Parkinsonism and Related Disorders 61 (2019) 82–87

Contents lists available at ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

Quantification of brain-derived extracellular vesicles in plasma as a biomarker to diagnose Parkinson's and related diseases

Takuma Ohmichi[a], Masato Mitsuhashi[b], Harutsugu Tatebe[a,c], Takashi Kasai[a], Omar M. Ali El-Agnaf[d,e], Takahiko Tokuda[a,f,*]

[a] Department of Neurology, Kyoto Prefectural University of Medicine, Kyoto, 602-0841, Japan
[b] NanoSomiX, Inc., Irvine, CA, 92618, USA
[c] Department of Zaitaku (Homecare) Medicine, Kyoto Prefectural University of Medicine, Kyoto, 602-0841, Japan
[d] Neurological Disorders Research Center, Qatar Biomedical Research Institute (QBRI), Qatar Foundation, PO Box 5825, Doha, Qatar
[e] Life Sciences Division, College of Science and Engineering, Hamad Bin Khalifa University (HBKU), Qatar Foundation, PO Box 5825, Doha, Qatar
[f] Department of Molecular Pathobiology of Brain Diseases, Kyoto Prefectural University of Medicine, Kyoto, 602-0841, Japan

ARTICLE INFO

Keywords:
Neuron-derived exosome
Astrocyte-derived exosome
Oligodendrocyte-derived exosome
Plasma biomarker
Parkinson's and related diseases

ABSTRACT

Introduction: There is still a substantial unmet need for blood-based biomarkers to make an objective diagnosis of Parkinson's disease (PD) and the parkinsonism-plus syndromes. This study is aimed to determine whether enumeration of brain-derived exosomes (BDEs) in plasma is informative in the diagnosis of those diseases.

Methods: We have developed a specific method to enumerate the plasma levels of neuron-derived, astrocyte-derived, and oligodendrocyte-derived exosomes (NDEs, ADEs and ODEs, respectively), and quantified them individually in patients with PD (n = 15), multiple system atrophy (MSA, n = 15), progressive supranuclear palsy (PSP, n = 7) and disease controls (n = 15). Our assays employ specific antibodies against molecules expressed by neurons, astrocytes and oligodendrocytes, respectively, combined with an antibody to the exosome common marker CD81.

Results: The plasma levels of NDEs showed significant increase in PD compared to control ($p < 0.01$) and MSA ($p < 0.05$) (one-way ANOVA, Bonferroni post hoc test). The plasma levels of ODEs and the ratio of ODE/NDE showed a significant correlation with UPDRS part III scores in the patients with MSA with predominant parkinsonism (MSA-P) ($r^2 = 0.57$, n = 6, p = 0.048) and in the patients with PD ($r^2 = 0.51$, n = 14, p = 0.0041), respectively.

Conclusions: This is the first paper that enumerated NDE, ADE, and ODE in human plasma and showed the usefulness of those levels as biomarkers for PD. Our results suggest the capability of the plasma levels of NDE and ODE as a diagnostic and surrogate biomarker for PD and MSA-P, respectively.

1. Introduction

Parkinson disease (PD) is a common neurodegenerative disorder in the elderly, and the earliest clinical features of PD, including constipation, hyposmia and REM sleep behavior disorder, are typically reported retrospectively and not specific. The motor symptoms of PD do not develop until 70–80% of the dopaminergic terminals in the striatum and ~50% of the dopaminergic neurons in the substantia nigra have already been lost [1,2]. However, the diagnosis of PD is still made primarily by its motor symptoms following the UK Brain Bank Criteria [3], and a patient's response to dopaminergic medication. Thus, a major problem in clinical practice for PD is substantial difficulty in making an accurate and early clinical diagnosis that differentiates PD from various diseases that manifest parkinsonism, including multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) [1,2]. Development of specific and noninvasive biochemical markers for those diseases, especially blood-based biomarkers, would be useful in early diagnosis of PD before substantial neuronal loss occurs, to facilitate differential diagnosis from other diseases with parkinsonism, and monitoring of disease progression.

Exosomes are small (40–150-nm) membranous vesicles originating from multivesicular bodies that contain intraluminal vesicles in the endosomal system. Various types of human cells, including those of the brain, release exosomes into not only cerebrospinal fluid (CSF) but

* Corresponding author. Department of Molecular Pathobiology of Brain Diseases, Kyoto Prefectural University of Medicine, Kyoto, 602-0841, Japan.
E-mail addresses: t-omichi@koto.kpu-m.ac.jp (T. Ohmichi), mmitsuhashi@nanosomix.com (M. Mitsuhashi), tatebe@koto.kpu-m.ac.jp (H. Tatebe), kasaita@koto.kpu-m.ac.jp (T. Kasai), oelagnaf@hbku.edu.qa (O.M. Ali El-Agnaf), ttokuda@koto.kpu-m.ac.jp (T. Tokuda).

https://doi.org/10.1016/j.parkreldis.2018.11.021
Received 8 May 2018; Received in revised form 3 November 2018; Accepted 19 November 2018

blood under physiological and pathological conditions [4,5]. It is known that exosomes carry cell-type specific proteins, lipids and various RNA species, depending on their different cellular ancestries and various conditions of those parent cells [6,7]. This implies that exosomes in the blood that have originated from various brain cells could reflect pathogenic intracellular processes developed in their ancestral brain cells in brain diseases. In fact, brain-derived exosomes (BDEs) were found in blood and several proteins are identified [8,9].

In order to make BDEs applicable to diagnostics, the first step is to quantify the levels of BDEs in blood. Thus, in this study, we developed a specific sandwich immunoassay using 2 different antibodies against the exosome common marker CD81 and brain cell-specific proteins, respectively. After the assay was fully optimized, only a single moiety having both CD81 and brain markers showed positive signals. Furthermore, we confirmed that all the neuron-, astrocyte-, and oligodendrocyte-markers captured extracellular vesicles with the size range from 100 to 400 nm, and therefore double positive signals of both CD81 and one of those brain markers indicate neuron-, astrocyte-, and oligodendrocyte-derived exosomes (NDEs, ADEs, and ODEs). We report the levels of NDEs, ADEs, and ODEs in the peripheral plasma obtained from patients with PD, MSA, PSP and control subjects.

2. Materials and methods

2.1. Study design, subject characterization, and sample collection

All subjects enrolled in this study were recruited at Department of Neurology, Kyoto Prefectural University of Medicine, Kyoto, Japan, and provided written informed consent to participate in the study, which was approved by the University Ethics Committee (Kyoto Prefectural University of Medicine, Kyoto, Japan; the reference number RBMR-C-559-4). The study procedures were designed and performed in accordance with the Declaration of Helsinki. A total of 52 subjects, including 15 patients with PD (ages 46–79, mean ± SD of 64.7 ± 10.8 years, 9 male and 6 female), 15 patients with MSA (ages 44–74, mean ± SD of 63.3 ± 8.16 years, 10 male and 5 female), 7 patients with PSP (ages 58–81, mean ± SD of 71.6 ± 9.8 years, 2 male and 5 female), and 15 disease controls (ages 47–77, mean ± SD of 64.7 ± 8.5 years, 10 male and 5 female) were included in this study. There was no significant difference of age among those 4 groups. The clinical diagnoses of definite PD, probable MSA and probable PSP were made according to internationally standardized criteria [3,10,11], and complemented with neuroimaging studies and information on the responsiveness to levodopa therapy. All participants underwent MRI to exclude structural causes of illness other than neurodegenerative diseases. Assigned diagnoses were independently reviewed by a board-certified neurologist with subspecialty training in movement disorders (author TT). Fifteen patients with MSA consisted of 3 patients with MSA with predominant parkinsonism (MSA-P) and 12 patients with predominant cerebellar ataxia (MSA-C). Severity of the patients with PD, MSA and PSP was evaluated commonly with modified Rankin scale (mRS). Severity of parkinsonism was individually evaluated with Hoehn and Yahr scores in all the PD patients (n = 15), and with the Unified Parkinson's Disease Rating Scale (UPDRS) part III (motor scores) [12] during off-periods in 14 PD, 3 MSA-P, and 3 MSA-C patients. Severity of ataxia was evaluated with International Cooperative Ataxia Rating Scale (ICARS) [13] in 10 MSA-C patients. Control patients consisted of patients with non-neurodegenerative diseases without any evident brain lesions or parkinsonism (n = 15), namely called as “disease controls”, including patients with myelopathy (n = 7), peripheral neuropathy (n = 6), epilepsy (n = 1), and hyperventilation syndrome (n = 1).

Plasma samples were taken through venous puncture, and a total of 8 mL of blood was collected in EDTA-containing tubes. After collection, plasma was separated by centrifugation for 15 min at 2,000 g and distributed in polypropylene vials, then stored at −80 °C until analysis.

2.2. Development of enzyme-linked immunosorbent assays (ELISAs) for brain-derived exosomes

Development of specific ELISAs for NDEs, ADEs, and ODEs existing in human plasma are shown in Supplementary methods and Supplementary Fig. 1. Briefly, in order to find antibodies against NDEs, ADEs, and ODEs in plasma, various dilutions of plasma were applied to anti-CD81 monoclonal antibody (clone JS-81, BD Biosciences, Sparks, MD)-immobilized ELISA plates to capture whole exosomes. After extensive washing, biotinylated antibodies against various target proteins were applied, and ELISA was carried out as described in the next paragraph. From these exploratory ELISAs, we found a monoclonal antibody (clone B-8, Santa Cruz Biotechnology) against synaptosomal-associated protein 25 (SNAP25), polyclonal antibodies (purchased from Bioss Antibodies, Woburn, MA) against excitatory amino acid transporter 1 (EAAT1), which is also called glutamate aspartate transport (GLAST), and oligodendrocyte-myelin glycoprotein (OMG), showed very strong signals on CD81-immobilized plates, but not on control mouse IgG-immobilized plates. After discovery of appropriate antibodies, anti-SNAP25, anti-EAAT1, and anti-OMG were immobilized onto ELISA plates, then probed with biotinylated anti-CD81 (Clone 1.3.3.22, LS Bio, Seattle, WA) in our present assay for NDEs, ADEs, and ODEs, respectively.

2.3. Procedures of the ELISAs and quantification of ELISA data

The details are shown in the Supplementary methods. In brief, forty μL each of standards, controls, and plasma samples were applied to each well of ELISA plates with immobilized monoclonal antibodies against SNAP25, EAAT1, or OMG, and incubated overnight. Next day, after washing, 40 μL of biotinylated detection antibodies suspended in phosphate buffered saline, pH 7.4, containing 0.1% tween-20 (Sigma-Aldrich), 1% blocker BSA (Thermo Fisher), 8 μg/mL mouse IgG (Equitech-Bio, Kerrville, TX). After incubation, bound antibodies were reacted with streptavidin-horseradish peroxidase (Thermo Fisher), and then quantified with a chemiluminescent substrate (SuperSignal, Thermo). The chemiluminescent signals (relative light units, RLU) was determined in a luminometer (ANSH Labs, Webster, TX).

Since our sandwich ELISA used a combination of 2 different target antibodies, such as anti-CD81 and anti-SNAP25, recombinant protein was not applicable as a quantification standard. Thus, we first screened various plasma samples obtained from commercial sources (Innovative Research, Novi, MI, and EquiTech Enterprise, Kerrville, TX), and found plasma samples with high concentrations of all the BDEs. By assigning 100 units/mL to this plasma, a dilution study was carried out in each ELISA to obtain RLU in each dilution. Then using 4 parameter logistic analysis, RLU of each sample was converted to units/mL.

2.4. Statistical analyses

Logarithmic transformations of measured values of BDE were used as appropriate to a particular analysis to reduce excessive skewing and outlier influence in analyses for group differences and regressions between parameters. The mean differences in BDE between diagnostic groups were analyzed by one-way ANOVA, Bonferroni's multiple comparison test (Prism, GraphPad, La Jolla, CA), and the regressions between the levels of BDE and clinical parameters were assessed by Spearman rank correlation test (Prism, GraphPad). We also derived receiver operating characteristics (ROC) curves for the diagnosis of PD using the levels of BDE as the predictor, and estimated the area under the curve (AUC; AUC = 0.5 indicates no discrimination and AUC = 1 would indicate a perfect diagnostic test) to evaluate a diagnostic ability of each predictor (the plasma levels of NDE, ADE and ODE). We tested correlations between the levels of plasma BDEs and scores of clinical severities in the patients with PD and MSA by using Spearman's rank correlation test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Specificity and performance of the ELISA assays

Analyses of specificity and performance of our newly developed ELISA assays are shown in Supplementary results and Supplementary Figs. 2-7.

3.2. Analysis of the size and morphology of captured particles with the immobilized antibodies

Nanoparticle Tracking Analysis (NanoSight, AlphaNano Tech, Research Triangle Park, NC) confirmed the presence of extracellular vesicles with the size range from 100 to 400 nm in all samples eluted from ELISA plates with immobilized monoclonal antibodies against SNAP25 (clone B-8), EAAT1 (clone A-3), and OMG (clone E-8; all from Santa Cruz Biotechnology) that had been exposed to human plasma samples (Supplementary Fig. 3). In atomic force microscopy (AFM) analysis, there were many round or elliptic protrusions of various sizes observed on the surface of ELISA wells with immobilized monoclonal antibodies against SNAP25, EAAT1, or OMG that had been exposed to human plasma samples. The size of each protrusion ranged from 22.1 to 45.7 nm in height and 178–660 nm in width. We consider that these size ranges of round or elliptic protrusions observed by AFM correspond to the size of the extracellular vesicles including exosomes (~40–150 nm), although the height and width of protrusions was not considered a precise measurement due to geometrical constraints of the AFM probe tip (Supplementary Fig. 4).

Furthermore, final enumeration of BDEs was accomplished by the detection and quantification of CD81 (an exosomal marker)-positive extracellular vesicles among all extracellular vesicles captured with those antibody-immobilized ELISAs.

Enumeration of plasma BDE and comparison between patients and controls with non-neurodegenerative diseases (disease controls).

When only PD and control samples were compared by Mann-Whitney *U* test, NDE, ADE, and ODE were all significantly higher in PD than controls. However, when all data were used for the analysis by one-way ANOVA, only NDE showed significant increase in PD samples than in control ($p < 0.01$) and MSA ($p < 0.05$; Bonferroni's test; Fig. 1). Patients with PSP also showed significantly higher levels of plasma NDE, and ADE compared with the controls by Mann-Whitney *U* test, but such significant difference was not confirmed by one-way ANOVA.

Fig. 2 shows the ROC curves for the classification of patients with PD and disease controls based on the levels of plasma NDE, ADE, and ODE. The AUC of the ROC curve for plasma NDE, ADE and ODE were 0.82, 0.75, and 0.78, respectively, suggesting that plasma levels of NDE, ADE, and ODE may be applicable to diagnostic tests for PD with substantial accuracy.

3.3. Correlation between plasma BDE and clinical severity of the patients with PD and MSA

We investigated the correlation between the levels of plasma BDE and scores of clinical severities in the patients with PD. As shown in Fig. 3, plasma levels of all BDE (NDE, ADE and ODE) were significantly higher in the patients with advanced PD, namely in both groups with mRS grade 4 ($n = 4$; $p = 0.008$ for NDE, $p = 0.006$ for ADE, and $p = 0.006$ for ODE; Bonferroni's test; Fig. 3A, B, and 3C, respectively), and those with Hoehn and Yahr (HY) stage 4 ($n = 5$; $p = 0.003$ for NDE, $p = 0.003$ for ADE, and $p = 0.003$ for ODE; Bonferroni's test; Fig. 3D, E, and 3F, respectively), compared with the control group. Furthermore, plasma levels of NDE were significantly higher even in the mild PD patients, who were in mRS grade 1–2 ($n = 5$; $p = 0.002$; Bonferroni's test; Fig. 3A) or in HY stage 1–2 ($n = 4$; $p = 0.004$; Bonferroni's test; Fig. 3D), compared with the controls. Less remarkably, but nevertheless significantly, plasma levels of ODE were also higher even in the mild PD patients with mRS grade 1–2 ($p = 0.005$; Fig. 3C) or in HY stage 1–2 ($p = 0.011$; Bonferroni's test; Fig. 3F) compared with the controls. Moreover, plasma levels of ADE were also higher even in the mild PD patients with mRS grade 1–2 ($p = 0.023$; Bonferroni's test; Fig. 3B) compared with the controls. These results indicate that the plasma levels of NDE and ODE could be useful as a diagnostic biomarker for PD patients especially in early disease stages. Although the levels of ODE fluctuated during early disease courses of mRS and Yahr score 1–3, the levels of ODE were significantly higher in mRS and Yahr score 4 than score 1–3 ($p = 0.011$ for mRS, and $p = 0.0024$ in Yahr, Mann-Whitney *U* test). This suggests that ODE may be a potential surrogate biomarker of the monitoring of disease progression of PD.

We further investigated the correlations between the levels of BDE or the ratios between them (ADE/NDE, ODE/NDE, and ODE/ADE) and various clinical parameters in the PD group (Supplementary Table 1). The ratio of ADE/NDE showed statistically significant high r^2 values for disease duration of the patients ($r^2 = 0.51$; $n = 15$; $p = 0.0028$), grades of mRS ($r^2 = 0.47$; $n = 15$; $p = 0.0048$), and UPDRS part III scores ($r^2 = 0.52$, $n = 14$, $p = 0.0036$), as well as ODE/NDE for UPDRS part III scores ($r^2 = 0.51$; $n = 14$, $p = 0.0041$; Supplementary Fig. 8). These results also suggest that the plasma levels of BDE could possibly be useful for monitoring severity of patients with PD.

Next, we investigated the correlations between the levels of BDE

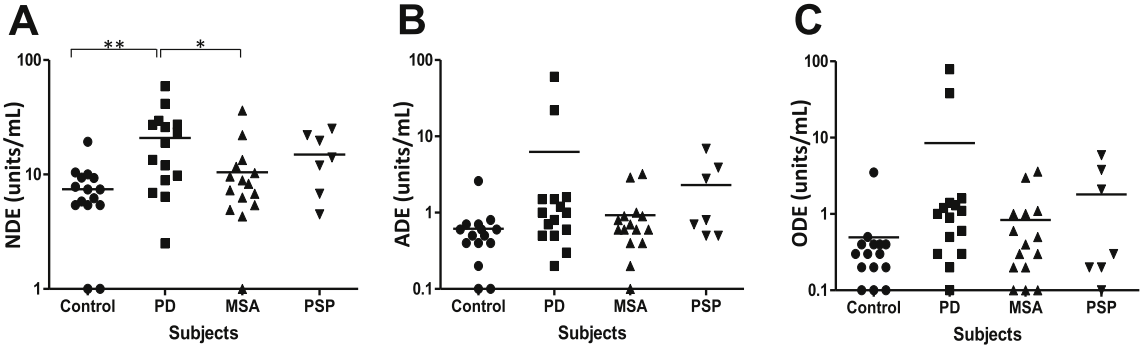

Fig. 1. Enumeration of plasma BDE and comparison between patients and controls. NDE (A), ADE (B), and ODE (C) were enumerated in 15 each of disease controls, PD, MSA, and 7 PSP patients. Each dot represents a single individual, and p values (*: $p < 0.05$, **: $p < 0.01$) were determined by one-way ANOVA, Bonferroni's multiple comparison test.

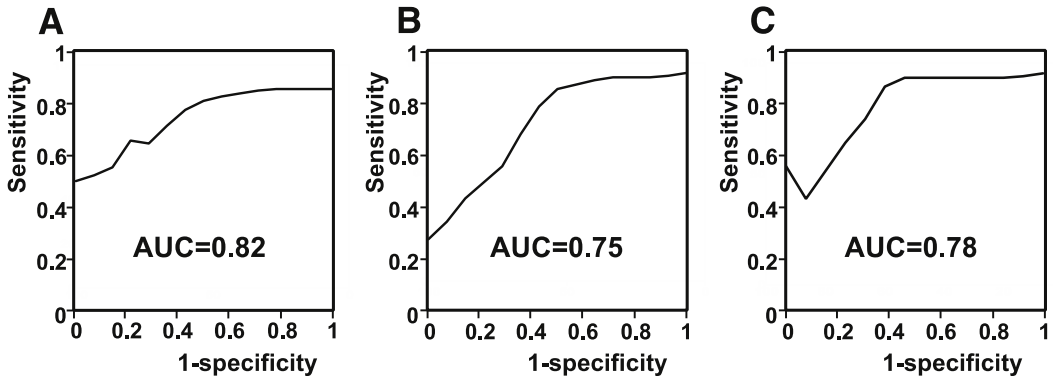

Fig. 2. Receiver Operating Characteristic (ROC) curves of NDE, ADE and ODE for the differential diagnosis of PD and controls. ROC was calculated using NDE (A), ADE (B) and ODE (C) data of PD and disease controls. Area under curve (AUC) was 0.89, 0.83% and 0.88 for NDE, ADE and ODE, respectively.

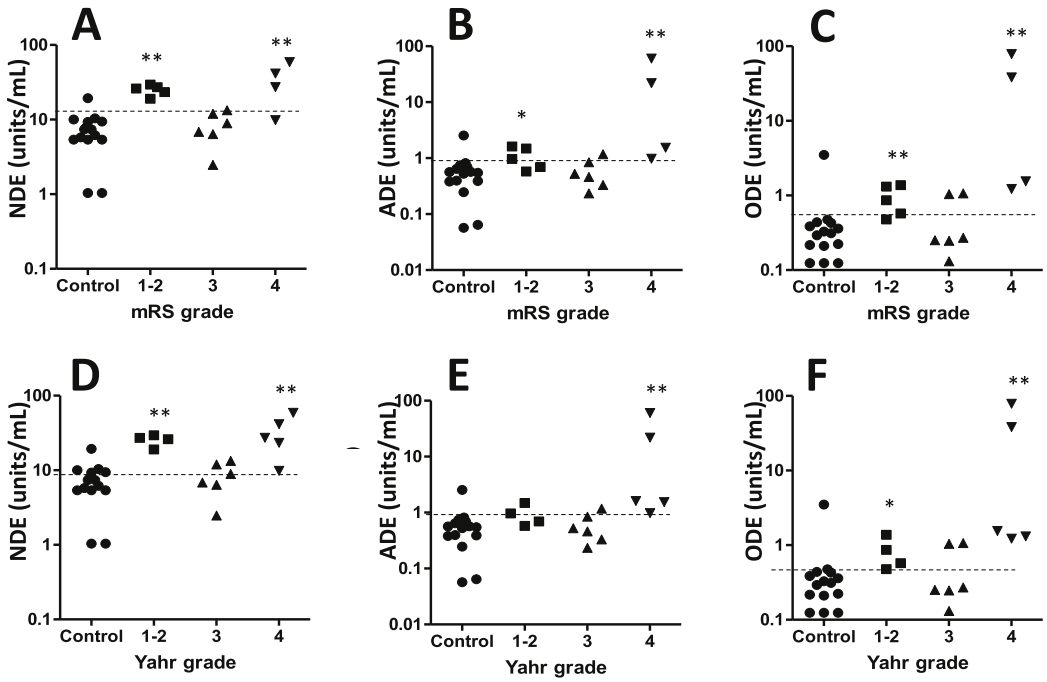

Fig. 3. Correlation between the levels of plasma BDE and disease severity in PD. NDE (A, D), ADE (B, E), and ODE (C, F) of PD patients were classified with the severity scores of mRS (A–C) and Hoehn-Yahr (D–F) and shown with those of disease controls. Each dot represents a single individual, and statistical p-values were between each severity group and control. P values (*: $p < 0.05$, **: $p < 0.01$) were determined by one-way ANOVA, Bonferroni's multiple comparison test.

and various clinical parameters in the MSA group. We focused on ODE this time because accumulation of insoluble α-synuclein (α-syn) as cytoplasmic inclusions in oligodendrocytes, so-called glial cytoplasmic inclusions (GCI), has been recognized as a pathognomonic feature of all forms of MSA [14,15]. As shown in Fig. 4, plasma levels of ODE did not correlate with the grades of mRS (Fig. 4A) or disease duration (Fig. 4C) in the patients with MSA as a whole, or not with ICARS scores in MSA-C (Fig. 4D), but had a significant correlation with UPDRS part III scores in MSA-P ($r^2 = 0.57$, $n = 6$, $p = 0.048$, Fig. 4B).

4. Discussion

In this study, we demonstrated the use of an assay system for the enumeration of NDE, ADE, and ODE in human plasma because we consider that our results shown as “the levels” of BDEs should reflect the difference in the number of BDEs (refer to Supplementary Fig. 1 and 7, and Supplementary results). Although we identify NDE, ADE, and ODE, it is extremely difficult to completely validate the origin of each exosome. However, due to the double positive nature of both the exosome common marker CD81 and neuron-specific SNAP25, astrocyte-specific EAAT1, and oligodendrocyte-specific OMG, the system disclosed in this study is a practical platform to analyze NDE, ADE and ODE, respectively. Although NDE [8,16] and ADE [9] were isolated, or at least enriched, from plasma and exosome cargo proteins analyzed in previous published papers, there is no corresponding report available for ODE. Also, those previous reports did not show the quantity of NDE and ADE in the original plasma before isolation. To the best of our knowledge, this is the first paper that enumerated NDE, ADE, and ODE in human plasma and showed the relevance to human diseases.

SNAP25 is a 25-kDa synaptosomal-associated intracellular protein involved in the regulation of neurotransmitter release [17]. In previous studies that isolated NDE from plasma, NDE was immunocaptured with anti-L1CAM (L1 cell adhesion molecule) antibody [8,16]. L1CAM protein however, is expressed not only in the brain but also in kidney and adipose tissue, and even secreted from cells as a plasma protein. By contrast, SNAP25 protein is almost exclusively expressed in the brain and exists in neurons as a presynaptic plasma membrane protein. Also, SNAP25 is a membrane protein. We therefore consider SNAP25 to be a much more suitable target protein on the exosomal surface when isolating brain-specific NDEs from peripheral plasma, than L1CAM. EAAT1

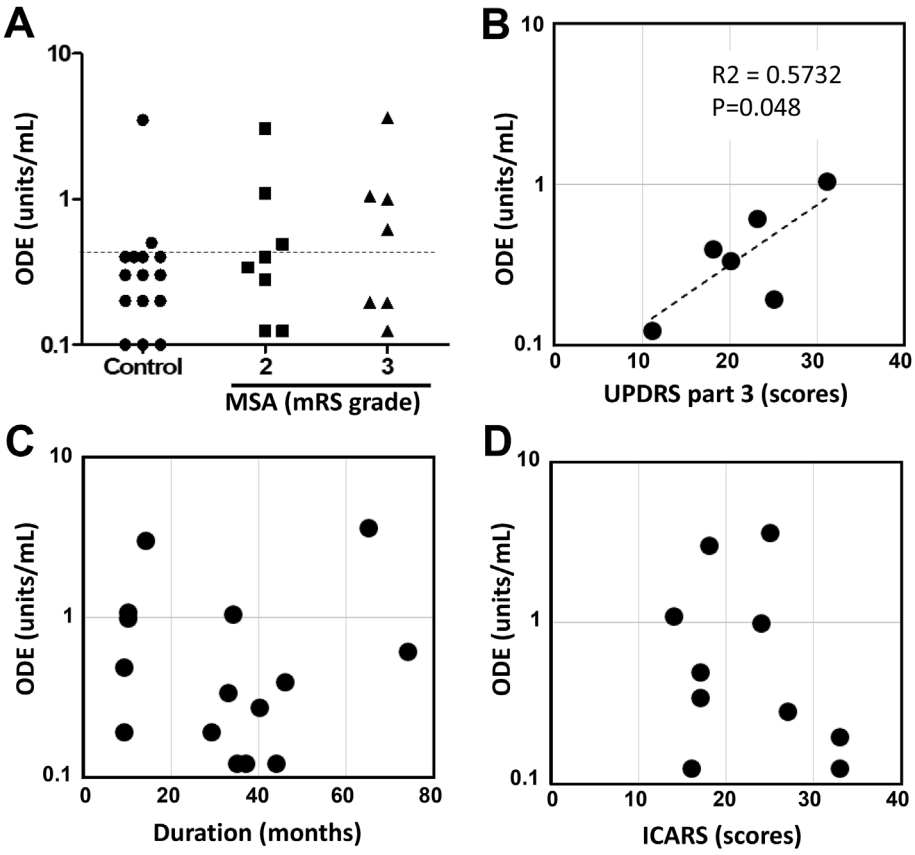

Fig. 4. Correlation between the levels of plasma BDE and disease severity in MSA. A: ODE of MSA patients (n = 15) was classified with the severity scores of mRS and shown with those of disease controls (n = 15). Each dot represents a single individual. B: ODE of each patient with MSA (3 MSA-P and 3 MSA-C) was compared with Unified Parkinson's Disease Rating Scale (UPDRS) 3 (motor). C: ODE of each MSA patient (n = 15) was compared with disease duration in months. D: ODE of each patient with MSA-C (n = 10) was compared with Scores of International Cooperative Ataxia Rating Scale (ICARS). The r^2 and p values were shown in E.

(also named GLAST) protein is almost exclusively expressed in the brain [18] and is highly expressed in astrocytes and Bergmann glia in the cerebellum [19]. OMG is a glycosylphosphatidylinositol-anchored protein exclusively expressed by neurons and oligodendrocytes in the central nervous system, and involved in the formation and maintenance of myelin sheaths [20]. Moreover, both EAAT1 and OMG are membrane proteins. From these reports and related information, we consider that EAAT1 and OMG are rational and suitable target proteins to isolate ADE and ODE from plasma, respectively.

In this study, we showed that plasma levels of both NDE and ODE are significantly increased in patients with PD, MSA or PSP when compared to the disease controls (Fig. 1), even in the early stages of PD (Fig. 3A and D), and that the levels of ODE increased with the progression of disease severity in PD (Fig. 3C and F). We consider that those results not only support the capability of NDE and ODE as biomarkers, but suggest an underlying defense mechanism of those brain cells which clears out neurotoxic substances that are intracellularly produced and accumulated in the cytosol. Recently, exosomes have attracted much interest in the research of neurodegenerative diseases, in part because they have been considered to propagate and spread the disease pathology, acting as potential carriers of pathogenic molecules in neurodegenerative diseases such as prion disease, AD and PD [5,8,9,16]. Although the intercellular transfer of those deleterious substances via exosomes might spread disease pathology, it would be good, from another viewpoint, for brain neurons and glial cells to clear out and reduce intracellular detrimental metabolites and proteins, such as aggregated α-syn in PD or MSA, by their externalization via exosomes. It has been shown that exosomes derived from PD patients are protective in models of neuronal stress, providing further evidence for the neuroprotective efficacy of exosomes [21].

We here showed the possibility that the plasma levels of BDE could be diagnostic biomarkers (Figs. 1 and 2) and severity-level biomarkers (Fig. 3) for PD and related diseases. Blood-based biomarkers would be clinically desired because they are non-invasive and widely available.

Regarding biochemical markers for PD, levels of CSF total α-syn and α-syn oligomers have been accepted as being significantly lower and higher, respectively, in patients with PD compared with controls [22–24], with moderate performance in aiding PD diagnosis [22–24]. Meanwhile, reports on blood levels of α-syn species, have been less consistent [25,26], mainly because of abundant peripheral production of α-syn especially in red blood cells [27] and interference of heterophilic antibodies in plasma [28]. It has been recently reported that BDEs can be detected in peripheral blood and that the levels of α-syn contained in plasma BDE are substantially higher in PD patients [8], suggesting that plasma exosomal α-syn can serve as a candidate of blood-based biomarker for PD. However, evidence is still far from sufficient due to its scarceness and many methodological insufficiencies; there is no standard method to isolate BDE from the blood. In addition, there is no standardized methods for solubilization of exosomes to analyze their contents, and efficiency in solubilizing and isolating proteins bound to exosomal membrane is greatly influenced by experimental conditions and many other factors, resulting in inconsistencies. Therefore, in this study we enumerated plasma BDE per se, not their contents, and demonstrated their potential usefulness as diagnostic and severity biomarkers for PD, MSA, and PSP. As shown in Supplementary Figs. 2-7, the assay is specific and reproducible, and ELISA format is easily expandable to a high throughput platform.

Neurons [29], astrocytes [30], and oligodendrocytes [6] have been confirmed to secrete exosomes, and BDEs from these brain cells can cross the blood-brain barrier. Our present ELISA to quantify the individual levels of plasma NDE, ADE, and ODE may provide us multiplexed biomarker assays highly useful in the diagnosis and monitoring of PD and related diseases. Moreover, we also believe that the values of NDE, ADE, and ODE in the original plasma will become a powerful tool for the development of universalized BDE isolation procedure by assessing the yield of each step.

5. Conclusions

We have developed original methods that enumerated NDE, ADE, and ODE in human plasma and reported their usefulness as urgently required blood-based biomarkers for the diagnosis and severity monitoring of PD, MSA and PSP. Moreover, plasma levels of ODE could be used as a good blood-based biomarker to monitor the severity of parkinsonism in MSA-P.

Author contributions

Mitsuhashi and Tokuda designed the study (1-A, B). Ohmichi, Mitsuhashi, Tatebe and Tokuda performed the experimental works (1-C). Kasai and El-Agnaf supported experimental design and data analysis (1-A, 2-A, B, C). Mitsuhashi and Tokuda wrote the manuscript (3-A). All authors read and approved the final manuscript (3-B).

1) Research project: A. Conception, B. Organization, C. Execution;
2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique;
3) Manuscript: A. Writing of the first draft, B. Review and Critique.

Conflicts of interest

The authors (except MM) declare that they have no conflicts interests relevant to this study. MM declares competing interest because he is an employee of NanoSomiX, Inc., where various exosome-related patent applications are pending.

Financial disclosure

This study was funded by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (KAKENHI: 15K09322 to T.T.); and Agency for Research and Medical Development (AMED) (17dk0207030h0002 and 17ek0109222h0001; to T.T.).

Acknowledgements

We would also like to thank Dr. Douglas Galasko (University of California, San Diego) for his critical review of this manuscript, and Masao Matsuda and Taketo Muroya (Shimadzu Corporation, Kyoto, Japan) for their technical assistance in AFM analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2018.11.021.

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