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Original Article

Alpha-synuclein alterations in red blood cells of peripheral blood after acute ischemic stroke

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Abstract: Post-stroke induction of alpha-synuclein (AS), a neuronal protein implicated in the pathogenesis of Parkinson's disease (PD), has been demonstrated to induce secondary brain damage after cerebral ischemia. Therefore, understanding the expression and pathogenic modifications of AS is clinically meaningful for evaluating the prognosis of stroke. Here, 54 patients with acute ischemic stroke (AIS) and 55 controls were enrolled. Different forms of AS in red blood cells (RBCs), including hemoglobin-bound AS (Hb-AS), oligomeric AS (O-AS), and serine 129-phosphorylated AS (pS-AS), were measured using ELISA methods. Compared with controls, significantly increased levels of Hb-AS, O-AS, and pS-AS were observed in AIS patients. The levels of O-AS and pS-AS were both positively correlated with that of Hb-AS. However, no correlation was observed between O-AS and pS-AS. The levels of all three forms of AS were associated with increased risk of AIS diagnosis. Receiver operating characteristic (ROC) curves revealed that the three forms of AS yielded a moderate discriminative power (AUC ranging from 0.67 to 0.71 in discriminating AIS patients from controls, with varying sensitivity (0.41~0.61), specificity (0.78~0.90), PPV (0.73~0.81), and NPV (0.61~0.68)). These findings suggest that RBC AS can be a potential biomarker for evaluating AS changes in the brain of AIS patients.

Keywords: Alpha-synuclein, acute ischemic stroke, red blood cell, brain

Introduction

Alpha-synuclein (AS) is a 140 amino acid protein normally enriched in presynaptic terminals of neurons [1]. Genetic and pathologic studies suggest that AS is strongly implicated in the pathogenesis of Parkinson's disease (PD) [2]. In the brain of PD patients, AS aggregates into fibrils that deposit in the special pathological lesions termed Lewy bodies and Lewy neurites [2, 3]. Although AS in the pathologic lesions is fibrillated, accumulating evidence suggests that the oligomeric form of AS is toxic to neurons [4-7]. Mitochondrial deficiency, oxidative stress, endoplasmic reticulum stress, autophagic dysfunction, and inflammation, can all promote intracellular AS accumulation, aggregation, and phosphorylation [8, 9]. Interestingly, the above conditions not only present in the brain of patients with PD, but also occur in the brain of patients with acute ischemic stroke (AIS), although the time frame and intensity differ between PD and AIS, with the latter being more acute and stronger [9]. This indicates that AS may also participate in the pathogenesis of AIS. Indeed, increased AS expression, phosphorylation, and aggregation have been reported in the brain of various animal models with ischemic stroke [4, 10-12], in which AS has been shown to be a major PD-related protein mediating secondary brain damage after cerebral ischemia [9, 13].

It is well-established that AS can be secreted into the extracellular space from brain neurons [14] and further transported across the blood brain barrier into peripheral blood [15, 16]. This raises a possibility that detection of blood AS may help us understand the AS changes in the brain. However, studies aiming at detecting AS in the plasma of PD patients have yielded conflicting results, possibly due to the low concentration of AS in the plasma and contamination of AS released from red blood cells (RBCs)

resulting from hemolysis [17], since 99% of blood AS presents in RBCs [18]. Therefore, in order to reliably and stably detect blood AS, the above interfering factors should be avoided.

Since RBCs contain abundant AS and detection of RBC AS is not interfered with by plasma proteins and hemolysis, RBC AS might be an alternative biomarker to understand the AS changes in the brain. In support of this possibility, previous studies have shown that oligomeric AS is significantly increased in the RBCs of patients with PD and AIS [19, 20] and in both diseases, there is AS accumulation in the brain [9]. We also found that AS could bind to hemoglobin (Hb) to form Hb-AS complex, and the levels of Hb-AS complex in RBCs were correlated with those in the brain of aging monkeys, where AS was accumulated [21]. However, if the Hb-AS complex in RBCs also change in AIS patients remains to be investigated.

In the present study, we measured the levels of Hb-AS complex in peripheral blood RBCs for both AIS patients and non-AIS control subjects. In addition, we also measured the levels of oligomeric AS (O-AS) and serine-129-phosphorylated AS (pS-AS) in RBCs, since the O-AS and pS-AS were reported to be increased in the ischemic brain. We analyzed the relationships between different forms of AS and their associations with the risk of AIS diagnosis and also assessed the performance of each form of AS in discriminating AIS patients from non-AIS controls.

Materials and methods

Study subjects

A total of 54 patients with AIS were recruited from the in-patients at Department of Neurology, Peking University Shenzhen Hospital. In addition, a total of 55 age- and sex-matched control subjects were enrolled from the Center of Medical Examination, Peking University Shenzhen Hospital, after excluding those with the following medical histories and diseases: 1) patients with history of intracranial hemorrhage or ischemic stroke; 2) patients with PD and other Parkinson plus syndromes such as dementia with Lewy bodies, multiple system atrophy, progressive supranuclear palsy, Alzheimer's disease, diabetes, and chronic respiratory disease.

The study was approved by the Ethics Committee of Xuanwu Hospital and Peking University Shenzhen Hospital. Written informed consent was obtained from all participants or their legal guardians before inclusion in the study.

Blood sample collection

Blood samples of the AIS patients and control subjects were collected into EDTA-2Na tubes. The samples were centrifuged at 1500 × *g* for 15 minutes. The upper plasma and the intermediate white blood cell layer were aspirated, and the lower RBCs were collected, aliquoted, and stored at -80°C until use.

Measurement of Hb-AS complex

The Hb-AS complex was measured using the ELISA method established before [21]. Briefly, a 96-well microtiter plate was coated by overnight incubation with 1 μg/ml non-biotinylated anti-hemoglobin antibodies (ab77125; Abcam, Cambridge, MA, USA). After blocking with blocking buffer [phosphate-buffered saline (PBS, pH 7.4) containing 0.3% Triton X-100 and 10% BSA], 100 μL of RBC samples (disrupted by repeated freeze-thaw cycles and diluted 1:10 with PBS) were added to each well and, incubated at 37°C for 2 hours. After completion of the immunoreaction, the wells were incubated with 100 μl of ExtrAvidin alkaline phosphatase (E-2636; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:20,000 in blocking buffer followed by the enzyme substrate p-nitrophenyl phosphate (N1891; Sigma-Aldrich). The reaction was allowed to proceed at 37°C for 30 minutes. The plate was read at 405 nm using a microplate reader.

Measurement of O-AS and pS-AS

Levels of O-AS and pS-AS in RBCs were measured by ELISAs described before [22]. For detection of O-AS, the non-biotinylated and biotinylated 3D5 mouse monoclonal antibodies against human AS were used for capture and detection, respectively. For detection of pS-AS, an anti-pS129 α-syn polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for capture, and the biotinylated 3D5 mouse monoclonal antibody was used for detection. The remaining steps were as same as those for the detection of Hb-AS complex.

Table 1. Demographics and levels of AS of study subjects (n = 109)

	Stroke (n = 54)	Control (n = 55)	*p*-value
Age, years, mean ± SD	62.56 ± 13.31	60.93 ± 10.62	0.482
Male, n (%)	35 (64.8)	29 (52.7)	0.200
Hb-AS, μg/mg, median (IQR)	65.62 (47.75)	41.27 (29.33)	0.003
O-AS, μg/mg, median (IQR)	17.83 (7.45)	14.96 (6.09)	0.001
PS-AS, μg/mg, median (IQR)	4.80 (1.93)	3.92 (1.09)	< 0.001

Abbreviations: SD, standard deviation; IQR, inter-quartile range; AS, α-synuclein; Hb-AS, hemoglobin-bond α-synuclein; O-AS, oligomeric α-synuclein; pS-AS, phosphorylated α-synuclein.

Statistical analysis

Descriptive statistics were performed for demographic characteristics and levels of O-AS, pS-AS and Hb-AS complex between AIS patients and control subjects. Student's t-test or Mann-Whitney U-test were used for numerical variables based on distribution normality and chi-squared test was performed for categorical variables. The relationships between different forms of AS were examined by Pearson correlation coefficient. The associations of Hb-AS, O-AS and pS-AS with the risk of AIS diagnosis were examined using univariate and multivariate logistic regression. An OR > 1 meant increased risk of being diagnosed as AIS patients, while OR < 1 meant the opposite. Effect modifications of the levels of AS by age and gender on the risk of AIS were assessed by interaction terms. A *P*-value ≤ 0.05 was considered significant. Receiver operating characteristic (ROC) curves were constructed for Hb-AS, O-AS and pS-AS to estimate their powers in discriminating AIS patients from control subjects using area under curve (AUC). The thresholds were chosen based on the highest Youden's index (the maximum value of 'sensitivity-(1-specificity)'). The analyses were performed using STATA version 13.1.

Results

Demographics and levels of RBC AS

Table 1 summarizes the demographics and RBC AS levels of the AIS patients and control subjects. The mean ages of the AIS patients and controls were 62.56 ± 13.31 and 60.93 ± 10.62, respectively. Among the 54 AIS patients, 35 (64.8%) were male, which were matched by 29 (52.7%) males among the 55 control subjects. The means of age and gender proportions were not significantly different between the two groups (for mean age, *P* = 0.482; for sex, *P* = 0.200). The levels of Hb-AS, O-AS and pS-AS were measured and compared between the AIS and control groups. The levels of all the forms of AS in the AIS group were significantly higher than those in the control group (*P* < 0.01 for all forms of AS).

Correlation between different forms of AS

Correlation analyses were performed to explore the potential links between Hb-AS and O-AS or pS-AS. The levels of Hb-As were positively correlated with the levels of O-AS (r = 0.22; *P* = 0.019) and pS-AS (r = 0.31; *P* = 0.001) (**Figure 1A**, **1B**). However, the levels of O-AS and pS-AS were not correlated (**Figure 1C**).

Association between the level of RBC AS and the risk of AIS diagnosis

Logistic regression was conducted to analyze the association between each form of AS and the risk of AIS diagnosis. The results are shown in **Table 2**. Each unit of increase in the level of Hb-AS was associated with marginally elevated risk of AIS diagnosis in both the univariate and multivariate analysis (Crude OR: 1.02, 95% CI: 1.00-1.03, *P* = 0.007; Adjusted OR: 1.01, 95% CI: 1.00-1.03, P = 0.009). The level of O-AS was also positively associated with the risk of AIS diagnosis in the univariate analysis (Crude OR: 1.14, 95% CI: 1.05-1.24, *P* = 0.001) and this association remained significant after adjusting for age and gender (Adjusted OR: 1.15, 95% CI: 1.06-1.25, *P* = 0.001). Similar association was observed for pS-AS in the univariate analysis while it was attenuated slightly in the multivariate analysis (Crude OR: 2.05, 95% CI: 1.36-3.07, *P* = 0.001; Adjusted OR: 2.01, 95% CI: 1.34-3.02, *P* = 0.001). No interaction between any form of AS and age or gender was identified (all *P*-values > 0.1, data not shown).

ROC curve analysis

Table 3 and **Figure 2** show the results of ROC curve analysis. Among the three forms of AS, pS-AS showed the highest power in discriminating AIS patients from control subjects (AUC

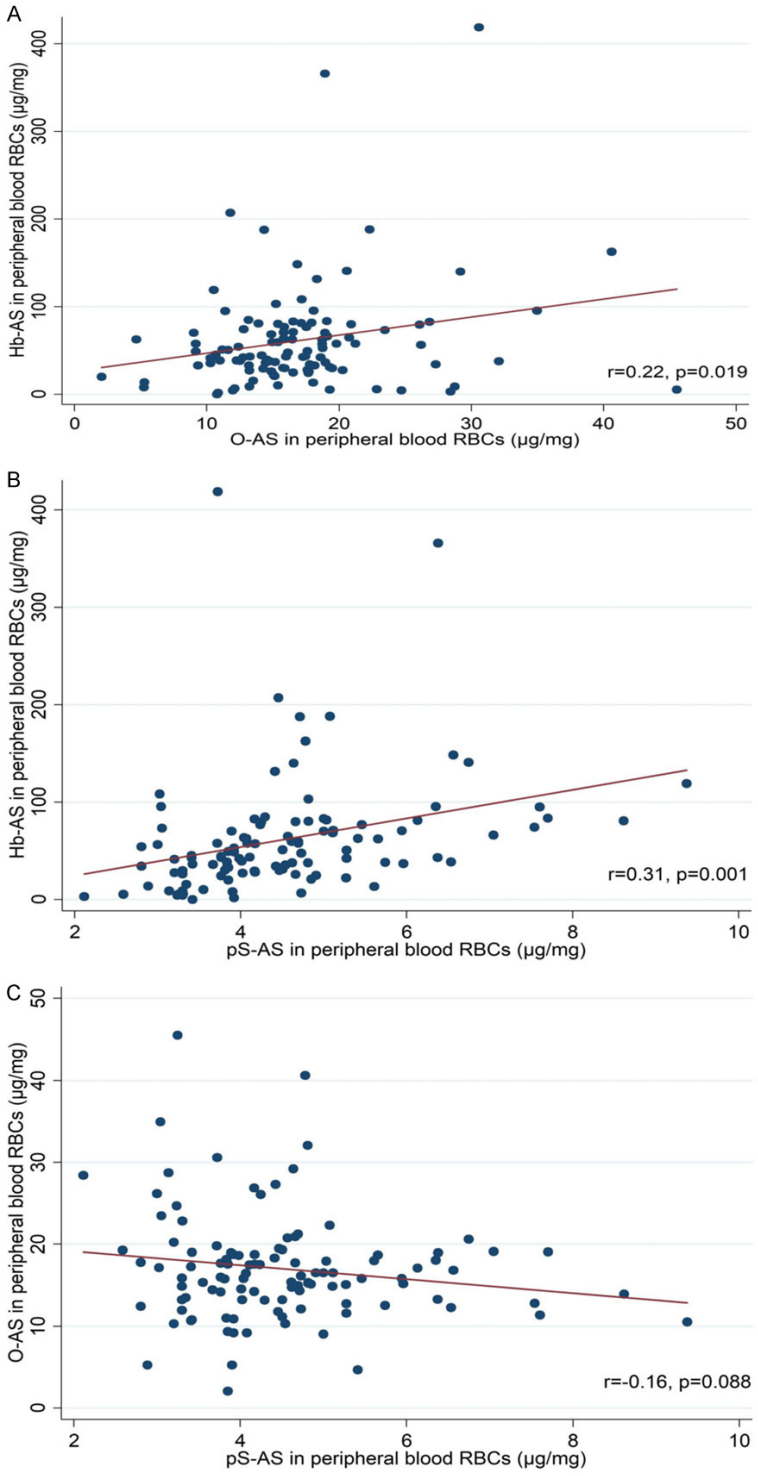

Figure 1. Scatterplots of AS levels in peripheral blood RBCs (n = 109). Pearson correlation coefficient was used to analyze the correlations between the levels of different forms of AS in peripheral blood RBCs. (A) Correlation between Hb-AS level and O-AS level (B) Correlation between Hb-AS level and pS-AS level (C) Correlation between O-AS level and pS-AS level. Note: r denotes the Pearson correlation coefficient. Abbreviations: AS, α-synuclein; Hb-AS, hemoglobin-bound α-synuclein; O-AS, oligomeric α-synuclein; pS-AS, phosphorylated α-synuclein; RBCs, red blood cells.

0.71, 95% CI: 0.61-0.80) with a sensitivity of 0.61 (95% CI: 0.51-0.71) and specificity of 0.82 (95% CI: 0.73-0.89). The discriminative performances of Hb-AS and O-AS were also fairly acceptable (Hb-AS: AUC 0.67, 95% CI: 0.57-0.76; O-AS: AUC 0.69, 95% CI: 0.59-0.78), with a sensitivity of 0.61 for Hb-AS (95% CI: 0.51-0.71) and 0.41 for O-AS (95% CI: 0.31-0.51) and a specificity of 0.78 for Hb-AS (95% CI: 0.69-0.86) and 0.90 for O-AS (95% CI: 0.82-0.95). Although O-AS provided the highest positive predictive value (PPV) (Hb-AS: 0.73, 95% CI: 0.63-0.81; O-AS: 0.81, 95% CI: 0.72-0.88; pS-AS: 0.77, 95% CI: 0.68-0.85), its negative predictive value (NPV) was lower compared to Hb-AS and pS-AS (Hb-AS: 0.67, 95% CI: 0.57-0.76; O-AS: 0.61, 95% CI: 0.51-0.71; pS-AS: 0.68, 95% CI: 0.58-0.77).

Discussion

The present study showed that AIS patients had increased levels of Hb-AS, O-AS, and pS-AS in peripheral blood RBCs compared with control subjects. Because RBCs lack nuclei, the increased component of AS was more likely exogenous. We speculate that the elevation of the three forms of AS in the RBCs of AIS patients might be a result of brain-to-blood transport of the AS accumulated in focal ischemic brain regions. First, increased expression of AS, including O-AS and pS-AS, has been demonstrated in the ischemic brain regions of various animal models with ischemic stroke [4, 9-12]. Second, previous studies have shown that AS can be secreted from

Table 2. ORs and 95% CI of Hb-AS, O-AS, and pS-AS for the risk of AIS diagnosis (n = 109)

	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value*
Hb-AS	1.02 (1.00-1.03)	0.007	1.01 (1.00-1.03)	0.009
O-AS	1.14 (1.05-1.24)	0.001	1.15 (1.06-1.25)	0.001
PS-AS	2.05 (1.36-3.07)	0.001	2.01 (1.34-3.02)	0.001

Abbreviations: OR, odds ratio; CI, confidence interval; AIS, acute ischemic stroke; Hb-AS, hemoglobin-bond α-synuclein; O-AS, oligomeric α-synuclein; pS-AS, phosphorylated α-synuclein. *P-values are based on multivariate logistic regression adjusting for age (numerical, years) and gender (male/female).

Table 3. Discriminative ability of Hb-AS, O-AS and pS-AS between AIS patients and control subjects (n = 109)

	Hb-AS	O-AS	pS-AS
AUC (95% CI)	0.67 (0.57-0.76)	0.69 (0.59-0.78)	0.71 (0.61-0.80)
Threshold	57.87	13.35	4.64
Sensitivity (95% CI)	0.61 (0.51-0.71)	0.41 (0.31-0.51)	0.61 (0.51-0.71)
Specificity (95% CI)	0.78 (0.69-0.86)	0.90 (0.82-0.95)	0.82 (0.73-0.89)
PPV (95% CI)	0.73 (0.63-0.81)	0.81 (0.72-0.88)	0.77 (0.68-0.85)
NPV (95% CI)	0.67 (0.57-0.76)	0.61 (0.51-0.71)	0.68 (0.58-0.77)

Abbreviations: Hb-AS, hemoglobin-bond α-synuclein; O-AS, oligomeric α-synuclein; pS-AS, phosphorylated α-synuclein; AUC, area under curve; AIS, acute ischemic stroke; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

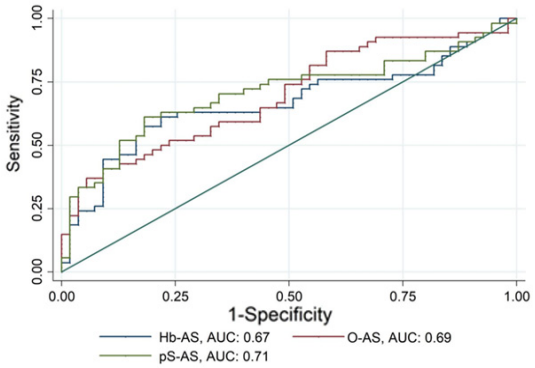

Figure 2. ROC curves of Hb-AS, O-AS, and pS-AS for discriminating AIS patients from control subjects (n = 109). ROC curves were constructed to estimate the power of the levels of Hb-AS, O-AS, and pS-AS in peripheral blood RBCs in discriminating AIS patients from control subjects. Abbreviations: ROC curves, receiver operating characteristic curves; Hb-AS, hemoglobin-bound α-synuclein; O-AS, oligomeric α-synuclein; pS-AS, phosphorylated α-synuclein; AUC, area under curve; AIS, acute ischemic stroke.

the living neurons or released from the damaged neurons [14], which can be further transported across the blood brain barrier into the blood plasma [15]. Third, the N-terminal part of AS contains several KTVEGV repeats, and these repeats can mediate the penetration of AS through the cell membrane [23], raising a possibility that the AS released into the plasma can be taken into RBCs. Therefore, it is possible that RBC AS can be a potential biomarker reflecting the AS changes in the brain. To demonstrate this, ROC curves were constructed to analyze the performances of the three forms of RBC AS in discriminating AIS patients from control subjects. Our results showed that the three forms of RBC AS had a similar moderate discriminative power (AUC ranging from 0.67 to 0.71), with varying sensitivity (0.41~0.61), specificity (0.78~0.90), PPV (0.73~0.81), and NPV (0.61~0.68). Based on our findings, pS-AS seems to be the most powerful post-stroke modification of AS compared to Hb-AS and O-AS. As the further supporting evidence, we showed that each unit of increase in the levels of the three forms of AS was associated with elevated risk of being diagnosed with AIS in both the univariate and multivariate analysis. In addition, the present results for O-AS are consistent with the findings of one population-based comparative study in which increased O-AS provided moderate power in distinguishing AIS patients from healthy controls [20].

Because AS can bind to Hb to form Hb-AS complex [21], any change of AS may affect the level of Hb-AS complex. We therefore analyzed the correlations between different forms of AS in RBCs. We found that both the levels of O-AS and pS-AS were positively correlated with the level of Hb-AS complex, indicating that increased O-AS and pS-OS accumulation in RBCs can promote the binding between AS and Hb. However, no significant correlation was found between O-AS and pS-OS, although AS phosphorylation has been shown to promote AS oligomerization [8, 13]. This is probably because the formation of O-AS is affected

by multiple modifications, including nitration, ubiquitin, methylation, glycosylation. and truncation, in addition to phosphorylation [8, 24]. If other modifications may also contribute to the formation of O-AS remains to be investigated.

Currently, the clinical diagnosis of AIS is mainly based on physical and neurological examinations performed by neurologists together with brain imaging tests such as computed tomography (CT) scan and magnetic resonance imaging (MRI) [25]. Since poststroke induction can mediate secondary brain damage [9], it is meaningful to understand the AS changes in the brain of AIS patients. Although this attempt has been made previously by measuring O-AS in RBCs [20], the present study detected several forms of AS in RBCs, which can reflect the AS changes in the brain of AIS patients more comprehensively. However, before these detections can be applied in the clinic, more experiments using large cohorts of patients and controls are needed to examine the associations between changes of different forms of the RBC AS and volumes and localizations of the infarct lesions. In addition, the period of observation should be prolonged to explore the longitudinal changes of RBC AS and their associations with prognosis after stroke.

In conclusion, the present study provides evidence that the levels of different forms of AS in RBCs, including Hb-AS, O-AS, and pS-AS, are significantly increased in AIS patients and showed moderate power in discriminating AIS patients from control subjects. These findings suggest a possibility for the three forms of RBC AS to be potential biomarkers for evaluating different forms of AS changes in the brain of AIS patients, although further studies are needed to confirm this.

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Disclosure of conflict of interest

None.

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