000 coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The Q Exactive was operated in data-dependent analysis mode and up to the top 15 most abundant precursors were selected with an isolation window of 1.6 Thompsons and fragmented by higher-energy collisional dissociation. The raw files were processed using the MaxQuant[17] computational proteomics platform (version 1.2.7.0) for peptide identification and quantitation. The fragmentation spectra were searched against the UniProt human protein database (downloaded June 27, 2014). Both peptide and protein identifications were filtered at 1% false discovery rate (FDR).

Image analysis, statistical analysis, logistic regression analysis and contextual analysis

Western blot images were analyzed using IQTL software (GE Healthcare) and Microsoft excel tools were used for two-tailed Student's t-test. Predicted targets of miR-335-5p and miR-3613-3p were used as input queries for the Partek Genomics Suite software, version 6.6 Copyright © 2012 (Partek) to perform Gene ontology (GO) analysis and generate interactive maps and pathways. Label free quantification using mass spectrometry data was performed using the R language package (version 3.2.3). Significantly changed proteins between miRNA transfected and control cells were revealed by a t-test (threshold value $p > 0.01$ and two-fold ratio).

The discriminative ability of miRNAs with regard to PD diagnosis was assessed from ROC analysis using IBM SPSS Statistics, version 21.

Extended methods can be found in the Supplementary Information.

Results

Discovery of differentially expressed serum miRNAs in PD patients and controls

In the discovery phase of this project we performed a miRNA microarray analysis employing serum samples from a subset of PD patients and control individuals participating in the ParkWest study[11]. We performed small RNA isolation on serum from 16 drug naïve PD patients and eight control individuals (Table 1), and following quality-control the RNA samples were analyzed using Affymetrix GeneChip® miRNA 4.0 arrays. Using ANOVA we identified 23 human miRNAs (19 mature and 3 pre-miRNAs) with a fold change of ≥ 1.4 and a p -value cutoff of <0.05 (Supplemental Table 2). We selected a relatively low fold-change and p -value cutoff score to ensure that we captured a comprehensive list of differentially expressed miRNAs within the discovery phase of the project.

Confirmation of the differentially expressed miRNAs

As miRNA microarray screening may give rise to false positives the 24 serum samples used for the microarray screen were subjected to qRT-PCR assays using scaRNA17 as a reference small RNA and cel-miR-39-3p as a spike-in control. [The spike-in control was included to ensure the recovery of total small RNA was consistent across serum samples.](#) From the 23 putative miRNAs in the discovery phase we confirmed that human mature miRNAs hsa-miR-335-5p, hsa-miR-3613-3p, and hsa-miR-6865-3p (PARKmiRs) were significantly upregulated in the 16 PD patients as compared to the eight control individuals suggesting that the three PARKmiRs may represent a miRNA signature that can distinguish PD serum samples from control serum samples (Fig. 1A).

Verification of the PARKmiRs in a large longitudinal cohort

For the verification phase of this project we employed a qRT-PCR-based approach using 346 ParkWest serum samples (182 control and 164 newly-diagnosed, drug-naïve PD patients) (Table 1) and found that a combination of three PARKmiRs (hsa-miR-335-5p, hsa-miR-3613-3p, hsa-miR-6865-3p) were robust [classifiers](#) of PD. Indeed, Receiver Operating Characteristic (ROC) curve analysis shows that the combinations, hsa-miR-335-5p/hsa-miR-3613-3p (AUC 0.90, 95% CI 0.87 to 0.94), hsa-miR-335-5p/hsa-miR-6865-3p (AUC 0.90, 95% CI 0.87 to 0.93), miR-335-5p/miR-3613-3p/miR-6865-3p (AUC 0.90, 95% CI 0.87 to 0.94), and to a lesser extent hsa-miR-3613-3p/hsa-miR-6865-3p (AUC 0.74, 95% CI 0.69 to 0.80), can distinguish PD serum from control serum at the time of diagnosis (Fig. 1B-1F).

Validation of the PARKmiRs in an independent cohort

To validate the ParkWest discovery and verification phases we tested the PARKmiR signatures in 64 NYPUM study[12] serum samples (22 control and 42 newly-diagnosed, drug-naïve PD patients) (Table 1). All serum samples were subjected to identical assay conditions as in the verification phase and miRNA expression profiles were analyzed using ROC curve analysis. We validated that the PARKmiR combinations, miR-335-5p/miR-3613-3p (AUC 0.75, 95% CI 0.63 to 0.87), miR-3613-3p/miR-6865-3p (AUC 0.75, 95% CI 0.63 to 0.87), miR-335-5p/miR-6865-3p (AUC 0.71, 95% CI 0.59 to 0.84), and miR-335-5p/miR-3613-3p/miR-6865-3p (AUC 0.76, 95% CI 0.64 to 0.87),

significantly distinguished PD serum from control serum as observed in the ParkWest cohort (Fig. 2A-2E).

Interestingly, miR-335-5p can distinguish PD serum from control serum in the ParkWest cohort (AUC 0.90, 95% CI 0.87 to 0.93) whilst in the NYPUM cohort miR-335-5p was unable to differentiate PD from control serum (AUC 0.62, $p = 0.125$) (Supplemental Fig. 1). This suggests that single miRNA expression profile differences may not represent robust PD [classifier](#)s across different patient cohorts but rather that combinatory miRNA signatures are required.

The PARKmiR signatures exhibit different profiles in AD patient serum

To ensure that the PARKmiR signatures showed specificity towards PD and not towards general neurodegeneration we tested the PARKmiR profiles in AD serum samples from the DemWest study[13]. 48 AD serum samples (Table 1) were subjected to RNA isolation, cDNA synthesis and qPCR analysis as in the verification phase. Interestingly, and in contrast to PD serum, the PARKmiRs displayed decreased levels in AD serum suggesting that the identified PARKmiR signatures show specificity towards PD (Fig. 3). Although the PD and AD serum samples were collected, processed, stored and analyzed in an identical manner, at the same clinical site, we corroborated the findings by performing qPCR analyses on the AD serum samples using U6 and miR-455-3p as control targets. miR-455-3p was chosen as a control target as it did not show any significant differences in abundance between PD and control serum in the confirmation phase of the project. Our results demonstrate that both U6 and miR-455-3p show no

significant differences in abundance amongst control, PD and AD samples, confirming the integrity of the AD samples and dataset (Supplemental Fig. 2).

***In silico* target prediction of the PARKmiRs**

Some miRNAs are deregulated in both biofluids and in brain tissue suggesting that perhaps the increase in serum PARKmiRs in PD may reflect PARKmiR secretion and/or release from apoptotic cells in the brain[3]. We therefore performed *in silico* prediction analyses to identify putative protein targets of the PARKmiRs using the Partek Genomics Suite, TargetScanHuman 6.2, DIANA TarBase, miRTarBase, miRecords, comiR and miRDB. We found that the PARKmiRs were predicted to target numerous proteins associated with fundamental neuronal processes with high enrichment scores (ES) including neurogenesis (18.3), neuronal developmental (14.8), differentiation (9.9), neurotransmitter secretion (6.1), and neurotransmitter transport (4.4) (Supplemental Fig. 3).

Interestingly, hsa-miR-335-5p may target *LRRK2* and *Parkin* whereas hsa-miR-3613-3p may target *SNCA*. No significant neuronal-associated protein targets were predicted for miR-6865-3p. To verify this we transfected mimics and antagomirs of hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-3p into SH-SY5Y neuroblastoma cells (Fig. 4A, 4D and 4H) followed by Western blotting. We found that the hsa-miR-335-5p mimic downregulates LRRK2 whilst inhibiting hsa-miR-335-5p leads to LRRK2 upregulation (Fig. 4B, 4C). Similarly, the miR-3613-3p mimic upregulates a-syn whilst the antagomir moderately downregulates a-syn (Fig. 4E, 4F). To corroborate these findings we found that *a-syn* mRNA levels are regulated by the hsa-miR-3613-3p mimic and antagomir

(Fig. 4G). By contrast, we did not find any significant regulation of Parkin in SH-SY5Y cells expressing the miR-335-5p mimic or antagomir.

Protein target analysis of the PARKmiRs using LC-MS

To complement the *in silico* analysis we subjected PARKmiR mimic- and control mimic-transfected SH-SY5Y cells (Fig. 4A, 4D and 4H) to LC-MS analysis. Out of 2,658 proteins identified (Supplementary Dataset 1) 52 proteins (Supplemental Fig. 2, Supplemental Table 3) showed significant differences in abundance in response to PARKmiR expression compared to mimic control cells. In hsa-miR-335-5p, hsa-miR-3613-3p, and hsa-miR-6865-3p mimic cells 18, 19, and 20 proteins showed statistically significant differential levels, respectively (Supplemental Table 3).

miR-335-5p overexpression resulted in increased levels of E3 ubiquitin ligase hectd1 (Supplemental Table 3) and miR-6865-3p overexpression resulted in increased abundance of the ubiquitin pathway proteins ube2h, ube3c, ube4b and ube2a (Supplemental Table 3). Ubiquitin ligases polyubiquitinate substrates directed for 26S proteasome degradation and inappropriate proteasomal degradation may be one of the causative effects of neurodegeneration[18, 19]. miR-335-5p and miR-6865-3p may affect the ubiquitin pathway having an impact on neurodegeneration.

The PARKmiRs also appear to regulate other key cellular enzymes. For example, miR-335-5p and miR-6865-3p overexpression downregulate phosphatase ppp1r18[20], whereas miR-6865-3p and miR-3613-3p upregulate ppp4r1, a serine/threonine-protein phosphatase 4 regulatory subunit 1[21] (Supplemental Table 3). Numerous phosphatases

and regulatory subunits contribute to cell survival and PARKmiRs may regulate phosphatases influencing neurodegeneration[22].

miR-6865-3p overexpression also increases fkbp10 levels (Supplemental Table 3), a peptidyl-prolyl cis-trans isomerase influencing protein folding[23], and decreases hspb8 (Supplemental Table 3) which stimulates clearance of proteins prone to aggregation in neurodegenerative diseases[24]. miR-6865-3p overexpression downregulates syntaxin-16 levels, a t-SNARE family member (Supplemental Table 3) and SNARE complex assembly is promoted by a-syn[25].

Our data suggests that the serum PARKmiR signatures may not only be valuable as [classifiers](#) of PD but that these signatures may indeed reflect cellular changes in the brain.

Discussion

Our study has shown that combinatory miRNA signatures in serum can act as robust [classifiers](#) of PD and that these miRNA signatures regulate PD-associated proteins, including a-syn and LRRK2. We have shown that a combination of miRNAs hsa-miR-335-5p, hsa-miR-3613-3p, and hsa-miR-6865-3p (PARKmiRs) can differentiate between PD and control serum in a large 370 PD patient and control longitudinal cohort at baseline. Furthermore, these PARKmiR signatures were validated in an independent 64 PD patient and control longitudinal cohort. In addition, the PARKmiR signatures were shown to have specificity towards PD as they exhibit different characteristics in AD serum samples. It is important to note that sequence similarities between the miR-520 and miR-548 families caused unsuccessful qRT-PCR analysis. Further the qRT-PCR data for some of the initial 23 candidate miRNAs in the affymetrix screen showed intergroup variation that resulted in statistically insignificant fold changes. These factors highlight the refinement of the initial 23 putative miRNAs into the more robust 3 PARKmiRs.

Previous studies that have shown differential abundance of miRNAs in biofluids from PD and control samples are inconsistent with very few common candidate miRNAs amongst these studies[3, 6, 8-10]. This is mainly because of the small cohort sizes employed, the characterization of single miRNAs, and importantly the lack of independent validation studies[3, 10]. The importance of using samples at the time of diagnosis (baseline) from at least two independent prospective population-based longitudinal cohorts, in defining and characterizing miRNAs as potential biomarkers, cannot be over emphasized. In general, PD patients undergo a wide variety of pharmacological treatments involving multiple drugs in varying dosage[26] and biofluids

collected for biomarker discovery post-treatment tend to produce inconsistent results that cannot be validated in independent cohorts. The most compelling study to date used 102 whole blood PD samples reporting altered expression of miRNAs, however the study only screened for miRNAs associated with LRRK2[27]. Interestingly, a few studies have detected interesting miRNAs but the sample origin and type of biofluid makes it challenging to connect the findings [3]. [Furthermore, Martins and colleagues found that miR-335-5p is down-regulated in peripheral blood mononuclear cells \(PBMCs\) as compared to the present study showing upregulation in serum \[9\]. The observed differences in miR-335-5p levels can most likely be attributed to the different clinical samples used for the miRNA extraction.](#)

Our study has also highlighted that changes in single miRNA profiles, between control and PD patient serum samples, may not act as robust [classifiers](#) of disease. For example, although miR-335-5p alone distinguished PD serum from control serum in the ParkWest cohort (AUC 0.90, 95% CI 0.87 to 0.93), miR-335-5p was unable to differentiate PD from control serum (AUC 0.62, $p = 0.125$) in the NYPUM cohort (Supplemental Fig. 1). In contrast, PARKmiR combinations acted as good [classifiers](#) across different cohorts suggesting that combinatory miRNA signatures show more promise as complementary diagnostic tools for PD (Fig. 1 and Fig. 2).

Based on our PARKmiR protein target discovery (Fig. 4) it may be that miRNA serum profiles in PD could give insight into cellular abnormalities in brain tissue. Indeed, previous studies have suggested that altered miRNA levels in brain tissue are associated with disease pathogenesis[3, 4, 28]. The experimental verification of the predicted targets a-syn and LRRK2 by miR-335-5p and miR-3613-3p, respectively, complements this

notion (Fig. 4). The LC-MS analysis also revealed the differential expression of many neurodegeneration-associated proteins in response to the PARKmiR mimics, which may shed light on new protein targets in PD pathogenesis. [It is important to note that the exact source \(tissue or organ\) of miRNAs present in biofluids is unclear. Because of this it is challenging to correlate serum miRNA changes in biofluids with functionality in the brain..](#)

Further work will be required to determine how robust the identified miRNA signatures are across additional geographically and ethnically diverse cohorts. It is likely that additional screening is needed to identify supplementary signatures in order to capture the full heterogeneity of PD. [Furthermore the PARKmiRs should be tested in other related synucleinopathies like DLB, to ensure the specificity.](#) Also, because of the heterogeneity of PD we envisage a matrix approach for diagnostic biomarkers where miRNA signatures will represent one class of molecules alongside current diagnostic practices and other biochemical and cellular components.

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Authors' roles

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Simon Geir Møller	Study supervision or coordination, Study concept or design, Analysis or interpretation of data, Drafting/revising the manuscript for content, obtaining of funding

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Figure legends

Fig. 1. Confirmation and verification of PARKmiRs by qRT-PCR. (A) qRT-PCR analysis of miR-3613-3p, miR-6865-3p and miR-335-5p in 16 PD patients compared to eight control serum samples. ROC analysis of qRT-PCR data from 164 PD patients compared to 182 control serum samples from the Norwegian ParkWest study for combination of miR-335-5p/miR-3613-3p (B), miR-3613-3p/miR-6865-3p (C), miR-335-5p/miR-6865-3p (D) and miR-335-5p/miR-3613-3p/miR-6865-3p (E). (F) Statistical analysis (ROC) for PARKmiR combinations. Error bars indicate SEM $n = 8$ (control), 16 (PD); *, $p < 0.05$, **, $p < 0.01$.

Fig. 2. Validation of PARKmiRs by qRT-PCR. ROC analysis of qRT-PCR data from 42 PD patients compared to 22 control serum samples from the NYPUM study for combination of miR-335-5p/miR-3613-3p (A), miR-3613-3p/miR-6865-3p (B), miR-335-5p/miR-6865-3p (C) and miR-335-5p/miR-3613-3p/miR-6865-3p (D). (E) Statistical analysis (ROC) for PARKmiR combinations.

Fig. 3. Specificity of PARKmiR combinations. Plot for qRT-PCR data showing distinct expression patterns observed for PARKmiR combinations in 164 PD patient and 48 AD patient serum samples as compared to 182 control serum samples.

Fig. 4. Overexpression and inhibition of PARKmiRs and their effect on LRRK2 and a-syn. (A, D, H) qRT-PCR analysis showing miR-335-5p (A), miR-3613-3p (D) and miR-6865-3p (H) in response to mimic and antagomir. (B, C) Western blot analysis showing

effect of miR-335-5p on LRRK2 expression in SH-SY5Y cells. (E - G) Western blot analysis (E, F) and qRT-PCR analysis (G) showing effect of miR-3613-3p on a-syn (SNCA) expression in SH-SY5Y cells. Error bars indicate SEM (n = 3 (F, G), n = 4 (A, C, D, H) *, p < 0.05, **, p < 0.01; ***, p < 0.001.