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Circulating exosomal miRNAs as diagnostic biomarkers in Parkinson's disease

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Abstract. – OBJECTIVE: This research has showed that exosomal miRNAs from cerebrospinal fluid could act as biomarkers for Parkinson's disease (PD). However, no analysis has been conducted to explore the potential value of exosomal miRNAs from plasma.

PATIENTS AND METHODS: 52 patients with PD were included in study group. 48 healthy adults were included in control group. Blood samples were collected from all those people and then exosomes were extracted from the plasma.

RESULTS: Compared with controls, patients with PD showed a significantly higher expression of circulating exosomal miR-331-5p. ROC curve showed that the area values under the curve of miR-331-5p and miR-505 were 0.849 and 0.898, respectively.

CONCLUSIONS: Exosomal miRNAs, including miR-331-5p and miR-505, could potentially act as biomarkers for PD.

Key Words:

Exosomal miRNAs, Parkinson's disease, Plasma, Biomarkers.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder with the clinical characteristics of bradykinesia, tremor and rigidity[1,2]. The prevalence of this disease was estimated at about 1% of the population who was 60-year-old or above in industrialized countries in 2004[3]. However, the ratio increased to 5% in 2016[4]. Thus, it is indeed an increasingly developed disease. However, to date, it is still difficult to diagnose PD due to the subjective examinations[5]. The disease has been under development for several years before the clinical diagnosis[6]. Therefore, to find useful biomarkers that have potential diagnostic value for the early stage of PD is urgent. Exosomes transferring proteins, lipids, DNA, mRNA and microRNAs (miRNAs), have been well recognized as an essential vehicle in many diseases[7,8]. Garcia et al[9] reported exosomes could be used as biomarkers and therapeutic tools for type 1 diabetes mellitus. PD has been linked to several genetic mutations[10] and various environmental factors[11]. As the most extensively researched non-coding RNA, miRNAs are considered to have an impact on the pathogenesis or progression of PD [12]. miRNAs, which are 18-25 nucleotides in length, regulate protein translation through their interaction with 3'-untranslated region of the mRNAs of the target genes[13,14]. A lot of miRNAs from plasma are reported to express significantly higher or lower in PD than healthy people[15]. For example, miR-626 and miR-505 from the plasma were shown to be downregulated in PD, while miR-181c, miR-331-5p, miR-193a-p, miR-196-p, miR-454, miR-125a-p and miR-137 were shown to be upregulated in PD.

Exosomal miRNAs in cerebrospinal fluid have been shown to have the potential diagnostic value for PD[16]. However, little is known about exosomal miRNAs from plasma. Therefore, this study was to determine if exosomal miRNAs from plasma could be new biomarkers for PD.

Patients and Methods

Patients

52 PD patients as study group and 48 healthy people as control group were recruited from Cangzhou Central Hospital (Cangzhou, China). PD patients were diagnosed according to U.K. Parkinson's Disease Society Brain Bank criteria [17]. All patients had a Mini-Mental State Examination (MMSE) score of > 24 and had taken current PD medications for at least 2 weeks. Subjects were excluded if they met any of the following criteria: (1) history of ischemic or hemorrhagic stroke or severe head trauma; (2) cognitive impairment as assessed by Mini-Mental State Examination score of 24 or less; (3) regular intake of

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antioxidant drugs or vitamin supplements in the last 6 months; (4) history of chronic systemic diseases such as malignant tumor, diabetes mellitus, lung diseases, hepatic or hematologic disorders, or chronic renal failure requiring dialysis. Before recruitment, written informed consent was obtained from all participants, and an evaluation that consisted of medical history, physical and neurological examinations, laboratory tests, and neuropsychological assessments, was performed. All the procedures of this study were approved by the Ethics Committee of Cangzhou Central Hospital (Cangzhou, China). All of patients signed informed consent.

Blood Sample Collection

Peripheral blood samples were obtained using 10 ml EDTA tubes. All tubes were put on ice immediately and centrifuged at 4°C, 1,000 g for 15 min. Then, plasma supernatant was collected into 1.5 ml tubes and stored immediately at -80°C for the following analysis.

Exosomes Isolation and Identification

Exosomes Isolation

Exosomes were extracted from the obtained plasma according to the PureExo® Exosome Isolation Kit (101Bio, Palo Alto, *CA*, USA) for serum or plasma. Briefly, the plasma sample was centrifuged at 3,000 g for 15 min to remove cell debris; then, a total volume of 0.9 ml ExoQuick exosome precipitation solution was added to 500 µl plasma and was mixed well. After being incubated at 4°C for 30 min, the mixture was centrifuged at 5,000 g for 3 min. Then, the upper supernatant was removed and the residual fluid was centrifuged again at 5,000 g for 3 min. The upper supernatant and the colorless bottom layer were removed. At this point, only the fluffy middle layer where there were exosomes was left in the tube. Finally, the final exosome pellet was resuspended in 100 µl phosphate-buffered saline (PBS) and stored at -80°C for further studies.

Exosomes Identification

The morphological features of exosomes were examined using transmission electron microscopy (TEM). Briefly, 100 µl of the exosome resuspension were added to a clean wax plate after a copper mesh was placed. 4 minutes later, the copper mesh was removed and put in 2% phosphotungstic acid for 5 min. After the mesh was dried on the filter paper, the size and morphology feature of exosomes were observed by TEM.

The characteristics of exosomes were identified using Western blot analysis. Briefly, proteins were extracted from exosomes resuspension solution with RIPA lysis buffer and separated on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before transferring to a polyvinylidene difluoride (PVDF) membrane. The blotting membrane was incubated with CD9 antibody (1:1000 dilution, Abcam, Cambridge, MA, USA)/CD63 antibody (1:200 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)/TSG101 (1:500 dilution, Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with the corresponding secondary antibodies at room temperature for 1 hour. The proteins were determined using SuperSignal chemiluminescence system (ECL, Pierce Biotechnology, Waltham, MA, USA).

Plasma RNA Isolation

The plasma was centrifugated at 3000 g for 15 min to eliminate cell debris. The plasma RNA was isolated from the plasma using the TRI reagent RT-blood protocol (Molecular Research Center, Cincinnati, OH, USA) [18].

Exosomal RNA Isolation

Exosomal RNA including miRNA was isolated from the exosome resuspension using Exsomal RNA and Protein Extraction kit (101Bio, Palo Alto, *CA*, USA) according to the manufacturer's protocol. Briefly, this protocol included sample preparation, homogenization, phase separation, precipitation, washing, and elution. Finally, the exosomal RNA pellet was dissolved in 10-15 µl RNA elution buffer and the extracted exosomal RNA was stored at -80°C for downstream assay.

RT-qPCR Analysis for Plasma miRNAs and Exosomal miRNAs

The expressions of both plasma miRNA and exosomal miRNAs were analyzed by reverse transcriptase quantitative Real-time PCR (RT-qPCR).

For cDNA synethesis, 1 ng of total RNA was added and reversely transcribed in a 20 µl reaction using a miScript kit (Qiagen, Hilden, Germany). The expressions of miRNAs, including miR-626, miR-505, miR-181c, miR-331-5p, miR-193a-p, miR-196-p, miR-454, miR-125a-p, miR-137, were tested by Real-time PCR using SYBR Green master mix

(Exiqon, Vedbaek, Denmark) on 7900 Real-time PCR machine (Applied Biosystems, Danvers, MA, USA). The reaction conditions were as follows: 95°C for 10 min to denature DNA templates, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The relative expressions of miRNAs were normalized to the endogenous control miRNA expression.

Statistical Analysis

IBM SPSS Statistics version 22 (IBM, Armonk, NY, USA) was used to analyze the difference of exosomal miRNAs between the two groups. Normally distributed data were expressed as the mean ± standard deviation (SD) and receiver operating characteristic (ROC) curves was used to evaluate the diagnostic value of exosomal miRNAs. Results were considered to have statistically significant difference if $p < 0.05$.

Results

Characteristics of the Study Subjects

In this study, 100 subjects were finally recruited, including 52 PD patients and 48 healthy people. No significant differences were shown in the following clinical characteristics between these two groups: age, gender, BMI. Other clinical characteristics including MMSE score and medication were shown in the Table I.

The Identification of Circulating Exosomes

TEM analysis of exosomes revealed that the particles were 30-100 nm in size and round-shaped or oval-shaped in morphology (Figure 1A). Western blot analysis revealed the expression of CD9, CD63, and Tsg101 (Figure 1B). From this evidence, we demonstrated the existence of exosomes in plasma.

Expression of Plasma miRNAs and Exosomal miRNAs

As shown in Figure 2A, the expression of all observed plasma miRNAs showed no significant difference between PD patients and healthy people. However, among all observed exosomal miRNAs, as shown in Figure 2B, exosomal miR-331-5p was significantly higher in PD group than controls, while exosomal miR-505 was significantly lower.

Besides, we compared the expression of plasma miRNAs and exosomal miRNAs in PD patients. It was shown that miR-331-5p was mainly packaged in exosomes (Figure 3). Similarly, miR-505 was barely expressed in exosomes but in the plasma (Figure 3). For the rest of exosomal miRNAs, there were no significant differences between two groups.

Exosomal miRNAs as Potential Biomarkers for PD Patients

In order to determine the diagnostic value and predictive ability of exosomal miRNAs in distinguishing PD patients from healthy population, we performed ROC curve on exosomal miRNA-331-5p and miR-505. As shown in Figure 4, we found that the areas under the curve were high, which were 0.849 and 0.898, respectively.

Discussion

As novel, non-invasive biomarkers, circulating exosomal miRNAs have been proposed to

Table I. Characteristics of the Study Subjects.

Clinical features	Patients with PD (n=52)	Healthy controls (n=48)	*p*-value
Gender (% male)	53.8	54.2	> 0.05
Age (years)	65.6 ± 10.5	61.2 ± 9.0	> 0.05
BMI categories	23.7 ± 2.5	23.1 ± 1.5	> 0.05
Disease duration (y)	5.3 ± 1.6	N/A	
MMSE score	28.5 ± 2.2	N/A	
Medication			
Levodopa/carbidopa	52 (100%)	N/A	
Entacapone	20 (38.5%)	N/A	
Selegiline	10 (19.2%)	N/A	
Ropinirole	9 (17.3%)	N/A	
Pramipexole	5 (9.6%)	N/A	
Amantadine	4 (7.7%)	N/A	

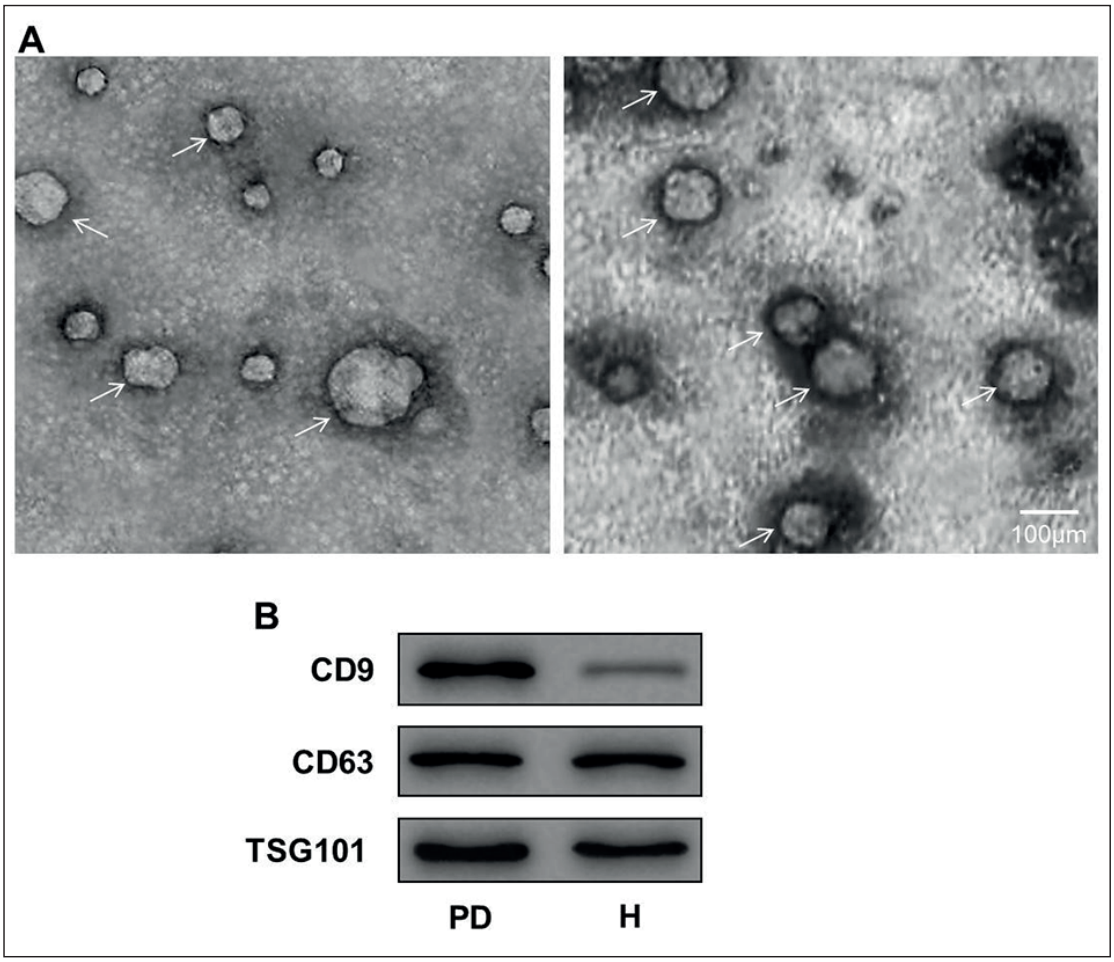

Figure 1. Detection of exosomes in plasma. ***A:*** TEM analysis of exosomes revealed that the particles were 30-100 nm in size and round-shaped or oval-shaped in morphology, bar = 100 μm; ***B:*** Western blot analysis revealed the expression of CD9, CD63, and Tsg101.

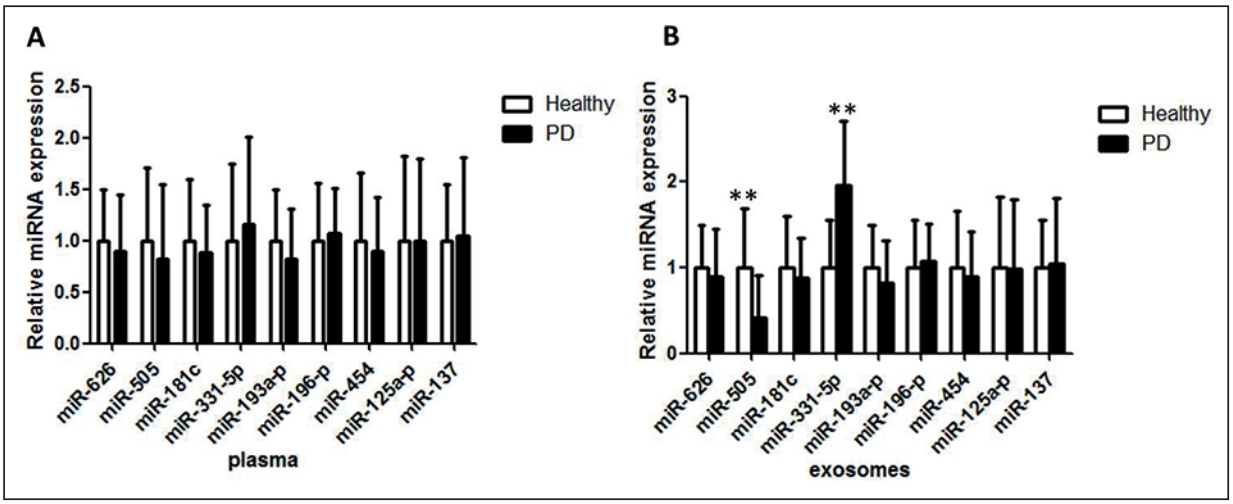

Figure 2. Expression of plasma miRNAs and exosomal miRNAs in PD and health patients. ***A:*** the expression of all observed plasma miRNAs showed no significant difference between PD patients and healthy people. ***B:*** exosomal miR-331-5p was significantly higher in PD group than controls, while exosomal miR-505 was significantly lower. $^{**}p < 0.01$.

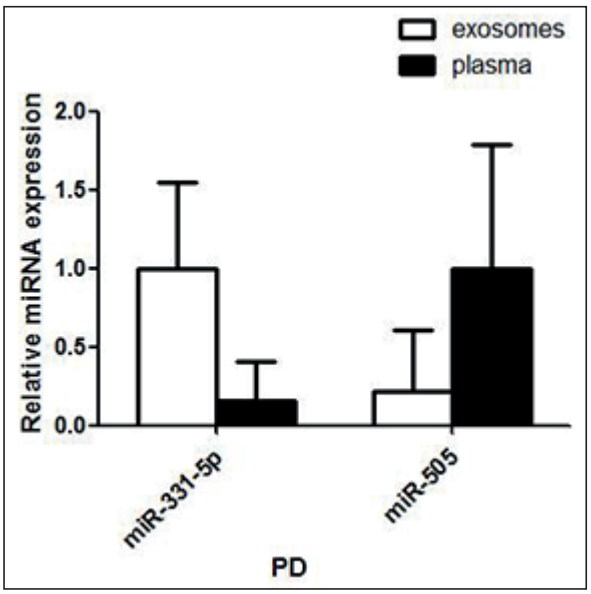

Figure 3. The expression of plasma miRNAs and exosomal miRNAs in PD patients. ***$p < 0.001$, **$p < 0.01$.

have diagnostic value for Alzheimer disease [19,20]. MiR-146a-5p level in serum exosomes predicts therapeutic effect of cisplatin in non-small cell lung cancer[21]. However, to our knowledge, no researches have been reported to explore the role of circulating exosomal miRNAs in PD. Based on previous evidence, we performed an analysis on several exosomal miRNAs, which expressed significantly differently from the plasma between patients with PD and healthy subjects. For the first time, we found that both exosomal miR-331-5p and exosomal miR-505 showed significant difference. On the one hand, Cardo et al[22] reported that 384 miRNAs,miR-181c, miR-331-5p, miR-193a-p, miR-196-p, miR-454, miR-125a-p and miR-137 in PD patients were significantly overrepresented. However, compared to the controls, only miR-331-5p was significantly higher in PD group when those miRNAs were analyzed in 31 patients and 25 healthy subjects. According to our investigation, it was shown interestingly that among those miRNAs from circulating exosomes, only exosomal miR-331-5p showed a significant difference. We also demonstrated that miR-331-5p were mainly packaged in the exosomes rather than in the plasma. We logically guess that miR-331-5p might be transferred to PD-related cells through exosomes, thus getting involved in the pathological process of PD.

Moreover, Khoo et al[18] reported even though circulating miR-505 showed the high predictive power for PD in microarray data, low predictive values which were shown in the validation set. In order to reexamine the role of plasma miR-505, and to examine the role of exosomal miR-505, we performed an analysis on all samples. In accordance with the previous study, we found that the expression of circulating miR-505 showed no significant difference between PD patients and healthy subjects. However, the expression of exosomal miR-505 was significantly lower in PD patients than controls. Combined with the evidence that miR-505 was barely expressed in exosomes but in the plasma, we guess that circulating miR-505 might not be transferred to PD-related cells through exosomes. Therefore, whether miR-505 is transferred via other vesicles like micro particles needs to be further verified.

Of note, to further determine the important role of exosomal miR-331-5p and miR-505 and evaluate their diagnostic values, we performed ROC curve. We found that the area values un-

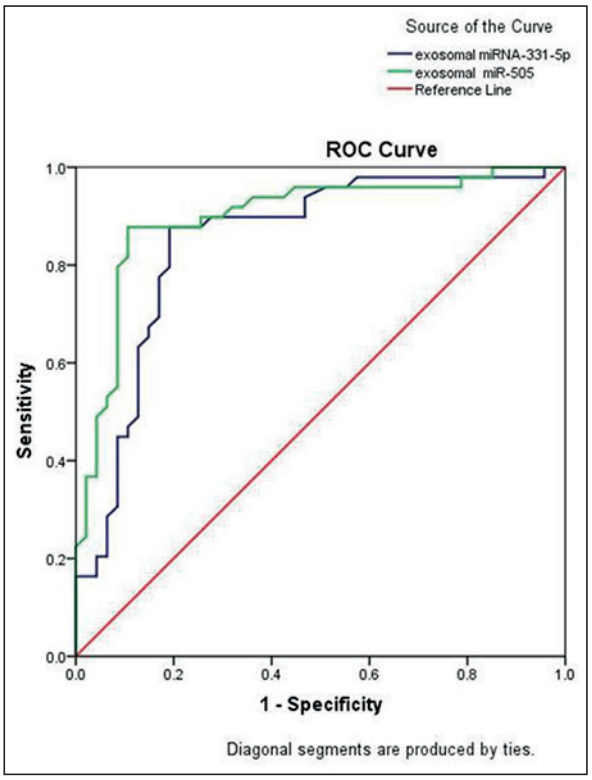

Figure 4. ROC curve on exosomal miRNA-331-5p and miR-505. The areas under the curve were high, which were 0.849 and 0.898, respectively.

der the curve of miR-331-5p and miR-505 were 0.856 and 0.899, respectively. This was the solid evidence that exosomal miR-331-5p and miR-505 could be biomarkers for PD, which was potentially important for clinical use in the early diagnosis of PD.

Conclusions

This study demonstrated that exosomal miR-331-5p and miR-505 have diagnostic values for PD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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