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Altered regulation of serum lysosomal acid hydrolase activities in Parkinson's disease: A potential peripheral biomarker?

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ABSTRACT

Introduction: Recent studies have indicated that lysosomal dysfunction contributes to the development of idiopathic Parkinson's disease (PD). It is uncertain whether dysregulation of serum lysosomal acid hydrolase activity exists in sporadic PD patients compared with normal controls (NCs) and parkinsonian syndrome (PS) patients.
Methods: Sporadic PD patients without *GBA1* mutations (n = 68) were matched with normal controls (n = 45), and parkinsonian syndrome patients (n = 32) in terms of family history, age, and sex. We measured the activities of lysosomal enzymes, α-galactosidase, β-galactosidase, and β-hexosaminidase and examined the possible correlations between lysosomal acid hydrolase activities with age in NCs, PD, and PS patients.
Results: β-Galactosidase activity was significantly higher in the PD and PS than in the NC group ($P < 0.001$). The β-galactosidase to α-galactosidase and β-hexosaminidase to β-galactosidase activity ratios were more useful for distinguishing PD and PS patients from NCs ($P < 0.0001$). Furthermore, α-galactosidase activity was significantly higher in PS patients than both PD and NC groups (p = 0.04). β-Galactosidase and α-galactosidase activities exhibited a statistically significant negative correlation with age in NCs, and β-hexosaminidase activity showed a positive correlation with age in PS. However, PD patients did not show any of these correlations.
Conclusion: Our results suggest the presence of an unknown regulatory mechanism(s) of serum acid hydrolase activities with aging in the normal population and abnormalities in their regulation in PD and PS patients. However, the pattern of dysregulation in these two groups is different. Thus, serum lysosomal acid hydrolase activity can be used as a peripheral biomarker for PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease [1]. A meta-analysis showed that the incidence of PD increases with age and its prevalence is increasing in proportion to the growth of the aging population [1]. As PD causes progressive disability and reduced quality of life, the health and social impact of PD will continue to rise alongside the aging of the world's population. There is growing evidence that early intervention may help in preserving neuronal functioning, reducing symptoms, slowing disease progression, improving patients' quality of life, and reducing the overall costs associated with PD. Early treatment is not easy because it depends on early diagnosis, which can be difficult to achieve because of the complexity of the disease. A reliable and simple biomarker is required for the early diagnosis of PD.

The underlying cause of PD has yet to be determined; however, mutations in the gene encoding glucocerebrosidase1 (*GBA1* [OMIM 606463]) are a common genetic risk factor for idiopathic PD [2]. *GBA1* encodes a lysosomal acid hydrolase that is also deficient in Gaucher's disease, one of the common lysosomal storage diseases. In Gaucher's disease, insufficient glucocerebrosidase activity causes the accumulation of glucocerebroside and glucosyl sphingosine in many organs, including peripheral immune cells, and the PD phenotype is more frequently observed in patients with Gaucher's disease than in the normal population [3]. Although it remains to be elucidated how *GBA1* mutations are linked to the development of PD, lysosomal impairment contributing to the aggregation and degradation of α−synuclein is thought to be an important neuropathological hallmark of PD [4,5]. Recent findings indicate that autophagy-lysosome dysfunctions are also present in *GBA1*-deficient cells [6]. The most recent work by Burbulla

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et al. further suggests that dopamine oxidation causes lysosomal dysfunction in PD [7]. Thus, lysosomal impairment is increasingly recognized as the central event in the pathological process of PD.

In light of recent reports demonstrating changes in lysosomal acid hydrolase activity in the cerebrospinal fluid (CSF) and leukocytes of PD patients [8–11], we explored whether lysosomal dysfunction is present in the periphery such as serum. We measured the activities of the lysosomal acid hydrolases α-galactosidase, β-galactosidase, and β-hexosaminidase in sera from PD patients, normal controls (NCs), and patients with parkinsonian syndrome (PS) because no data are available on serum lysosomal enzyme activities. We chose these enzymes because statistically significant differences are commonly found in brain tissues, CSF, and peripheral leukocytes between PD patients and NCs. In common clinical settings, it is easier to obtain sera from patients and healthy volunteers than to obtain other biological samples such as CSF and leukocytes because the collection of CSF is more invasive and leukocyte collection requires isolation.

2. Material and methods

2.1. Subjects

This study was approved by the ethics committees of Fujita Health University Hospital and Kobe University Hospital, and all subjects provided written informed consent before participation.

Patients without a positive family history of neurological disorders were recruited consecutively between April 2013 and March 2016 at Fujita Health University Hospital. Seventy-one PD patients were recruited for the study, three of whom were excluded because they carried *GBA1* mutations. Thirty-two PS patients were recruited, including 14 with multiple system atrophy (MSA), 7 with corticobasal degeneration (CBD), and 11 with progressive supranuclear palsy (PSP). Thus, this cohort study included 68 PD patients, 32 PS patients, and 45 NCs. The patients were diagnosed with PD according to the clinical diagnostic criteria of the UK Parkinson's Disease Society Brain Bank [12]. PS patients were diagnosed according to the diagnostic criteria for each disease [13–15]. Disease stage in the 'on' condition was rated using the modified Hoehn and Yahr classification. The NC subjects participated voluntarily. None had a history of psychiatric or neurological disorders, and the age and sex of the NC group did not differ significantly from those of the PD and PS groups recruited during the same period.

2.2. Sequencing and genotyping of the GBA1 gene

Genomic DNA was extracted from all PD patients using a standard salting-out method. DNA samples were subjected to polymerase chain reaction (PCR) and direct-sequence analyses to identify *GBA1* mutations (R120W, N188S, and L444P (RecNci1)), which account for 82% of the pathogenic variants in the Japanese population. Primer sequences for PCR and sequencing were the same as described in our previous report [16].

2.3. Assays for the activity of lysosomal acid hydrolases

To obtain sera from all recruited subjects, blood samples were collected in the morning and centrifuged for 15 min at 3000 g, and 1.0-mL aliquots were immediately frozen at −80 °C until ready to be assayed. We divided each aliquot to avoid repeated freezing and thawing. The activities of three lysosomal enzymes, α-galactosidase, β-galactosidase, and β-hexosaminidase, were assayed using the 4-methylumbelliferyl (4-MU) derivative of the respective substrates (Calbiochem, La Jolla, CA, USA), as described previously [17]. Briefly, to assay α-galactosidase activity, *N*-acetyl-D-galactosamine (Sigma-Aldrich, St. Louis, MO) was used as an inhibitor of α-*N*-acetylgalactosaminidase (NAGA, MIM104170, EC3.2.1.49), formerly called α-galactosidase B. For the α-galactosidase and β-galactosidase enzyme assays, 20 μL of sera were incubated with 40 μL of substrate solution for 4 h at 37 °C. The reactions were stopped by adding ice-cold 0.2 M glycine-NaOH buffer, pH 10.4, to a final volume of 260 μL. The incubation time for the β-hexosaminidase assay was 1 h. Fluorescence of the liberated 4-methylumbelliferone was measured (excitation, 355 nm; emission, 460 nm). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per hour at 37 °C (nmol/h/ml). All of the blood samples from PD patients were obtained during the 'on' condition. Enzyme assays were performed in duplicate and the acceptance specification for the within-run coefficient of variation was fixed at < 10%.

2.4. Statistical analysis

R, an open-source software environment for statistical computing and graphics (https://www.r-project.org/), was used for statistical analyses. Fisher's exact test was used to compare the sex distribution among groups. Kolmogorov–Smirnov and F-tests were used to test the normality of the variables and the equality of variances, respectively. The Tukey-Kramer test was used for comparisons of β-hexosaminidase with other variables because the variables were normally distributed with equal variances. The Steel-Dwass test was used to examine other enzymatic activities and their ratios because these variables showed unequal variances regardless of distribution. Steel-Dwass tests were also used to compare the investigation of disease severity and enzyme activity or the ratio of enzymatic activities in the PD group because these variables were not normally distributed and did not have equal variances. Spearman's correlation coefficients were used to evaluate the relationship between age and enzymatic activities or their ratios in all groups, while in the PD group we also analyzed the correlation between disease duration and lysosomal enzyme activity or the ratios of enzymatic activities. Significant differences were defined as $P < 0.05$. To investigate the factors that contributed to the diagnosis of PD, we performed a logistic regression analysis with categorization of age into 10-year intervals, α-galactosidase and β-galactosidase activity categorized by every 1 nmol/h/ml, and β-hexosaminidase by every 10 nmol/h/ml, respectively. A receiver operator characteristic (ROC) curve was used to calculate the cut-off values between groups according to the enzymatic activities or their ratios.

3. Results

3.1. Clinical features

The clinical characteristics of each subject are summarized in Table 1. Although there were slightly more females in the PD group, there was no statistically significant difference in sex among the PD, PS, and NC groups. It is important to note that only two patients were classified as Hoehn and Yahr disease severity stage I.

3.2. Lysosomal acid hydrolase activity

Given that age could influence lysosomal hydrolase activity, we investigated the relationship between age and the activities of each enzyme in all groups. We found a significant negative correlation between age and α-galactosidase or β-galactosidase activities in the NC group and a positive correlation between age and β-hexosaminidase activities in the PS group (Fig. 1). Hence, to limit the effects of age on our analysis, we calculated the ratio of enzymatic activities for pairs of lysosomal hydrolases: β-hexosaminidase/α-galactosidase, β-galactosidase/α-galactosidase, and β-hexosaminidase/β-galactosidase. Table 2 shows the lysosomal acid hydrolase activities and the ratios of every pair of enzymes. Of all of the ratios examined, we found a significant positive correlation between age and the β-hexosaminidase/α-galactosidase ratio in the NC group and a negative correlation between age and the β-galactosidase/α-galactosidase ratio in the PS group, although

Table 1
Clinical features of the subjects. PD, Parkinson's disease; NC, normal controls; PS, Parkinsonian Syndrome; MSA; Multiple System Atrophy; PSP, Progressive Supranuclear Palsy; CBD, Corticobasal Degeneration, H&Y stage, Hoehn and Yahr stage.

Characteristics	NC	PD	PS
Number	45	68	32
Age, mean ± SD (range, years)	68.5 ± 9.4 (44–83)	71.6 ± 8.0 (51–82)	70.5 ± 6.5 (50–81)
Male/female	22/23	29/39	17/15
Duration, mean ± SD (range, years)		7.2 ± 5.8 (1–31)	
Median H&Y stage,		III	
stage grade (patient number)		I(2), II(9), III(31), IV(17), V(9)	
Disease Detail (patient number)			MSA (14), PSP(11), CBD (7)

these correlations were not found in the PD group (data not shown). We observed statistically significant differences between the PD and NC groups, with higher β-galactosidase activity in PD patients ($p = 0.0001$). Interestingly, there were more significant differences in the β-galactosidase/α-galactosidase ($p = 0.0000004$) and β-hexosaminidase/β-galactosidase ratios ($p = 0.00009$) between the PD and NC groups.

In the PS group, we found that α-galactosidase ($p = 0.04$), β-galactosidase ($p = 0.0000006$), and the β-galactosidase/α-galactosidase ratio ($p = 0.0000007$) were significantly higher than in the NC group, and the β-hexosaminidase/β-galactosidase ratio ($p = 0.000001$) was lower in the PS group. Moreover, there was a significant difference in the α-galactosidase activities ($p = 0.006$) and the β-hexosaminidase/α-galactosidase ratio ($p = 0.02$) between the PD and PS groups (Fig. 2). The α-galactosidase activity was much lower and the β-hexosaminidase/α-galactosidase ratio was higher in PD patients. In addition, we conducted ANCOVA study with age as a covariate. Significant differences remained after age adjustment. And the β-hexosaminidase/α-galactosidase ratio was significantly lower in the PS than the NC group ($p = 0.02$) when analyzed with ANCOVA (data not shown).

In the PD group, there were no statistically significant correlations among disease duration and the activities of individual lysosomal acid

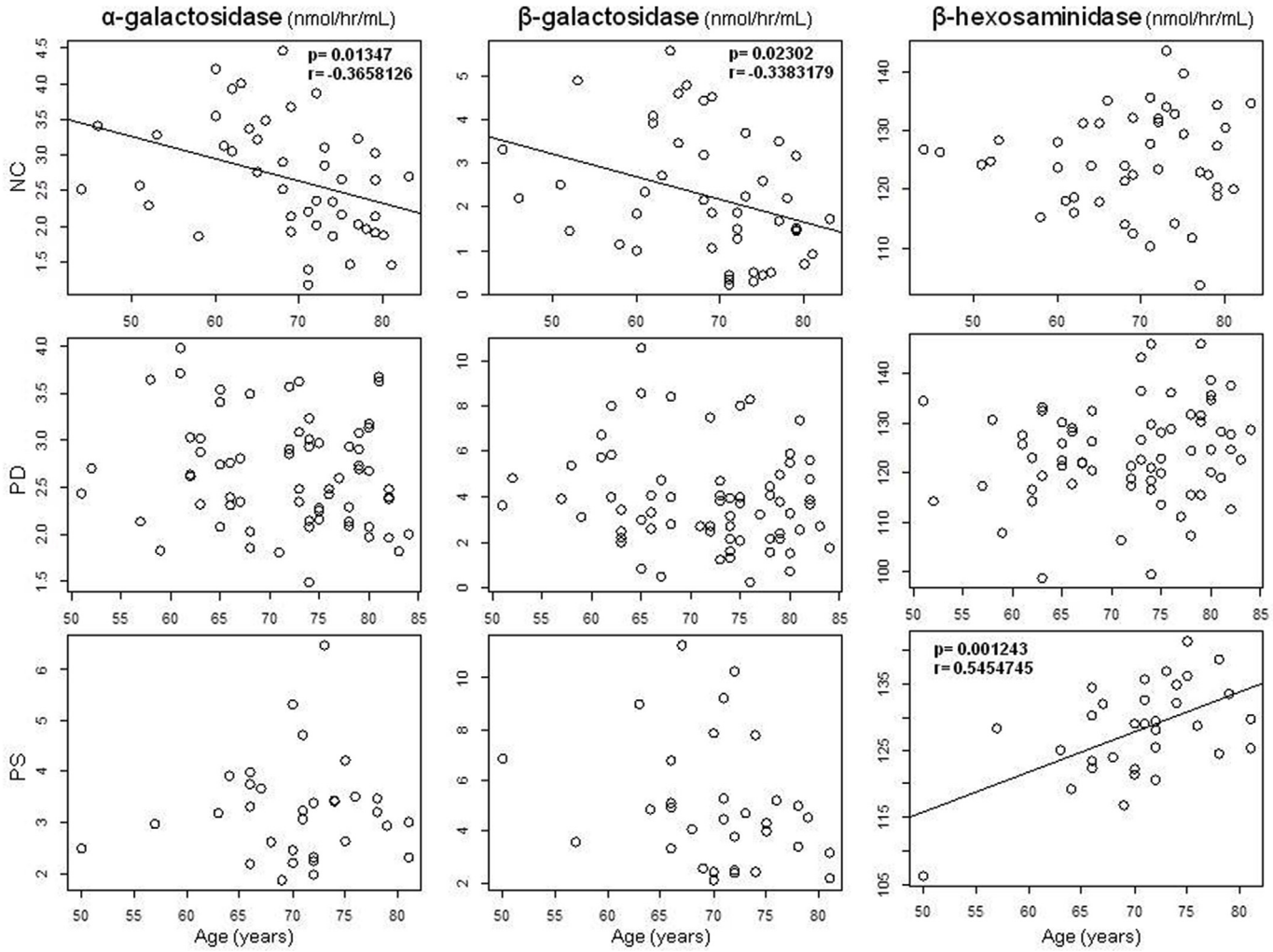

Fig. 1. Relationship between age and serum lysosomal hydrolase activities in the NC, PS, and PD groups. α-Galactosidase and β-galactosidase activities showed significant inverse correlations with age in NCs (correlation coefficient $r = -0.365$, $P = 0.013$ for α-galactosidase, $r = -0.338$, $P = 0.023$ for β-galactosidase). Furthermore, β-Hexosaminidase activity showed a significant positive correlation with age in PS cases (correlation coefficient $r = 0.545$, $P = 0.0012$. No correlation was seen in PD cases. NC, normal control; PS, Parkinsonian syndrome; PD, Parkinson's disease.

Table 2
Serum lysosomal acid hydrolase activity and the ratio of each enzyme among groups.

	NC	PD	PS
α-galactosidase (nmol/hr/mL. mean ± SD)	2.67 ± 0.80	2.65 ± 0.56	3.23 ± 0.98 [#1, 2]
β-hexosaminidase (nmol/hr/mL. mean ± SD)	124.83 ± 8.33	124.04 ± 9.78	128.02 ± 7.16
β-galactosidase (nmol/hr/mL. mean ± SD)	2.24 ± 1.45	3.86 ± 2.16 [#3]	4.98 ± 2.46 [#4]
β-hexosaminidase/α-galactosidase ratio (mean ± SD)	50.9 ± 15.80	48.72 ± 10.62	42.42 ± 10.55 [#5]
β-galactosidase/α-galactosidase ratio (mean ± SD)	0.79 ± 0.42	1.44 ± 0.70 [#6]	1.55 ± 0.65 [#7]
β-hexosaminidase/β-galactosidase ratio (mean ± SD)	110.57 ± 128.63	53.41 ± 74.29 [#8]	31.74 ± 14.13 [#9]

PD, Parkinson's disease; NC, normal controls; PS, Parkinsonian Syndrome.
[#1], p = 0.04 (NC-PS); [#2], p = 0.006 (PD-PS); [#3], p = 0.0001 (NC-PD); [#4], p = 0.0000006 (NC-PS)); [#5], p = 0.02; [#6], p = 0.0000004 (NC-PD); [#7], p = 0.0000007 (NC-PS); [#8], p = 0.00009 (NC-PD); [#9], p = 0.000001 (NC-PS).

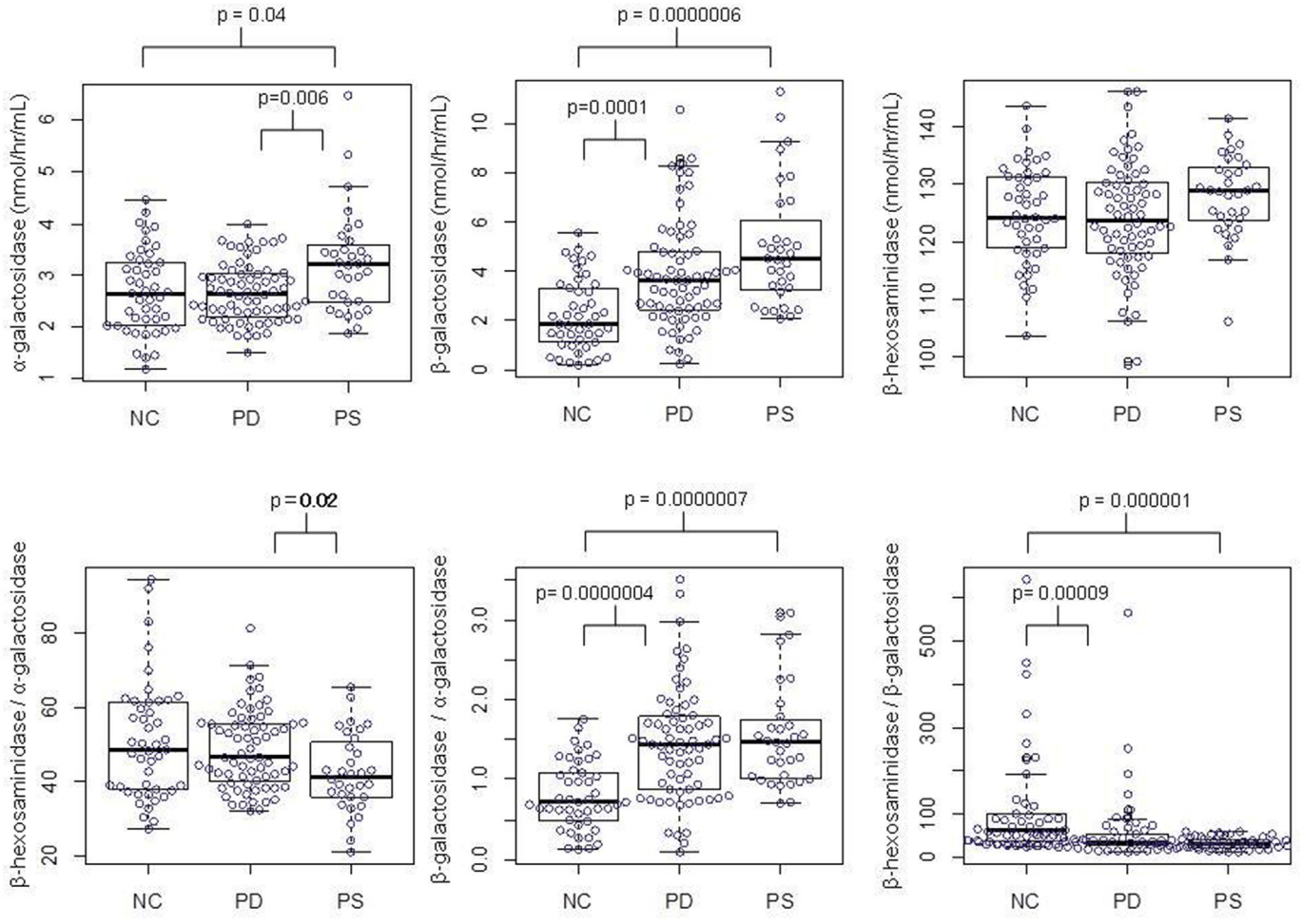

Fig. 2. Comparison of serum lysosomal hydrolase activities and their ratios in the NC, PS, and PD groups. β-galactosidase activities and the β-galactosidase/α-galactosidase ratio was significantly higher in PD and PS patients than in NCs. The β-hexosaminidase/β-galactosidase activity ratio was significantly lower in PD and PS patients than in NCs. α-Galactosidase activity was significantly higher in PS than in PD and NC. The β-hexosaminidase/α-galactosidase activity ratio was significantly lower in PS patients than in PD. NC, normal control; PS, Parkinsonian syndrome; PD, Parkinson's disease.

hydrolases or the ratios of enzymatic activities. Regarding disease severity, there were no significant differences among the Hoehn and Yahr stages and the activities of any of the enzymes tested or the ratios of enzymatic pairs, as shown in Supplemental Fig. 1.

Logistic regression analysis revealed that PD patients had significantly increased odds of age (odds ratio (OR (95% confidence interval (CI))): 2.11 (1.22–3.95), p = 0.029) and β-galactosidase activity (OR (95% CI): 2.13 (1.53–3.18), p = 0.000031). The odds ratio of β-galactosidase activity revealed that its contribution to the disease was independent of age.

We also performed ROC curve analyses of β-galactosidase activity and the ratios of pairs of enzymatic activities to determine the best cut-off value to differentiate PD patients from NCs. Among these analyses, the β-galactosidase/α-galactosidase ratio showed the highest value of the area under the ROC curve (AUC) (0.794). Its cut-off value was calculated as 1.372 with a sensitivity of 57.4%, specificity of 91.1%, positive predictive value of 41.4%, and negative predictive value of 9.3% for PD cases. Based on the AUC values reported elsewhere, these calculated values are moderately accurate. Furthermore, we conducted the same analyses on α-galactosidase activities to determine the cut-off value for distinguishing PD from PS. The cut-off value was calculated as 2.936 with a sensitivity of 65.6%, specificity of 72.1%, positive predictive value of 18.3%, and negative predictive value of 47.5%. The AUC value was 0.689, indicating low accuracy.

4. Discussion

To the best of our knowledge, this is the first study to compare serum lysosomal acid hydrolase activities in PD patients with those in normal (NC) and disease control (PS) groups. Aging is the greatest risk factor for developing PD [18], and our logistic regression analysis showed that serum lysosomal hydrolase activities contribute for the development of PD as much as aging. We found that β-galactosidase activity was significantly higher in the PD group than in the NC group. In accordance with our current data, Van Dijk et al. also reported that PD patients had significantly higher CSF β-galactosidase activity levels than normal controls [10]. Although a deficiency of β-galactosidase activity causes GM1 gangliosidosis, and the potential for parkinsonism [19–22], it remains unclear why β-galactosidase activity in the CSF and serum were upregulated in PD patients compared with the NC group. Van Dijk et al. hypothesized the link between elevated β-galactosidase activity and α-synuclein fibril formation, because GM1 interacts with α-synuclein and inhibits its fibril formation. This result supports that the dysfunction of lysosomal acid hydrolase in PD is not limited to glucocerebrosidase. Parnetti et al. also reported reduced α-galactosidase activity but elevated β-hexosaminidase activity in CSF from PD patients [8,9]. However, parkinsonism can also be observed in patients with Fabry's disease [23], one of the lysosomal storage disorders, in which deficiency of α-galactosidase activity plays a key role in the development of neurological impairments. This evidence suggests the systemic dysfunction of lysosomal acid hydrolases in PD.

No previous reports on serum lysosomal acid hydrolase activities in PS patients such as PSP, CBD, and MSA can be found in the literature. Intriguingly, the present study revealed that PS cases exhibit a more pronounced increase in serum β-galactosidase activities than PD patients. They also exhibit significantly upregulated α-galactosidase activities compared with both NC and PD groups. PS patients uniquely showed a significant positive correlation between β-hexosaminidase activities and age, as observed in other lysosomal enzyme activities in the NC group. Although the study recruited a limited number of PS patients, these unique findings may suggest that there is some abnormality in the regulating mechanism(s) of serum lysosomal acid hydrolase activities in PS patients, but this abnormality differs from that in PD patients.

At present, the source of lysosomal acid hydrolases present in bodily fluids such as CSF and even serum remain unclear. Tasegian et al., however, recently reported that α-mannosidase activity in CSF seems to mirror pathological changes in the brain [24]. Serum lysosomal acid hydrolase is thought to reflect the activity of vascular endothelial cells [25]. It is also noteworthy that β-galactosidase and α-galactosidase activities showed significant negative correlations with age in the NC group, and β-hexosaminidase showed a positive correlation with age in the PS group. However, we did not find any such correlation in the PD group. As age-related changes in lysosomes are used as biomarkers for aging, a variety of age-associated changes in lysosomal hydrolase activities have been reported [26]. Lysosomes are important organelle controlling cellular and organismal life and death, and also influence aging-related processes [27]. The correlations between age and lysosomal enzymatic activities found in this study might reflect some regulatory mechanism(s) of whole steady-state levels of serum acid hydrolase activities in the NC and PS groups. Therefore, our results imply that such mechanisms in PD patients differ significantly from those in the normal aging population and other parkinsonian syndromes.

Furthermore, it is important to keep in mind that the activities of lysosomal enzymes present in exosomes derived from sera may be included in the serum enzymatic activities measured in the present study. In fact, a previous study indicated that some exosomes contained lysosomal enzymes [28]. Lysosomal exocytosis is thought to be involved in secretion processes that interact with aging-related intercellular signaling at the tissue and organismal levels through exosomes. Vella et al. argued that impairments of the autophagy-lysosome pathway enhance exosome release and the role of exosomes in protein trafficking in PD patients [29]. Our results imply the role of the autophagy-lysosomal system in the pathophysiology of PD.

In this study, we found that the ratios of the activities of specific enzymes, such as the β-galactosidase/α-galactosidase and β-hexosaminidase/β-galactosidase ratios, were significantly different in PD patients compared with the NC group. From a clinical point of view, the combined use of enzymatic activities and their pair ratios may provide more useful information for the diagnosis of PD than the direct use of the enzymatic activities themselves. Although these differences are also apparent in PS, serum α-galactosidase activity could be useful in differentiating PD from PS cases. Our ROC curve analysis of the β-galactosidase/α-galactosidase ratio for PD patients showed the highest specificity and moderate accuracy, although it was not sufficiently sensitive to be used as a single diagnostic biomarker. Therefore, for better differential diagnosis of PD from other PS patients, it might be important to use additional methods such as neuroradiological measures [30].

There are several limitations of the present study. First, the total sample size was rather limited, especially the number of patients at each Hoehn and Yahr disease stage of PD, and the number of recruited PS patients was not ideal. Moreover, our results indicated no correlation between enzymatic activity and disease duration or stage in PD patients, suggesting that altered serum lysosomal acid hydrolase activities are a trait of, but not a stage marker, of PD. To strengthen the present results, a subgroup analysis with more participants is needed. Second, we only examined enzymatic activities at a single time point and did not follow serial changes over time. Therefore, additional longitudinal investigations using larger samples of patients with PD and PS are necessary to confirm the applicability of the present results and further elucidate the contribution of lysosomal dysfunction in the pathogenesis of PD.

Although the source and molecular mechanisms underlying the expression of lysosomal acid hydrolases in serum remain to be elucidated, the abnormalities in β-galactosidase and α-galactosidase activities might reflect impairments in the trafficking and metabolism of lysosomal enzymes in the periphery of PD patients.

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Disclosure

All authors declare no conflicts of interest.

Author contributions

YN contributed to the data collection, data analysis, and statistical analysis, and drafted the manuscript. SI and YM contributed to the statistical analysis and data collection and joined in scientific discussions. KM, SS, and AU contributed to the data collection and intellectual contents. WS and TT contributed to the data analysis and to the intellectual contents. NH contributed to the intellectual contents. TM designed the study, coordinated its execution, scientific discussion, supervised the study, and revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2018.10.032.

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