

Differential expression of circulating miRNAs in Parkinson's disease patients: Potential early biomarker?

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Abstract

Background & Objective: Circulating microRNAs (miRNAs) expressions have been suggested as potential biomarkers for Parkinson's Disease (PD). Identification of early biomarkers for PD is important and crucial as PD symptoms occur at a late stage. Hence, these biomarkers could be used in molecular diagnosis for early detection. We therefore examined and compared the expression of circulating miRNAs between PD patients and controls. We also compared the miRNAs expression between early-onset PD (EOPD) and late-onset PD (LOPD). **Methods:** RNA was extracted from the plasma of EOPD (onset age <50 years; n=14), LOPD (onset age < 60 years; n=14) and healthy controls (n=11). The miRNAs expression was determined using the Affymetrix GeneChip microarray. Differential analysis was performed using the R software. Significantly differentiated miRNAs were subsequently analyzed for functional enrichment and biological pathway using the FunRich v1.3 software based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The Omics.net was used to determine the predicted target mRNAs of these miRNAs, and their interactions, based on the five most differentially expressed miRNAs. **Results:** In total, 273 miRNAs were upregulated in PD patients compared to controls. The most significant miRNAs were hsa-miR-301a-3p, 100-5p, 140-5p, 486-3p, 143-3p (fold change ranging from 11.2 – 32.0). A total of 140 circulating miRNAs were differentially expressed in EOPD compared to LOPD. Five of these miRNAs (one upregulated miRNA (hsa-miR-29b-3p) and four downregulated miRNAs (hsa-miR-297, 4462, 1909-5p and 346) belonged exclusively to the EOPD patients. The predicted gene targets of these miRNAs involved in dopaminergic synapse regulation, crucial to the pathogenesis of PD.

Conclusion: Circulating miRNAs differ between PD patients and controls, and between EOPD and LOPD patients. A validation study with a larger and more diverse multi-ethnic population should be conducted to confirm our results.

Keywords: Blood biomarker, miRNA, microarray, Parkinson's disease, early Onset, and Late onset Parkinson disease.

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disorder with increasing prevalence, disability, and deaths worldwide due to the rise of the aging population.¹ The Global Burden Of Disease Study on Parkinson's disease showed that the age-standardized prevalence rate of PD in Malaysia had increased from 20.9 % in 1990 to 32.7% in 2016, while the age-standardized mortality rate increased from 8.5% in 1990 to

32.7% in 2016.¹ Typically, PD affects individuals above the age of 50 years, with an estimated worldwide prevalence of 1% in those of 60 years of age. Young-onset PD (YOPD) occurs in individuals aged 40 years and below, and affects approximately 15% of all PD patients.^{2,3} Due to a wide variation in the age of onset, individuals who develop PD at 50 years of age or below are referred to as early-onset PD (EOPD).^{2,3} Sporadic PD occurs in 90% of cases, whereas in the 10% of cases, there is family history of either autosomal

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dominant or recessive inheritance.⁴ Diagnosis of PD at an early stage is often challenging due to the heterogeneity in clinical presentation, disease course and response to medication.⁴ Furthermore, as timing of seeking medical advice could be delayed from when symptoms first appear, the determination of onset age may not always be accurate. Additionally, with clinical phenotypic overlap between idiopathic PD and other parkinsonian syndromes, molecular biomarkers such as circulating miRNA could be helpful in establishing the diagnosis of PD and in distinguishing between EOPD and LOPD more accurately.

MicroRNA (miRNA) is a single-stranded non-coding small RNA (18 – 22 nucleotides in size) capable of negatively regulating the mRNAs expression by binding to the 3'-untranslated region.⁵ Since miRNAs are stably expressed in circulating biofluids⁵, their expression could be used as biomarkers in various stages of PD development and clinical manifestation. Previous reviews have summarized the role of these miRNAs as potential biomarkers for PD, including the miR-205, miR-34b, miR-34c, and miR-144-5p.⁶⁻⁸ There were only a few studies investigating the difference in miRNAs profiles between EOPD and LOPD patients.⁹⁻¹² Moreover, the PD genetics profile in the multi-ethnic population (Malaysia and Singapore) is reported to be different from other Asian population.¹³⁻¹⁶ Here, we conducted a case-control study to compare the differentially expressed miRNAs in the peripheral blood of PD patients and controls as well as EOPD and LOPD patients in Malaysia.

METHODS

Patient and control sample collection

A total of 28 idiopathic PD patients attending the neurology clinic at Hospital Chancellor Tunku Muhriz UKM (HCTM), Kuala Lumpur were recruited into this study. Idiopathic PD patients were diagnosed based on the UK PD Society Brain Bank Criteria by a movement disorder specialist. The Hoehn and Yahr staging was used to determine severity of PD.¹⁷ Patients were excluded if they had other neurodegenerative diseases or parkinsonism from other causes. Following recruitment, PD patients were divided into two groups: EOPD if their age of onset was < 50 years (n=14) and LOPD if their age of onset was >50 years (n=14). Healthy patients, aged between 24 to 60 years with no symptoms or family history of neurodegenerative

diseases or parkinsonism were recruited as healthy controls (n=11) and were stratified into two groups according to age. Those aged less than 50 years became EOPD controls (n=5), while those aged > 50 years became LOPD controls (n=6). The detailed clinical characteristics of PD patients and controls are presented in Supplementary Table 1. This study was approved by the UKMMC Ethics Committee of the Universiti Kebangsaan Malaysia (UKM) (Ethics No: UKM PPI/111/8/JEP-2016-136) and have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. Written informed consent was obtained from all participants before enrolment. For each subject, approximately 3 mL of blood was collected in the EDTA tube for miRNAs isolation immediately after the sampling.

Microarray miRNA expression analysis

miRNAs were isolated using the miRNeasy (Serum/Plasma) kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The purity and quantity of the extracted samples were measured using the Nanodrop Spectrophotometer ND-2000 (Thermo Fisher Scientific, Massachusetts, USA). The integrity of each sample was determined using the Bioanalyzer 2100 (Agilent Technology, California, USA), and samples with the RIN number greater than 7, were allowed to proceed for the microarray experiment. miRNAs expressions were determined using the Affymetrix GeneChip miRNA v.4.0 (Affymetrix, California, USA) according to the recommended protocol. Briefly, the ELOSA Quality Control (QC) plate was prepared according to the manufacturer's instructions and left overnight before the microarray experiment. About 500 ng of total RNA was labeled with biotin using the FlashTag Biotin HSR RNA labeling kit and hybridized to the microarray chip. The chips were then scanned using the GeneChip 3000 7G Scanner (Affymetrix, California, USA), and the raw data were exported out using the GeneChip Command Console Software v.4.0 (Affymetrix, California, USA).

Data preprocessing and differential expression analysis

For all the raw data, missing values from the assay output were imputed. The data was then pre-processed for background correction, normalization, and expression calculation using the R software via the Affy package.¹⁸ For each dataset, the expression level of each microRNA was log₂-transformed before proceeding to

Table 1: Summary of the demographic data of the PD patients involved

A descriptive summary of the PD patients (Early-Onset PD, EOPD, n=14, and Late-Onset PD, LOPD, n=14), and the healthy controls (n=11) that were involved in the present study. Data are expressed as count (n) and percentage (%), except for the Age parameter where the data are expressed as mean \pm standard error of the mean (SEM). Descriptive analysis was performed using χ^2 test and analysis with p-value <0.05 were considered statistically significant.

Parameters	Healthy control (n=11)		EOPD (n=14)	LOPD (n=14)	P-value
	EOPD Control (n=5)	LOPD Control (n=6)			
Gender					NS
Male	1 (9.09)	2 (18.2)	7 (50.0)	4 (28.6)	
Female	4 (36.4)	4 (36.4)	7 (50.0)	10 (71.4)	
Mean age of PD onset	-	-	42.4 \pm 1.47	61.4 \pm 1.41	<0.001
Mean age	37 \pm 2.26	54.8 \pm 1.42	53.9 \pm 1.78	68.3 \pm 1.28	<0.001
Ethnicity					NS
Malay	5 (45.5)	5 (45.5)	9 (64.3)	9 (64.3)	
Chinese	-	-	3 (21.4)	5 (35.7)	
Indian	-	-	2 (14.3)	-	
Other	-	1 (9.09)	-	-	
Disease Severity					0.038
Stage 1	-	-	-	2 (14.3)	
Stage 2	-	-	5 (35.7)	10 (71.4)	
Stage 3	-	-	7 (50.0)	2 (14.3)	
Stage 4	-	-	2 (14.3)	-	
Disease Duration					NS
< 10 years	-	-	6 (42.9)	11 (78.6)	
> 10 years	-	-	8 (57.1)	3 (21.4)	

PD, Parkinson's Disease, NS, Not significant.

differential analysis using the limma package,¹⁹ with an adjustment of the False Discovery Rate (FDR) value, based on the Benjamini and Hochberg method.²⁰ The log₂ fold-change (FC) was calculated based on the mean expression of normalized log₂ values for each group. For all comparisons, miRNA expression with adjusted p-value < 0.05 were considered significant. From these significant results, differentially expressed miRNAs were then ranked based on their FC values from the highest to the lowest, and those miRNAs with log₂ FC > 2 were considered as the top differentially expressed miRNAs.

Biological and functional enrichment analysis of differentially expressed miRNAs

In order to postulate the prospective functions of differentially expressed miRNAs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichment

analyses were performed using the FunRich v3.1.3.²¹ The lists of dysregulated miRNAs were separately uploaded into the software, and the enrichment analysis was then performed. For each comparison, the top five of the biological process (changes in the molecular interactions between the miRNAs-target genes and other molecules in a cellular biological process), and the biological pathways (changes in the miRNAs-target genes in a specific pathway) were selected. The miRNA target mRNA prediction and interaction were determined using the OmicsNet.²²

Statistical analyses

The categorical clinical data analyses were performed using the χ^2 test using GraphPad Prism software version 7 (GraphPad Prism, CA, USA). All analyses with p-value ≤ 0.05 were considered statistically significant.

RESULTS

Summary of the subjects involved

A total of 28 PD patients clinically diagnosed as either EOPD or LOPD were enrolled in the study (Table 1). The mean onset age for the EOPD patients was 42.3 ± 1.47 years, while the mean onset age of LOPD patients was at 61.4 ± 1.41 years. There were no differences in gender, ethnicity, and disease distribution between the two groups. The EOPD patients had more severe stage of the disease compared to the LOPD group (p -value= 0.038). The ethnic distribution of PD patients and healthy controls were as follows (EOPD; Malays =9 and Chinese= 5, LOPD: Malays= 8, Malay/Pakistan= 1, Indian = 2 and Chinese = 3, and healthy controls; Malay = 10, others = 1).

Identification of differentially expressed miRNAs

For within-group comparison, 273 differentially expressed miRNAs were up-regulated in PD patients (both EOPD and LOPD) when compared to healthy controls (Table 2). Using these miRNAs, we compared the expression profile between EOPD and LOPD. A total of 140 miRNAs had uniquely altered expressions when the two groups were compared, with all the miRNAs being down-regulated in the EOPD group (Table 2).

Functional pathway enrichment analysis

All the differentially expressed miRNAs were subjected to the GO and pathway analyses. We selected the top five biological processes and pathways based on the adjusted p -value. All the significant miRNAs were involved in the

Table 2: List of the significantly differentially expressed microRNAs (miRNAs) identified in PD versus healthy controls as well as Early-Onset (EOPD) and Late-Onset (LOPD) Parkinson's disease (PD) patients

Summary of the top significantly differentially expressed microRNAs (miRNAs) (P -value <0.05 and the five highest-ranked based on the Fold Change) identified in different comparison analyses from the Early-Onset (EOPD, $n=14$) and Late-Onset (LOPD, $n=14$) PD patients, as well as the healthy controls ($n=11$).

Comparison Analysis	MicroRNA	Fold change	Log2 Fold change
PD patients vs Healthy controls			
273 miRNAs are up-regulated	hsa-miR-301a-3p	32.02	5.001
0 miRNA is down-regulated	hsa-miR-100-5p	25.29	4.660
	hsa-miR-140-5p	24.35	4.606
	hsa-miR-486-3p	18.82	4.234
	hsa-miR-143-3p	11.24	3.491
EOPD vs Controls			
164 miRNAs are up-regulated	hsa-miR-301a-3p	27.54	4.783
1 miRNA is down-regulated	hsa-miR-101-3p	25.96	4.698
	hsa-miR-140-5p	20.92	4.387
	hsa-miR-486-3p	12.95	3.695
	hsa-miR-29b-3p	12.84	3.683
	hsa-miR-297	-2.92	-1.546
LOPD vs Controls			
386 mRNAs are up-regulated	hsa-miR-101-3p	40.71	5.347
0 miRNA is down-regulated	hsa-miR-301a-3p	36.17	5.177
	hsa-miR-100-5p	31.20	4.963
	hsa-miR-140-5p	28.11	4.813
	hsa-miR-486-3p	24.82	4.633
EOPD vs LOPD			
0 miRNA is up-regulated	hsa-miR-4462	-2.73	-1.449
140 miRNAs are down-regulated	hsa-miR-1909-5p	-2.51	-1.3288
	hsa-miR-346	-2.04	-1.029

PD, Parkinson's Disease, EOPD, Early-onset PD and LOPD, Late-onset PD, miRNAs, microRNAs.

regulation of nucleic acid metabolism in all comparison analyses, except in EOPD vs. LOPD groups (Figure 1). For the pathways involved, the majority of the significant miRNAs were enriched in the VEGF and VEGFR signaling network, glypican and proteoglycan syndecan-mediated signaling events, TRAIL (TNF-related apoptosis-inducing ligand) signaling pathway, integrin signaling and sphingosine 1-phosphate (S1P) pathway (Figure 2).

Prediction of the differentially expressed miRNAs and their target genes

Figure 3 shows the miRNA network for PD vs. healthy controls. From OmicsNet analysis, all predicted target genes of the five top dysregulated miRNAs in all PD cases compared to controls (hsa-miR-301a-3p, 100-5p, 140-5p, 486-3p, 143-3p), interacted with each other. Among these predicted target genes, 19 genes (*AKT1*, *CREBBP*, *CUL2*, *FLT1*, *IGF1R*, *LDHB*, *MTOR*, *EP300*, *VEGFA*, *BCL2*, *ENO4*, *HK2*, *MAPK1*, *SERPINE1*, *EDN1*, *EGLN3*, *CAMK2A*, *ERBB2*, *MKNK2* (p-value = 0.000308) are involved in neuroactive ligand-receptor interaction. Differentially expressed miRNAs were further analyzed to establish the miRNA-gene interaction, especially for the top five miRNAs that are exclusively changed in the EOPD group (hsa-miR-29b-3p, hsa-miR-297, hsa-miR-4462, hsa-miR-1909-5p, and hsa-miR-346). From the OmicsNet analysis, we identified except miR-4462, all the other miRNAs target genes were predicted to interact with each other (Figure 4). Interestingly, we found 10 of these genes (*PPP2R1B*, *GSK3B*, *CALM3*, *FOS*, *KCNJ3*, *PLCB1*, *CREB1*, *AKT3*, *GRIN2B*, and *KCNJ6*) are involved in dopaminergic synaptic regulation (Figure 4).

DISCUSSION

In this present study, several miRNAs have been identified in PD patients versus controls, as well as in EOPD vs. LOPD group. Although PD is the second most common age-related neurodegenerative disease,¹ the mechanisms involved in the pathogenesis of the disease remain poorly understood especially in differentiating and identifying EOPD from LOPD patients.^{2,4} Circulating miRNAs have great potential as biomarkers for early diagnosis of PD, as they are readily available in the circulating bio-fluids and most of their expressions correlate well with brain degeneration and PD pathogenesis.⁵ Previous studies showed that five plasma brain-miRNAs panel (miR-7-5p, miR-124-3p, miR-127-3p, miR-

139-5p, and miR-431-3p), and saliva (miR-153 and miR-223) could detect the idiopathic PD from the controls, though the potential ranges from 70.5% to 79%.^{23,24}

Comparison between PD cases and healthy controls showed that among the 273 differentially expressed miRNAs, three miRNAs (miR-486-3p, 301a-3p, 140-5p) and their predicted target genes were associated with Parkinson's disease.²⁵ Among these miRNAs, miR-486-3p plays a vital role in α -synuclein toxicity by targeting the *SIRT* gene, as shown in HEK293T, neuronal SH-SY5Y, and glioblastoma U87 cell lines.²⁶ In this study, miR-486-3p binds to the 3' UTR of *SIRT2* mRNA, affecting its protein translation. miR-486-3p mimics lead to the decrease of the SIRT2 level and reduce the α -synuclein-induced aggregation and toxicity.²⁶ Interestingly, a genetic variant of rs2241703 in *SIRT2* gene, may disrupt miR-486-3p binding sites in *SIRT2*.²⁶ Integrated *in-silico* analysis of the miRNA microarray data showed that five miRNAs (miR-199-3p, miR-126-5p, miR-29a-3p, miR-19b-3p, and miR-301a-3p) were upregulated in PD, consistent with our findings of miR-301a-3p.²⁷ Though, the exact mechanism of miR-301a-3p in PD progression remains unknown.

One of the predicted target genes from the three miRNAs (miR-486-3p, 301a-3p, 140-5p) network is the *AKT1*, which is involved in many signaling pathways, important in the regulation of proliferation, differentiation, cell survival, and apoptosis.^{28, 29} Inhibition of PI3K-AKT-mTOR signaling pathway leads to decreased *JNK3* expression, which subsequently protects the dopaminergic neurons with a potential to improve PD symptoms.²⁹ Moreover, several *AKT1* genetic variants have been reported in PD patients. In Han Chinese PD patients, the variant rs2498799 was significantly associated with PD, as the G allele of rs2498799 was shown to decrease the risk of developing PD.³⁰ This protective effect of *AKT1* genetic variants was also reported previously in a Greek PD population.³¹ In contrast, four other *AKT1* gene variants (rs2494743, rs2498788, rs2494746, and rs1130214) were not associated with PD in a Swedish Parkinson's disease cohort.³² These inconsistencies could be due to differing ethnic groups of the population studied, since all studies involved similar sporadic PD cases with the same methodological design.

Another predicted target gene is the *CREBBP*, which encodes for the CREB binding protein responsible for controlling cell growth, division, and differentiation for healthy development.³³

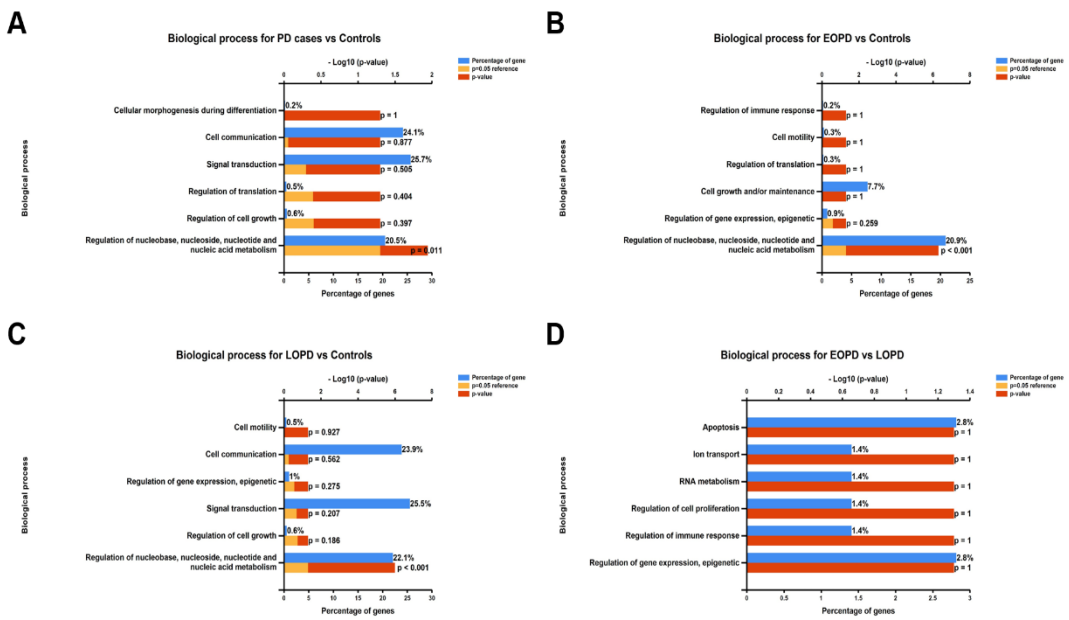


Figure 1. Functional enrichment analysis of differentially expressed microRNAs (miRNAs) in Parkinson's disease (PD) patients in terms of affected biological processes. Graphical presentation of the biological processes involved from the significantly dysregulated miRNAs in the analysis of A) All PD patients compared to healthy controls, B) All EOPD patients compared healthy controls, C) All LOPD patients compared to healthy controls, and D) All EOPD patients compared to LOPD patients. P-value < 0.05 is considered significant. EOPD, Early-onset PD, and LOPD, Late-onset PD.

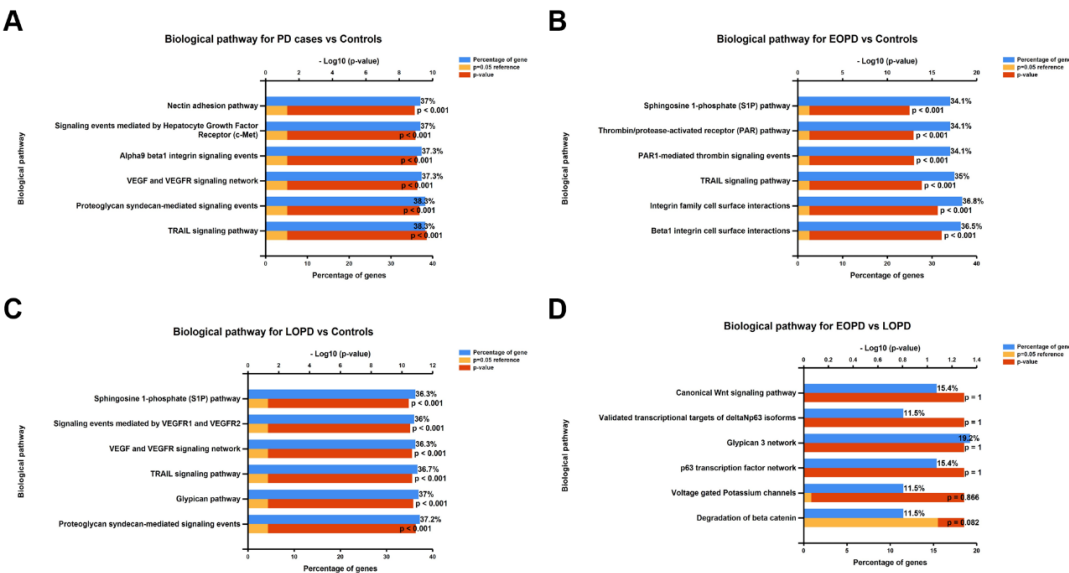


Figure 2. Functional enrichment analysis of differentially expressed microRNAs (miRNAs) in Parkinson's disease (PD) patients in terms of affected biological pathways. Graphical presentation of the biological pathways involved from the significantly dysregulated miRNAs in the analysis of A) All PD patients compared to healthy controls, B) All EOPD patients compared healthy controls, C) All LOPD patients compared to healthy controls, and D) All EOPD patients compared to LOPD patients. P-value < 0.05 is considered significant. EOPD, Early-onset PD, and LOPD, Late-onset PD.

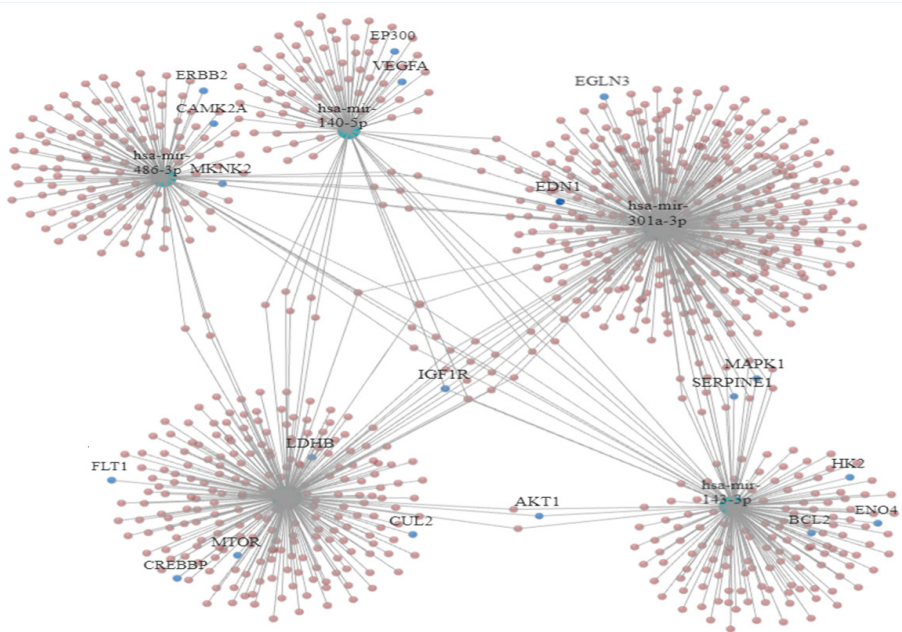


Figure 3. Interaction between the top dysregulated circulating miRNAs in Parkinson's disease (PD) patients with their predicted target genes.

Graphical presentation of the top five dysregulated miRNAs (hsa-miR-301a-3p, 100-5p, 140-5p, 486-3p, 143-3p) with their predicted target genes using Omics.Net. Green nodes, miRNAs, red nodes, target genes, and blue nodes, target genes that are involved in neuroactive ligand-receptor interaction.

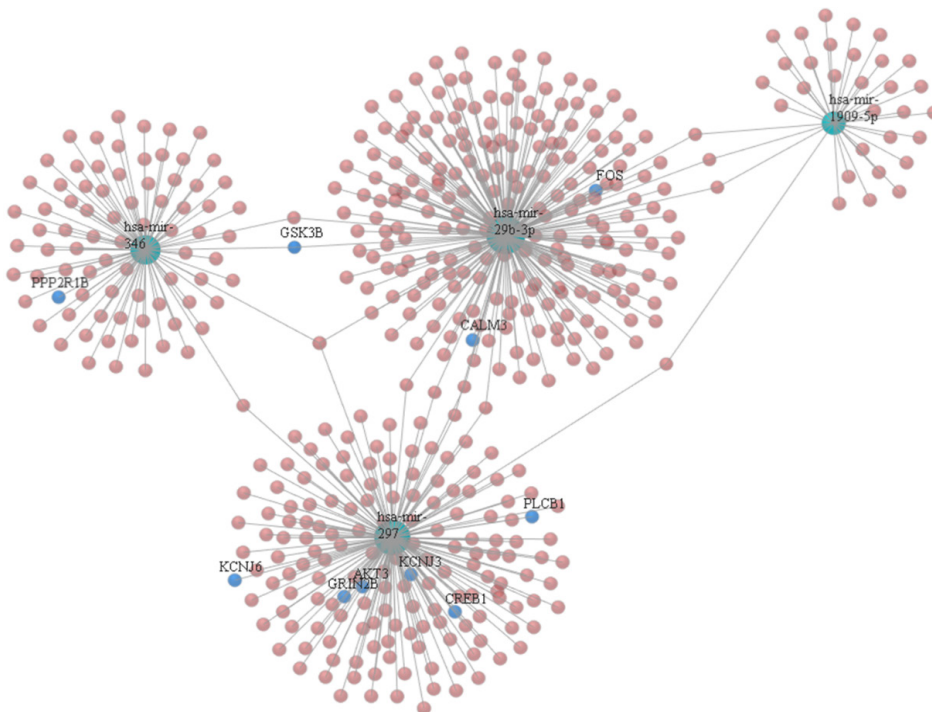


Figure 4. Interaction between the top dysregulated circulating miRNAs in the Early-onset of Parkinson's disease (EOPD) patients with their predicted target genes.

Graphical presentation of the top four dysregulated miRNAs (miR-29b-3p, miR-297, miR-1909-5p, and miR-346) with their predicted target genes using Omics.Net. Green nodes, miRNAs, red nodes, target genes, and blue nodes, target genes that are involved in dopaminergic synapse regulation.

CREBBP gene is important for brain development and in short-term and long-term memory formation.³⁴ Habib and colleagues showed that *CREBBP* gene was a significant node in gene-gene and protein-protein interaction network in neurodegenerative disorders such as Alzheimer's Disease (AD), PD, frontotemporal dementia and amyotrophic lateral sclerosis.³⁵ Another target gene is the Insulin-like growth factor-1 receptor (*IGF1R*), which is predicted to be regulated by hsa-miR-140-5p and hsa-miR-143-3p. *IGF1R* gene encodes a transmembrane receptor that has a tyrosine kinase activity, and it binds to the insulin-like growth factor (IGF1), known for its crucial role in cellular growth, function, and survival. Chi and colleagues performed an integrative analysis of circulating mRNA-lncRNA-miRNA expressions in 50 PD patients, compared to 20 healthy controls showed that *IGF1R* expression was significantly upregulated in PD and enriched in the endocytosis pathway, via its interaction with two miRNAs, hsa-miR-133b and has-miR-7.³⁶ Additionally, the target gene *MAPK1* (MAP kinases) controls many physiological processes such as cell differentiation and motility, metabolism, mitosis, stress response, and apoptosis.³⁷ Activation of the MAPK pathways via various stimuli such as stress, pathogens, and toxins induce phosphorylation of downstream targets such as JNKs, p38 MAP kinases (involved in apoptosis), and ERK1/2 (cell growth and differentiation).³⁸

Another important target gene is the *MTOR* gene, which is a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases and predicted to interact with miRNA, hsa-miR-100-5p. In PD animal and cell models, the interaction between miR-100 and *MTOR* was shown to be via the action of a long noncoding RNA, HAGLROS.³⁹ HAGLROS acted as a miR-100 sponge and removed the suppression of *ATG100* expression and consequently activated the PI3K/AKT/mTOR pathway, thus inhibiting apoptosis and autophagy.³⁹ The important role of *MTOR* gene in PD was further demonstrated by Fernandez-Santiago and colleagues, where the genetic variations in mTOR pathway genes modulated differential risk and the age at onset (AAO) of PD.⁴⁰ In that study,⁴⁰ they identified three loci with epistatic interaction of *RPTOR* rs11868112 and *RPS6KA2* rs6456121 with *SNCA* rs356219, which were associated with differential AAO of PD (odds ratio = 2.89; $P < .0001$). Another target gene that was involved in apoptosis and autophagy is the *BCL-2* that encoded an integral

outer mitochondrial membrane protein.⁴¹ *BCL-2* is a target gene for miR-34a, and in neuronal SH-SY5Y cells treated with MPP+ (PD cell model), miR-34a expression was upregulated, whereas the *BCL-2* expression was down-regulated, and leading to apoptosis induction.⁴¹

A total of 140 miRNAs had uniquely altered expressions when the EOPD vs. LOPD groups were compared. Five miRNAs were exclusively changed in the EOPD group (miR-29b-3p, -297, -4462, -1909-5p, and -346). Integrative and prediction analysis showed that these miRNAs target genes are involved in dopaminergic synaptic regulation. Our result is consistent with previous findings that the degeneration of substantia nigra dopaminergic neurons occurred at the onset of PD.⁴² Among these five miRNAs, miR-29b expression was higher in our EOPD patients. Unlike its family members miR-29a and miR-29c, the evidence for the role of miR-29b in PD progression is limited.^{6,7} A previous study of serum miRNAs profiling in PD Chinese patients found that expression of miR-29b was reduced in PD patients compared to the healthy controls, though their patients were older, with an average age of 64 years.⁴³ Interestingly, in that study,⁴³ the expression of miR-29b was consistently higher in females compared to males,⁴³ thus indicating a possible gender-based difference in this miRNA expression. The higher expression of miR-29b in our EOPD patients may suggest that with degeneration of neuronal cells, upregulation of miR-29b expression is necessary to prevent apoptosis.⁴⁴ We also observed a reduction in miR-297, -4462, -1909-5p and -346 expressions in the EOPD patients. Among these reduced miRNAs, miR-346 emerges as a strong candidate as an early biomarker for the neuronal dysregulation, as this miRNA is derived from the impulsivity locus (*Impu1*) which also contains the neuregulin 3 gene (*NRG3*).⁴⁵ *NRG3* expression is important in regulating the level of inhibitory control, and is also associated with the risk of developing Alzheimer's Disease.⁴⁶ We therefore propose that the loss of miR-346 expression in EOPD may indicate the loss of the *NRG3* gene, therefore implying that there may be an early dysregulation of impulse controls. Unfortunately, no information is available for miR-1909-5p, miR-4462, and miR-297 roles in PD or neuronal regulation, except that miR-297, is associated with hypoxic conditions.⁴⁷ In that glioblastoma study⁴⁸, the high expression of miR-297 exhibited toxic effects on the cells, but the hypoxic condition was able to reduce the toxicity effects of miR-297 by suppressing its

downstream target, DGK- α . Although there is no direct evidence of miR-297 role in PD, the fact that hypoxia can induce oxidative stress and inflammation, suggests that this miRNA could contribute to early neurodegenerative process.⁴⁹

Enrichment of the significant miRNAs in EOPD showed that most of the miRNAs were mainly involved in the regulation of VEGF and VEGFR signaling network and glypican/proteoglycans pathways. Vascular endothelial growth factors (VEGF) family of proteins have been shown to have significant neuroprotective effects.⁵⁰ The mechanism of VEGF neuroprotective is mainly by their regulatory effects using the neuropilins (NP1 and NP2) as co-receptor,⁵¹ thus leading to increased angiogenesis and anti-oxidant molecules,⁵² as well as providing anti-apoptotic effects by preventing the binding of apoptosis-inducing factor, SEMA3A to neuropilin⁵³ and via the PI3K/AKT pathway.⁵⁴ Dysregulation of the miRNAs in the VEGF signaling pathway may explain the neuronal degeneration in PD patients. Another pathway involved in the dysregulated miRNAs is through the glypican pathway. Glypicans constitute one of the two major families of heparin sulfate proteoglycans that located rather close to the cell membranes and are involved in the development of morphogenesis, and as regulators in several cell signaling pathways including Wnt and Hedgehog signaling.⁵⁵ There are six glypicans identified in mammals and are referred to as GPC1 through GPC6.⁵⁵ Fico and colleagues had reported that depletion of *Gpc4* expression enhanced DA neurons differentiation from ES cells *in vitro* and *in vivo* by positively modulating Wnt/B-catenin signaling pathway.⁵⁶ However, the exact mechanism of glypicans in the early onset of PD is still unknown and needs further study.

We acknowledge that there are a number of limitations to our study. Firstly, our sample size is smaller compared to other studies on miRNAs in PD.^{9-12,23,24} This is partially due to the limitation in budget and sample recruitment during the study, as the patients were all recruited from a single center. As such, the small sample size had prevented further sub-analysis to determine the effect of disease stages, gender or ethnicity on miRNAs expression,^{57,58} as the number of samples in one of the group was only two. Additionally, the small sample size, could have contributed to the variation in our findings of significantly expressed miRNAs, compared to other studies. Nevertheless, there were some miRNAs in our study which were also reported previously. The higher expression of plasma miR-29b in our

study was reported previously in brain samples of PD patients⁵⁹. In contrast, a reduced expression of miR-29b was previously reported in the peripheral blood mononuclear samples (PBMC) of PD patients.⁶⁰ These differences may due to different sampling tissues (brain tissues, plasma, and PBMC). Another limitation is that we did not measure the plasma level of miR-486,²⁶ which had been recently associated with neurotoxicity of α -synuclein.²⁶ Since we did not perform non-motor symptoms evaluation, we were unable to account if any of the non-motor features could have contributed to the miRNA expression. We also could not fully exclude the possibility of PD medications affecting the miRNA expression as all patients were on treatment. Still, we believe our study is the first to report on miRNAs among Malaysian PD patients and could add to the growing literature on PD-associated miRNAs expression.

In conclusion, our study showed that there were differentially circulating miRNAs and their downstream target genes in PD patients compared to healthy controls, and between EOPD and LOPD patients. This finding is quite interesting as it suggests that patients with EOPD and LOPD have distinct profiles of microRNAs, despite the same underlying pathology.^{2, 4} It would be interesting to see if these circulating microRNAs' expressions change over time, with the disease progression. However, our findings require further validation in a larger sample. Nevertheless, our study showed that dysregulated miRNAs expressions could potentially be used to differentiate between EOPD and LOPD patients. Since some of the previously reported miRNAs have shown a diagnostic potential to identify the PD patients from controls,^{23,24} future studies are needed to evaluate the usefulness of these miRNAs for diagnostic purposes. Furthermore, the stability of the miRNAs compared to their target genes in the circulating biofluids⁶¹ make the miRNAs as the more suitable biomarkers. The identification of various biological molecules as potential biomarkers for EOPD is important to improve the diagnosis and could further help in the treatment and disease management.

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This study was approved by the UKMMC Ethics Committee (Ethic No: UKM PPI/111/8/JEP-2016-136) and have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. For all the human subjects involved in this project, informed consent has been obtained from the participants involved.

DISCLOSURE

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