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Original Article

Levels of oligomeric alpha-synuclein in red blood cells are elevated in patients with Parkinson's disease and affected by brain alpha-synuclein expression

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Abstract: Detection of oligomeric α-synuclein (O-α-syn) in blood plasma as a potential diagnostic biomarker for Parkinson's disease (PD) has obtained inconsistent results. The abundance of α-syn in red blood cells (RBCs) provides a novel option for detecting the RBC-derived α-syn in PD diagnosis. Previous studies have shown levels of O-α-syn in RBCs (O-α-syn-RBC) are increased in patients with PD and multiple system atrophy (MSA). However, whether the levels of O-α-syn-RBC can reflect the change of α-syn in the brain and be used as a biomarker for differential diagnosis between PD and MSA remains poorly understood. In the present study, levels of O-α-syn-RBC in participants with PD (n = 333), MSA (n = 114), and healthy controls (n = 334) were measured using an enzyme-linked immunosorbent assay. The levels of O-α-syn-RBC were significantly higher in PD patients than in MSA patients ($P < 0.0001$) and healthy controls ($P < 0.0001$). Receiver operating characteristic curve (ROC) indicated that the O-α-syn-RBC level could clearly separate PD patients from healthy controls (sensitivity: 76.58%; specificity: 77.06; area under the curve (AUC): 0.82). The O-α-syn-RBC level could also differentiate PD from MSA patients (sensitivity: 85.29%; specificity: 61.40%; AUC: 0.78). However, no significant difference was detected in the levels of O-α-syn-RBC between PD and MSA patients. To investigate the correlation between O-α-syn-RBC levels and brain α-syn expressions, rat models with striatum overexpression of human α-syn were developed, and the effect of brain α-syn expression on the levels of O-α-syn-RBC was investigated. Overexpression of α-syn in one side of the striatum led to a significant increase of O-α-syn levels in the ipsilateral striatum, and simultaneously a dramatic elevation of O-α-syn-RBC levels in the periphery. Extracellular application of monomeric α-syn and O-α-syn also resulted in a rapid elevation of O-α-syn-RBC levels. Taken together, the present study suggests that O-α-syn-RBC levels are affected by brain α-syn expression and this could be used as a potential biomarker for separating PD from MSA patients and healthy controls.

Keywords: Oligomeric α-synuclein, red blood cells, nervous system, Parkinson's disease

Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized clinically by movement disorders (including resting tremor, rigidity, bradykinesia, and gesture instability) [1]. Currently, the diagnosis of PD is still a challenge, due to its complexity, heterogeneity and overlapping symptoms and mechanisms with other neurodegenerative diseases, such as multiple system atrophy (MSA) [2]. Therefore, in clinical practice, it is important to find a biomarker for the differential diagnosis of PD.

Pathologically, PD is characterized by intracellular deposition of aggregated alpha-synuclein (α-syn) in multiple brain regions, e.g. the substantia nigra, that primarily drives dysfunctional motor symptoms in PD patients [3]. Accumulating evidence suggests that small α-syn aggregates, especially its oligomeric form (O-α-syn), are the toxic pathogenic species of α-syn [4-7]. Therefore, alterations of O-α-syn in peripheral tissues are thought to be a promising diagnostic biomarker for PD. Efforts have been made to detect O-α-syn levels in cerebrospinal fluid (CSF), blood plasma, and saliva [8]. However, studies obtained so far have been inconsistent, presumably due to cofounding factors such as α-syn contamination by hemolysis, lipoproteins and heterophilic antibodies as well as inadequate age-matched controls, and differ-

ences in detection methods along with sensitivity or accuracy of the antibodies [9-11].

Red blood cells (RBCs) have been shown to have much higher α-syn than other peripheral tissues, accounting for around 99% of all the α-syn contained in whole blood [12]. The abundance of α-syn in RBCs provides a novel option for detecting the RBC-derived α-syn in PD diagnosis. Recent studies have provided evidence supporting the association of several species of α-syn with PD, including total α-syn, some post-translational α-syn modifications, and O-α-syn [13-15]. Due to the pathogenic role of O-α-syn, it is necessary to further investigate its utility as a biomarker for PD diagnosis and differential diagnosis and assess its correlation with brain α-syn expression.

In this study, an enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of O-α-syn in RBCs (O-α-syn-RBC) in patients with PD and MSA, and in heathy controls. The performance of O-α-syn-RBC in separating PD from MSA patients and healthy controls are analyzed. In addition, the correlation between O-α-syn-RBC levels and brain α-syn were investigated in a rat model over-expressing human α-syn in the striatum. The ability for the translocation of O-α-syn from plasma into RBCs was studied.

Methods and methods

Study design and participants

This study was designed to investigate the rationality and performance of O-α-syn-RBC as a diagnostic biomarker for PD, and involved both an animal study and the analysis of a human cohort. In the animal study, we constructed a rat model selectively over-expressing human α-syn in the striatum. Using this model, we assessed the correlation between levels of O-α-syn in the striatum and those in peripheral RBCs and plasma.

The human participants in the study were recruited from Xuanwu and Beijing Tiantan Hospitals of Capital Medical University, Dongfang Hospital of Beijing University of Chinese Medicine, from October 10, 2017 to December 31, 2018. Healthy controls were recruited from the community cohort of the Beijing Longitudinal Study on Aging [16]. Clinical data of both patient and control subjects were first reviewed by investigators from the each hospital, and re-evaluated by two senior movement disorder specialists, specifically for this project. All patients were diagnosed based on the MDS clinical diagnostic criteria for PD, released in 2015 [17] or MSA [18]. Patients with the following conditions were excluded: (i) Parkinsonian syndromes resulting from cerebrovascular, hypoxic, traumatic, infectious, metabolic or systemic diseases affecting the central nervous system (CNS); (ii) Parkinson's plus syndromes, including DLB, PSP and corticobasal degeneration (CBD); (iii) ambiguous diagnosis due to uncertain clinical or imaging features; (iv) a first degree relative with PD and MSA. Participants with incomplete or missing demographic and clinical information, or with low quality samples, were also excluded from the study. All subjects were comprehensively assessed for demographic information and clinical characteristics by the site investigators. Case and control subjects in both study stages were matched for age and sex.

The study was approved by the Institutional Review Board and Ethics Committees of the participating Hospitals. Written informed consent was obtained from each participant or their legal guardians before inclusion in the study.

Production and purification of recombinant O-α-syn

The preparation of recombinant α-syn monomer (wild type α-syn) was described previously [19]. In brief, the pET-15b-NACP plasmids expressing human α-syn were transformed into Escherichia coli BL21 cells, and then, the α-syn proteins were purified by sequential ion exchange, hydrophobic and reverse phase chromatography. Purity of the protein was identified by Coomassie Brilliant Blue staining and Western blot analysis. O-α-syn was prepared by incubating 100 μM of recombinant human α-syn in sterile phosphate-buffered saline (PBS, pH 7.4) with continuous shaking (650 rpm) at 37°C for 7 days on the Eppendorf Thermomixer Comfort [20], separated from the dimers and monomers by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and purified from the gel with a Micro Protein Recovery Kit (Sangon, Biotech, Shanghai, China) according to the protocol provided.

Rat model and histochemical staining

A rat model with the right side of the striatum selectively overexpressing human α-syn was developed by intra-striatal injection of a lentivirus plasmid expressing GFP-conjugated human α-syn (LV-GFP-α-syn) into male Sprague-Dawley (SD) rats (200 g). One and two weeks after injection, the rats were sacrificed, and 8 ml of blood was taken from the heart. Brain tissues were dissected from the striatum, thalamus, midbrain, and olfactory bulb, and homogenized as protein samples. For histochemical staining, rat brains were fixed with rats under deep anesthesia by perfusion of a fixative containing 4% paraformaldehyde and 0.35% glutaraldehyde through the aorta, and the brains were immediately removed and post-fixed for 24 h at 4°C with a fixative containing 4% paraformaldehyde. Then, the brains were cryoprotected by immersion into 20% sucrose before being sliced into a series of 20-μm-thick coronal sections. The sections containing the striatum region were subjected to histochemical staining with DAPI. Immunofluorescence was visualized by confocal microscopy.

Preparation of plasma and red blood cells (RBCs)

At the time of recruitment, whole blood samples (10 ml) from the peripheral vein were drawn into ethylenediaminetetraacetic acid (EDTA) tubes (1.8 mg per milliliter of blood), left to stand in a vaccine carrier (4-8°C) for 30 min, and then centrifuged at 4°C, 1,500 *g* for 15 min. The upper and middle layers, containing plasma and white blood cells, were removed, aliquoted and stored at -80°C for other uses, and the lower layer, containing RBCs, was washed three times with Hank's balanced salt solution (HBSS without Ca^{2+} and Mg^{2+}). Finally, the isolated RBCs samples were collected, aliquoted, and preserved in a freezer (-80°C), and quality-checked every three months using fresh samples as the control. Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) after storage and before the assay.

Western blot analysis

Proteins were separated by 12.5% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). The membrane was blocked for 1 h with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), and probed by 3D5 mouse monoclonal anti-human α-syn antibody (RRID: AB_2315787) (1:10,000) [21]. After washing with TBST, the membrane was allowed to react with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (1:5,000; Vector Laboratories, Burlingame, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence, and measured for densitometry with a Versadoc XL imaging apparatus (Bio-Rad). All experiments were conducted in triplicate.

Measurement of O-α-syn concentration by enzyme-linked immunosorbent assay (ELISA)

O-α-syn-RBC concentrations were measured according to the ELISA method described before [14, 22]. Briefly, a 96-well ELISA plate was coated with non-biotinylated 3D5 mouse monoclonal anti-α-syn antibody (1 μg/ml). After washing with PBS containing 0.05% Tween-20 (PBST) and blocked with 10% BSA in PBST, 100 μl of sample was added to each well and incubated at 37°C for 2 h. After washing with PBST, 100 μl of biotinylated 3D5 antibody (1 μg/ml) in blocking buffer was added to each well and incubated at 37°C for 2 h. After washing with PBST, the plate was incubated for 1 h with 100 μl of ExtrAvidin Alkaline Phosphatase (E-2636, Sigma-Aldrich, St. Louis, MO, USA) (1:5,000). Following washes with PBST, 100 μl of enzyme substrate p-nitrophenyl phosphate (pNPP, N1891, Sigma-Aldrich) was added per well, after which the absorbance was read at 405 nm using a Multiskan MK3 microplate reader (Thermo Scientific, UT, USA). To avoid bias, all the samples were assayed blinded to the diagnosis.

Statistical analysis

All data were compared between groups according to the normality of their distributions. Analyses of variance (ANOVA) or *t*-test, were appropriately used for normally distributed data, and the Kruskal-Wallis test was used for skewed data to compare differences among all the studied groups. The Chi-squared or Fisher's exact tests were performed to compare the distribution of categorical variables across groups. Receiver operating characteristic (ROC)

Table 1. Demographic data of the study subjects

Variable	Control	PD	MSA
Number	334	333	114
Sex (Male/Female)	174/160	192/141	65/49
Age	60.76 ± 10.99	60.37 ± 10.35	67.49 ± 8.72
Age at onset	NA	55.28 ± 11.78	58.15 ± 9.85
Disease duration, y	NA	5.03 ± 4.48	2.93 ± 2.02
Education, y	-	9.95 ± 4.82	9.91 ± 4.73
H&Y stage	NA	2 (2, 3)	3 (3, 4)
UPDRS III	NA	26.33 ± 12.70	NA
UMSARS II	NA	NA	25.13 ± 13.53

All data are presented as mean ± S.D. values unless otherwise indicated. PD, Parkinson's disease; MSA, Multiple system atrophy; LEDD, Levodopa equivalent daily dose; UPDRS III, Unified Parkinson's Disease Rating Scale part III motor score; H&Y stage, Hoehn & Yahr stage; UMSARS II, Unified Multiple System Atrophy Rating Scale part II motor score; NA, not applicable; -, not available.

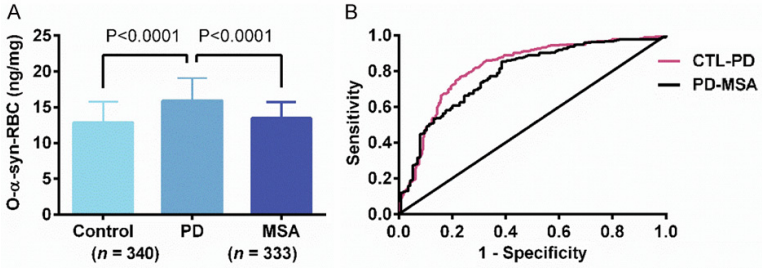

Figure 1. Utility of pS-α-syn-RBC in diagnosing PD. A: Concentrations of O-α-syn-RBC in PD vs controls and PD vs MSA in the cohort study. B: ROC curves for the diagnosis and differential diagnosis of PD. PD: Parkinson's disease; MSA: multiple system atrophy; ROC: receiver operating characteristic.

curves were constructed and the area under the curve (AUC) was calculated to evaluate the performance of the models. All statistical analyses were performed using IBM SPSS Statistics® v22·0·0·0 (SPSS Inc., Chicago, IL, 2013), Medcalc (Microsoft) and GraphPad Prism® v6·0 (GraphPad Software Inc., La Jolla, CA, 2009). *P*-values less than 0.05 were regarded as statistically significant.

Results

Alterations in O-α-syn-RBC levels in PD and MSA patients

To determine whether O-α-syn-RBC levels can serve as a peripheral biomarker for PD diagnosis and differential diagnosis, we recruited 333 potentially eligible PD patients, 114 MSA patients and 334 healthy controls between October 10, 2017, and December 31, 2018. The demographic and clinical data for all participants are shown in **Table 1**.

The levels of O-α-syn-RBC were measured by the ELISA assay specifically detecting α-syn oligomers. The levels of O-α-syn-RBC in PD patients (15.94 ± 3.12 ng/mg) were significantly higher than those in controls (12.84 ± 2.92 ng/mg; P < 0.0001) and MSA patients (13.44 ± 2.28 ng/mg; P < 0.0001) (**Figure 1A**). Receiver operating characteristic curve (ROC) indicated that the level of O-α-syn-RBC could separate PD patients from controls, with a sensitivity and specificity of 76.58% (95% CI: 71.65-81.02%), and 77.06% (95% CI: 72.22-81.42%), respectively, and an AUC of 0.82 (95% CI: 0.80-0.86) (**Figure 1B**). The level of O-α-syn-RBC could also differentiate between PD and MSA patients, with a sensitivity and specificity of 85.29% (95% CI: 81.02-88.91%), and 61.40% (95% CI: 51.83-70.37%), respectively, and an AUC of 0.78 (95% CI: 0.73-0.83) (**Figure 1B**). No significant difference was detected for the levels of O-α-syn-RBC between MSA (13.44 ± 2.28 ng/mg) and controls (12.84 ± 2.92 ng/mg) (P > 0.05).

Correlation between O-α-syn-RBC and brain α-syn expression

To examine the correlation of pS-α-syn-RBC with brain-expressed α-syn, a rat model selectively over-expressing human α-syn in the striatum was established by intra-striatal injection of LV-GFP-α-syn. Then the expression of total and oligomeric α-syn were examined 1 and 2 weeks after the injection. GFP-fused human α-syn (GFP-α-syn) started to express in the 1st week, and reached a high expression level in the 2nd week (**Figure 2A**). The α-syn total protein was selectively over-expressed in the thala-

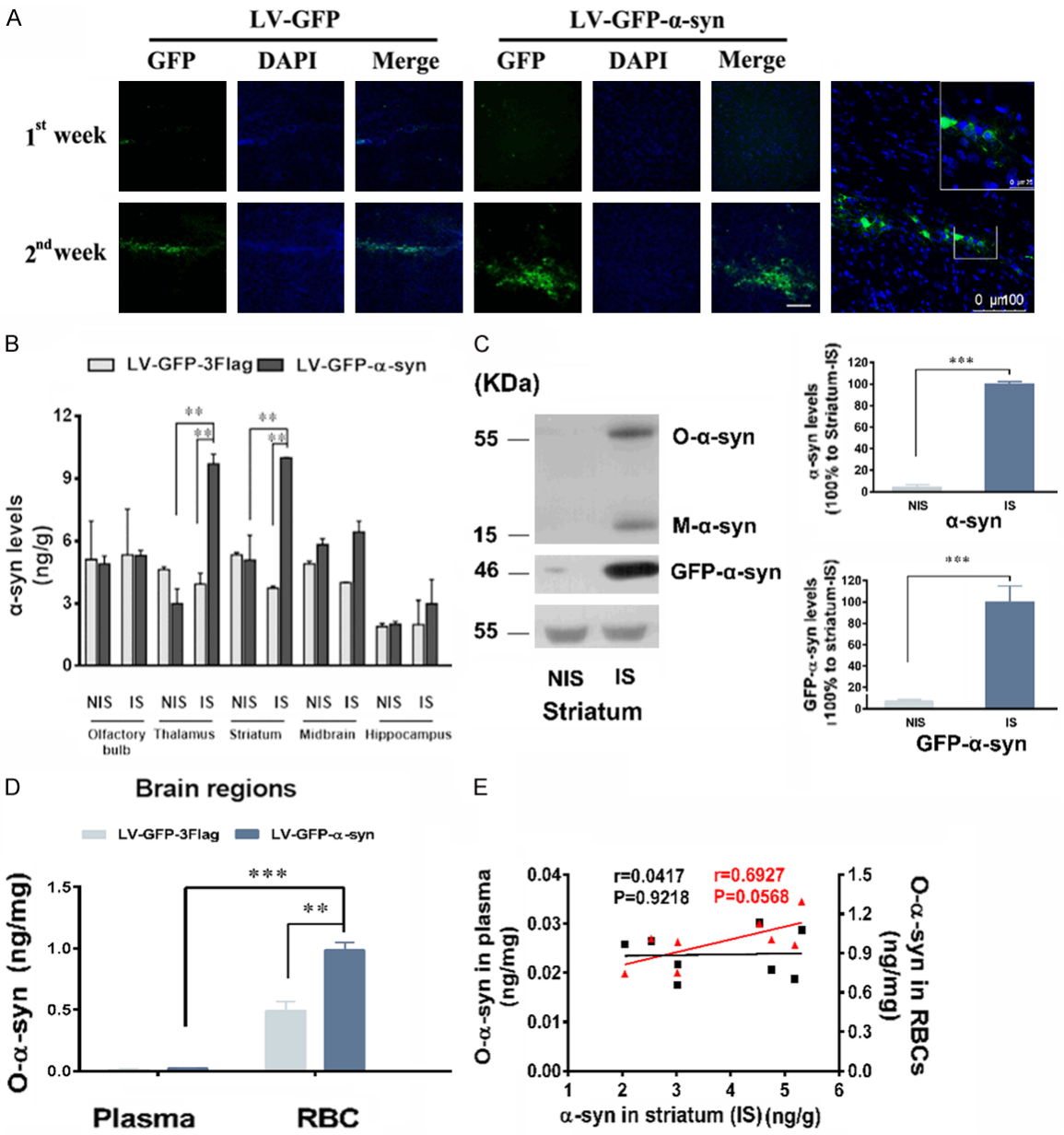

Figure 2. Brain-expressed α-syn is transported to and accumulates in RBCs. SD rats were intra-striatally injected with LV-GFP-α-syn or LV-GFP-3Flag expressing plasmids. After 1-2 weeks, α-syn expression was detected in brain tissues by immunofluorescence staining, ELISA assay and western blot (A-C). The levels of α-syn expressed in different tissues (olfactory bulb, thalamus, striatum, mid brain and hippocampus) from the IS and NIS of brain were measured and compared (B). α-syn and oligomeric α-syn (O-α-syn) expressed in the striatum in the IS and NIS of the brain were measured in rats injected with LV-GFP-α-syn (C). Simultaneously, α-syn level was measured and compared between the RBCs and plasma of the injected rats (D). The correlation between striatum-expressed α-syn and blood-detected O-α-syn was analyzed in the IS of rats injected with LV-GFP-3Flag (E). Red triangles: striatum-RBC; Black square: striatum-plasma. $^{**}P < 0.01$; $^{***}P < 0.001$. α-syn: α-synuclein; O-α-syn: oligomeric α-syn; RBCs: red blood cells; ELISA: Enzyme-linked immunosorbent assay; IS: injected side; NIS: non- injected side.

mus and striatum of the injected side (IS), but not the non-injected side (NIS), of the rats (**Figure 2B**). In particular, the levels of O-α-syn were also elevated in the injected striatum (**Figure 2C**). Simultaneously, an elevated level of O-α-syn was detected in the RBCs, but not in the plasma (**Figure 2D**). In addition, in rats injected with LV-GFP-α-syn, the levels of α-syn in the IS of the striatum were marginally correlated with the levels of O-α-syn detected in RBCs ($P = 0.057$), but not with that in plasma ($P > 0.05$) (**Figure 2E**).

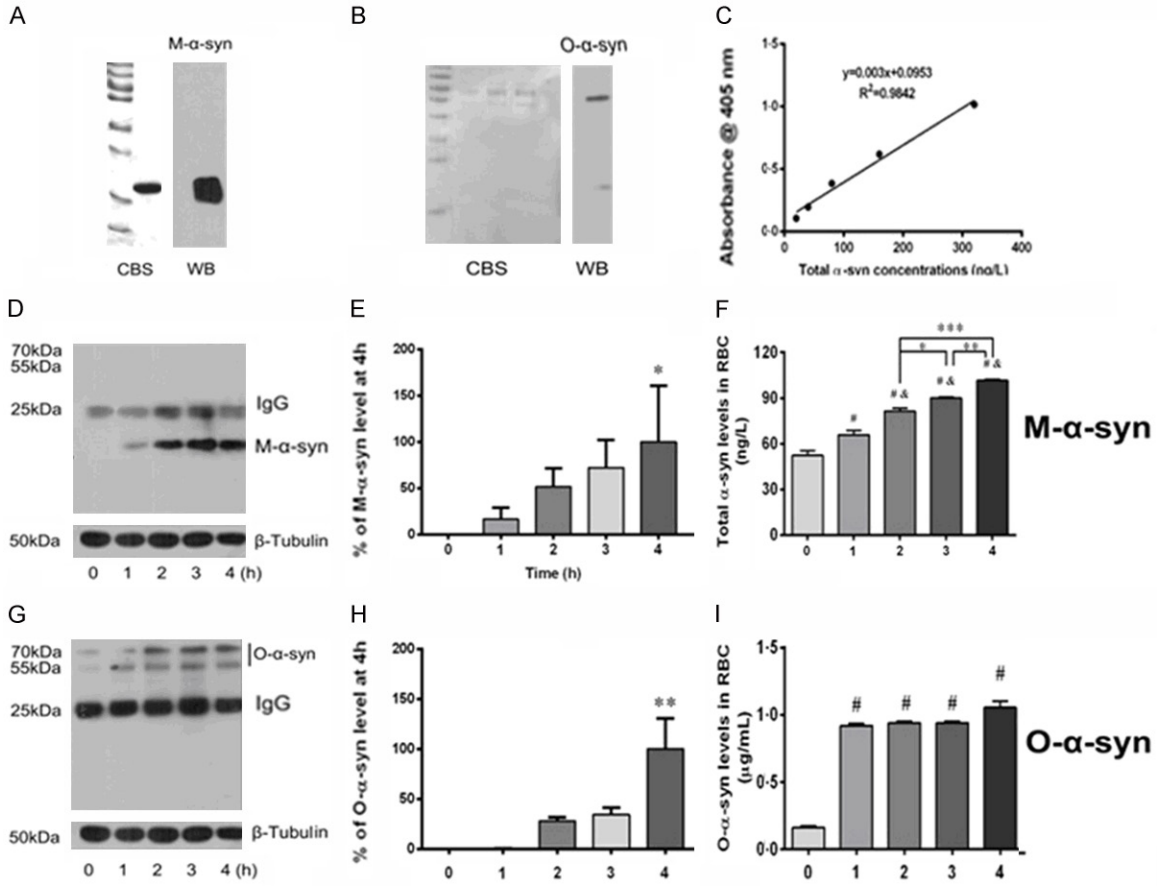

Figure 3. Peripheral α-syn was transported from the plasma to RBCs. Different forms of recombinant human α-syn were purified and validated by Coomassie blue staining (A, B). The standard curve of the ELISA assay detecting α-syn showed linear correlation with absorbance (C). After incubation with rat blood at different time points (0, 1, 2, 3 and 4 h), M-α-syn and O-α-syn in RBCs were detected by western blot and ELISA. (E, H) The intensity of western blot bands of for (D, G) respectively. (F, I) ELISA for the two forms of α-syn. Data represent results from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. M-α-syn: monomeric α-syn; CBS: Coomassie blue staining.

Translocation of O-α-syn from plasma to RBCs

The mechanism of translocation underlying the difference between plasma and RBC α-syn remains unresolved. One possibility is that some forms of α-syn in the plasma can freely penetrate or be transported into RBCs. To test this, purified M-α-syn and O-α-syn were separately incubated with rat blood *in vitro* for different times (0, 1, 2, 3 and 4 h), and the levels of different species of α-syn were assayed by western blot and ELISA. As shown in **Figure 3**, the levels of O-α-syn in RBCs were elevated after incubation in a time-dependent manner, similar to the M-α-syn.

Discussion

Although alterations of O-α-syn-RBC in peripheral blood as a potential biomarker for the diagnosis of PD and MSA patients have been investigated previously [14], whether or not this peripheral biomarker can differentiate PD from MSA patients and reflect brain α-syn remains poorly understood. The present study provides evidence that the levels of O-α-syn-RBC were increased in PD but not MSA patients as compared with heathy controls. Therefore, this biomarker can be used not only for separating PD patients from healthy controls (AUC = 0.82), but also for differentiating PD from MSA patients (AUC = 0.78). Because PD and MSA have many overlapped clinical manifestations, detection of O-α-syn-RBC could be useful in clinical practice to differentiate the two common Parkinsonian syndromes.

Previous studies have shown that levels of O-α-syn are significantly increased in the brain of patients with PD [23]. Since brain α-syn can

be transported across the blood-brain barrier (BBB) into blood plasma [24], there is a high possibility that the increased brain α-syn can make more α-syn that is released into blood plasma, where it is further translocated into RBCs. To demonstrate this possibility, we established a rat model overexpressing human α-syn in one side of the striatum. Our results showed that the total and oligomeric α-syn levels were significantly increased in the ipsilateral striatum. Simultaneously, we observed elevated levels of oligomeric α-syn in RBCs. This result indicates that brain α-syn overexpression can increase the levels of total and oligomeric α-syn in RBCs. To further support the possibility that α-syn oligomers can be translocated from plasma into RBCs, we added purified α-syn oligomers into the blood, and observed the changes of O-α-syn levels in RBCs after incubation. We found that O-α-syn levels in RBCs were quickly increased, indicating a plasma to RBC translocation of α-syn oligomers. The mechanism for the translocation is not clear. However, since the concentration of RBC α-syn is ~100 times higher than that of plasma [12], the entry of α-syn oligomers from plasma into RBCs should be an active process.

Although levels of O-α-syn are also significantly increased in the brain of patients with MSA [25], we only detected a slight increase in the levels of O-α-syn-RBC. A recent study reported that the levels of O-α-syn on the RBC membrane were significantly increased while those in the RBC cytosol were not changed in MSA patients [26]. Because the present study only measured the whole cell O-α-syn in RBCs, its elevation was not apparent. However, the present results, together with those previously reported, indicate a discrepancy between PD and MSA patients for the plasma to RBC translocation of O-α-syn.

In summary, our study demonstrates the utility of O-α-syn-RBC as a potential biomarker for diagnosis and differential diagnosis of PD. However, these findings need to be verified in drug-naïve or de novo patients, along with longitudinal follow-ups. In addition, the mechanism for the transportation of O-α-syn from the brain to peripheral RBCs remains to be clarified.

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Disclosure of conflict of interest

None.

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