ARTICLE

Identification of BAG2 and Cathepsin D as Plasma Biomarkers for Parkinson's Disease

Juhee Kang[1,†], Jae Whan Kim[1,†], Hansol Heo[1], Jihyun Lee[2], Kwan Yong Park[1], Jung Han Yoon[3,*] and Jaerak Chang[1,2,*]

The current diagnosis of Parkinson's disease (PD) mostly relies on clinical rating scales related to motor dysfunction. Given that clinical symptoms of PD appear after significant neuronal cell death in the brain, it is required to identify accessible, objective, and quantifiable biomarkers for early diagnosis of PD. In this study, a total of 20 patients with idiopathic PD and 20 age-matched patients with essential tremor according to the UK Brain Bank Criteria were consecutively enrolled to identify peripheral blood biomarkers for PD. Clinical data were obtained by clinical survey and assessment. Using albumin-depleted and immunoglobulin G-depleted plasma samples, we performed immunoblot analysis of seven autophagy-related proteins and compared the levels of proteins to those of the control group. We also analyzed the correlation between the levels of candidate proteins and clinical characteristics. Finally, we validated our biomarker models using receiver operating characteristic curve analysis. We found that the levels of BCL2-associated athanogene 2 (BAG2) and cathepsin D were significantly decreased in plasma of patients with PD ($P = 0.009$ and $P = 0.0077$, respectively). The level of BAG2 in patients with PD was significantly correlated with Cross-Culture Smell Identification Test score, which indicates olfactory dysfunction. We found that our biomarker model distinguishes PD with 87.5% diagnostic accuracy (area under the curve (AUC) = 0.875, $P < 0.0001$). Our result suggests BAG2 and cathepsin D as candidates for early-diagnosis plasma biomarkers for PD. We provide the possibility of plasma biomarkers related to the autophagy pathway, by which decreased levels of BAG2 and cathepsin D might lead to dysfunction of autophagy.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Although the current diagnostic method for Parkinson's disease (PD) shows high accuracy, it is frequently inefficacious to diagnose early PD or predict PD onset. Several studies showed that the autophagy-lysosomal pathway is altered in patients with early PD, suggesting autophagy-related proteins could be potential biomarkers for early PD.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ We aimed to identify plasma biomarkers for PD by quantitative analysis of proteins related to the autophagy-lysosomal pathway.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ This study showed that decreased levels of BCL2-associated athanogene and cathepsin D could be used as PD biomarkers with high accuracy.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ The diagnostic model using biomarkers identified in this study can be used for more accurate and convenient PD diagnosis. This study also supports that the autophagy-lysosomal pathway is fundamentally linked to the pathogenesis of PD.

Parkinson's disease (PD) is the second most common neurodegenerative disorder of insidious onset. PD is characterized by the presence of motor symptoms, including shaking, rigidity, bradykinesia, and postural disturbances, and non-motor symptoms, including gait, speech, and swallowing difficulties.[1] The motor symptoms of PD are caused by a significant decrease in dopamine levels in the brain due to the degeneration of dopaminergic (DA)

neurons.[2] Because the motor disturbance symptoms begin after a 60 to 80% loss of the DA neurons, it is critical to initiate the appropriate medical intervention at the early stage of disease progression.[3] Despite the rapid increase in PD prevalence, there are still no effective biological or imaging markers. The current diagnosis method of PD is made through the clinical criteria developed by the Brain Bank of the Parkinson's Disease Society in the UK.[4] Even though

[†]These authors contributed equally to this work.

[1]Department of Biomedical Sciences, Ajou University School of Medicine, Suwon, Korea; [2]Department of Brain Science, Ajou University School of Medicine, Suwon, Korea; [3]Department of Neurology, Ajou University School of Medicine, Suwon, Korea. *Correspondence: Jung Han Yoon (jhyoon@ajou.ac.kr) and Jaerak Chang (jaerakchang@ajou.ac.kr)

these criteria are with a high degree of accuracy, it is still not effective to predict PD onset or diagnose patients with early PD without motor symptoms. Thus, the development of early PD biomarkers to predict PD is of importance.

Identifying biomarkers is necessary as they can be administered in worldwide screening to predict PD progress and diagnose early PD. A biomarker should be applicable to all sexes and ages, easily accessible, noninvasive, and, most importantly, it should be a quantifiable value for clinical application. In this regard, using peripheral blood plasma is a promising way to develop biomarkers for PD.[5] Although several studies showed that the level of different types of α-synuclein in the plasma of patients with PD could be used as a biomarker, it is still controversial whether the α-synuclein level is a suitable biomarker for PD prediction or diagnosis, due to the inconsistency of the results.[6,7] Accordingly, the peripheral α-synuclein level does not seem to have potential as a biomarker. Nonetheless, a promising finding is that the level of DJ-1 decreased in the cerebrospinal fluid of patients with PD; however, DJ-1 levels in the sera of patients with PD did not differ from those of control patients.[6,8] In addition, the levels of uric acid in sera and epidermal growth factor in plasma are reported to be decreased in patients with PD.[6]

Thanks to the years of research on PD, it is now well-known that several factors, including α-synuclein, parkin, PINK1, LRRK2, and DJ-1, are deeply related to the pathogenesis of PD. The α-synuclein is a presynaptic neuronal protein, which is neuropathologically related to PD.[9,10] Several studies showed the implication of parkin and PINK1 in mitophagy that is thought to be one of the underlying pathogenic mechanisms of PD.[10–12] In addition, LRRK2 and DJ-1 are reported to play important roles in autophagy-mediated DA neuronal cell loss.[13,14] Most recently, Laperle *et al*. showed that lysosomal membrane proteins, such as LAMP1, were decreased in induced pluripotent stem cells of patients with young-onset PD.[15] These studies, all together, suggest the deep implication of autophagy in PD.

In this study, we aimed to identify autophagy-related proteins as potential biomarkers for PD by quantitative analysis with patient plasma samples. In addition, we investigated the relationship between the potential biomarkers and clinical characteristics of the patients with PD.

METHODS
Subjects and study approval
A total of 114 consecutive patients with parkinsonism who visited the Parkinson Center at Ajou University Hospital from March 2017 to June 2018, were prospectively recorded. Of these, 32 patients whose [18F] N-(3-fluoropropyl)-2b-carbon ethoxy-3b-(4-iodophenyl) nortropane photon emission tomography scans or 3T brain magnetic resonance imaging (MRI) were not checked at baseline assessment, and 21 patients who were lost to follow-up within 2 years were excluded from the study. We also excluded patients who showed signs of atypical parkinsonian syndromes (*n* = 24) or PD dementia (*n* = 14) during the follow-up period. Three patients with evidence of focal brain lesions in MRI were also excluded. Finally, a total of 20 patients with idiopathic PD were enrolled in this study. The diagnosis of PD was made using the United Kingdom PD Society Brain Bank clinical diagnostic criteria,[16] and parkinsonian motor symptoms were assessed using the Unified PD Rating Scale Part III (UPDRS-III). Twenty age-matched patients with essential tremor, who were diagnosed according to recent consensus statement,[17] were also enrolled during the same period to serve as the control group. Data on age, sex, medication history, smoking history, and lipid profiles were obtained for all participants. All enrolled patients with PD were subjected to 18F-N-(3-fluoropropyl)-2b-carbon ethoxy-3b-(4-iodophenyl) nortropane photon emission tomography and 3T brain MRI. This study was approved by the Ajou Institutional Review Board (AJIRB-MED-SMP-17-322) and was carried out in compliance with the Declaration of Helsinki and Korean good clinical practice guidelines. Before patients were enrolled, the detailed purpose of the study was explained to all patients who provided written informed consent, which followed the procedures authorized by the local institutional review board.

Sample preparation and immunoblotting
Albumin and immunoglobulin G (IgG) were depleted from plasma by using Hi-Bind Albumin-IgG depletion beads (Biovision Cat #7933). Briefly, beads were washed with 50 mM Tris-HCl buffer (pH 8.0) 5 times, followed by applying plasma to the bead and rotating for 2 hours at room temperature. Then, the supernatant was collected and mixed with 2× sodium dodecyl sulfate (SDS)-loading buffer and boiled at 95°C for 10 minutes. Immunoblotting was performed as described previously.[18] Briefly, samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked in 3% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). Blots were incubated with primary antibodies diluted in TBST with 3% bovine serum albumin, washed with TBST, and incubated with secondary antibodies conjugated with horseradish peroxidase in TBST containing 3% skim milk. Primary antibodies specific to BCL2-associated athanogene (BAG2; Bethyl Cat #A304-751A), BAG3 (Bethyl Cat #A302-807A), cathepsin B (Abcam Cat #ab58802), cathepsin D (Calbiochem Cat #IM-03), LAMP1 (Abcam Cat #ab25630), LAMP2 (Proteintech Cat #66301-1-Ig), WDFY3 (Bethyl Cat #A301-869A), and α-Tubulin (Abcam Cat #ab18251) were purchased. Immunoreactive proteins were detected with a ChemiDoc Gel Imaging System (Bio-rad). Band intensities were measured using Image Lab software (Bio-rad).

Antibody validation
For gene editing of BAG2 or cathepsin D (CTSD), targets were selected from the list recommended by E-CRISP.[19] The target sequences are as follow:

BAG2-CR1: 5′-ACATGAGGCAGATCAGTGA-3′
BAG2-CR2: 5′-CCTCATGTCCTGGCTATTT-3′
CTSD-CR1: 5′-TCCAGGTGGACCTGCCAGT-3′
CTSD-CR2: 5′-GCCTACTGGCAGGTCCACC-3′

After cloning guide RNAs (gRNAs) specific to the targets in pSpCas9(BB)-2A-Puro (PX459) vector (Addgene, #48139), the constructs were transfected into HeLa cells with Avalanche-Omni reagents (EZ Biosystems) for 36 hours, and then incubated with 2 µg/mL of puromycin for 24 hours. After

further growth, cells were lysed and boiled with 1× SDS lysis buffer and subjected to immunoblotting.

Statistical analysis

Results are expressed as mean ± SD or mean ± SEM. Logistic regression analysis and receiver operating characteristic (ROC) curve analysis, followed by Pearson's χ^2 test, were performed to assess the diagnostic accuracy of the biomarkers. GraphPad Prism 7 or Microsoft Excel Office 365 was used to analyze the statistical data and plot the graphs. The levels of significance for comparisons between groups were determined by Student's *t*-test. *P* value < 0.05 was considered significant.

RESULTS

Demography of subjects

Twenty patients who experienced PD onset in the last 3 years were recruited in the experimental group and correspondingly, 20 age-matched patients with essential tremor who were not clinically diagnosed with PD were recruited in the control group (**Table 1**). Based on clinical survey and assessment, patients in both groups had similar education experiences and displayed similar results from the cognitive function test (**Table 1**, top panel). Mid-50 years old was identified as the age of PD onset for the enrolled patients (55.1 ± 8.5 years old) and they experienced PD in the past 23.2 ± 10 months (**Table 1**, mid panel). Six of the 20 patients with PD had olfactory dysfunction and 7 had a history of rapid eye movement sleep behavior disorder (RBD), the major prodromal non-motor symptoms of PD. MRI scan results showed that approximately half of the patients had defects in the structure of deep white matter. Cardiac metaiodobenzylguanidine (MIBG) scans, which measure sympathetic cardiac denervation caused by Lewy bodies,[20] was also performed for 11 patients with PD.

Preparation of patient plasma samples for SDS-PAGE

Although enzyme-linked immunosorbent assay is a powerful method for quantifying proteins in body fluids, there are still some issues about their antigen specificity. Accordingly, we decided to analyze the levels of biomarker candidate proteins by immunoblot analysis, which is an easy and reliable quantitative measurement method. Blood plasma contains several components, such as dissolved salts, circulating RNAs, and proteins. Thus, before analyzing protein levels in plasma, we first depleted albumin, which is the major protein in plasma, and IgG from plasma samples to properly resolve selected proteins in the polyacrylamide gel. The albumin/IgG-depleted plasma samples from 40 subjects were successfully resolved and separated in the gel (**Figure 1a**).

Screening of biomarker candidate proteins implicated in the autophagy-lysosome system

To identify PD-specific biomarkers in plasma, we selected 30 proteins that were implicated in the autophagy pathway. We analyzed the levels of 30 proteins using pooled plasma samples that contain the same amount of plasma from each subject. Among 30 proteins, 7 proteins; BAG2, BAG3, cathepsin B, cathepsin D, LAMP1, LAMP2, and WDFY3 were detected as the levels of being able to quantify (**Figure 1b–i**; list of total proteins in **Table S1**).

By comparing the level of 7 candidate proteins, the average levels of BAG2 and CTSD in the pooled sample from the PD group were decreased > 20% relative to the control group (**Figure 1j,k**). Even though the levels of the other proteins also differ from those of control, we ruled out the proteins with a percent lower than 20% relative to control, for the purpose of our study was to develop novel biomarkers that had significant quantitative differences in the plasma of patients with PD.
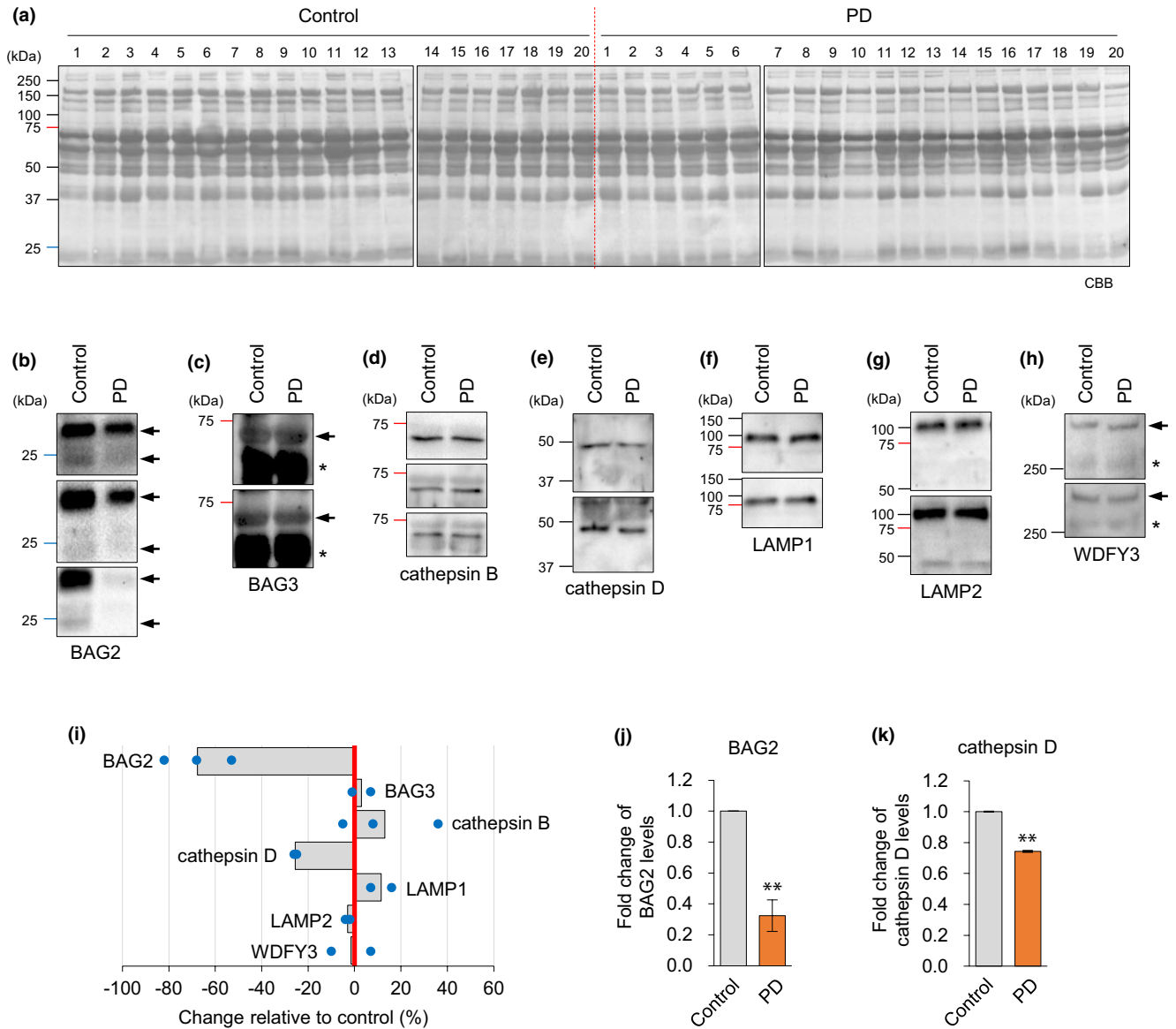
BAG2 levels are decreased in the plasma of patients with PD

One advantage of using immunoblot analysis to relatively compare protein levels is that we can directly examine the quality of the antibody, which is important for precise quantification of interesting protein levels. To validate the BAG2 antibody that we used for immunoblot analysis, we examined the specificity of the antibody to confirm the different levels of the proteins between control and PD groups in individual samples. First, we transfected BAG2 gene-specific gRNAs, which induce knock-out of the gene by the CRISPR-Cas9 system into HeLa cells, and performed immunoblot analysis to confirm whether BAG2 antibody could detect specific BAG2 band that would be decreased by the transfection. The antibody could detect a specific band (~ 25 kDa), which was significantly reduced by the transfection (**Figure 2a**). Although residual BAG2 proteins were still detected in the gRNA-transfected cells due to

Table 1 Demographic and clinical characteristics of subjects

	Age, years	Sex, male/female	Duration of education, years	MMSE
Control (*n* = 20)	59.3 ± 6.4	7/13	12.2 ± 4.3	28.5 ± 3.2
PD (*n* = 20)	56.6 ± 8.2	11/9	11.7 ± 5.5	26.1 ± 5.1
	Age of onset, years	Disease duration, months	UPDRS-III	MIBG (*n* = 11)
PD (*n* = 20)	55.1 ± 8.5	23.2 ± 10.0	18.3 ± 6.2	1.8 ± 0.4
	CC-SIT, normosmia/ hyposmia	History of RBD	Abnormal deep white matter	
PD (*n* = 20)	14/6	7	9	

CC-SIT, Cross-Cultural Smell Identification Test; MIBG, metaiodobenzylguanidine; MMSE, Mini-Mental State Examination; PD, Parkinson's disease; RBD, rapid eye movement sleep behavior disorder; UPDRS-III, Unified Parkinson's Disease Rating Scale.

Figure 1 Average protein levels of BAG2 and cathepsin D are decreased in the plasma of PD patients. (**a**) Preparation of the albumin/IgG-depleted plasma samples from control and PD patients (20 subjects each) was verified by SDS-PAGE. The plasma samples from the 20 control and patients with PD were each pooled for the quantitative analysis of the average levels of autophagy-related proteins. The pooled samples were immunoblotted with antibodies specific to BAG2 (**b**), BAG3 (**c**), cathepsin B (**d**), cathepsin D (**e**), LAMP1 (**f**), LAMP2 (**g**), and WDFY3 (**h**). Each analysis was repeated as necessary. Arrows and asterisks indicate specific and cross-reacting bands, respectively. (**i**) Intensities of specific bands of **b–h** were quantitatively analyzed, and the level of changes in the PD group was compared to that in the control group. Each blue dot indicates the result from each blot, and the grey bars represent mean values. (**j**) The fold change in BAG2 levels of the PD group. Values represent mean ± SEM of three independent experiments. (**k**) The fold change in cathepsin D levels of the PD group. Values represent mean ± SEM of two independent experiments. Molecular weight standards (in kDa) are shown to the left. **P < 0.01 (Student *t*-test). BAG, BCL2-associated athanogene; CBB, Coomassie Brilliant Blue; PD, Parkinson's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

genetic heterogenicity of the cells, this result indicates that the BAG2 antibody used in this study is adequately reliable and specific.

Immunoblot analysis of the albumin/IgG-depleted patient plasma showed that the level of BAG2 significantly decreased in the plasma from patients with PD (**Figure 2b,c**). Although the samples were loaded in four separate gels because of the limited number of lanes, all SDS-PAGE and immunoblot analyses were performed at

the same time and under the same conditions, including exposure time. The most intense BAG2 signal detected in plasma samples was 30 kDa, the intensity of the band was highly correlated with the 25 kDa band. Thus, we conclude that both bands are likely to be BAG2 present in plasma. Intriguingly, the BAG2 levels of control subjects were heterogeneous whereas those of PD subjects were relatively homogenous and significantly low. As a result, the relative mean value of BAG2 levels in the PD group was only 14%
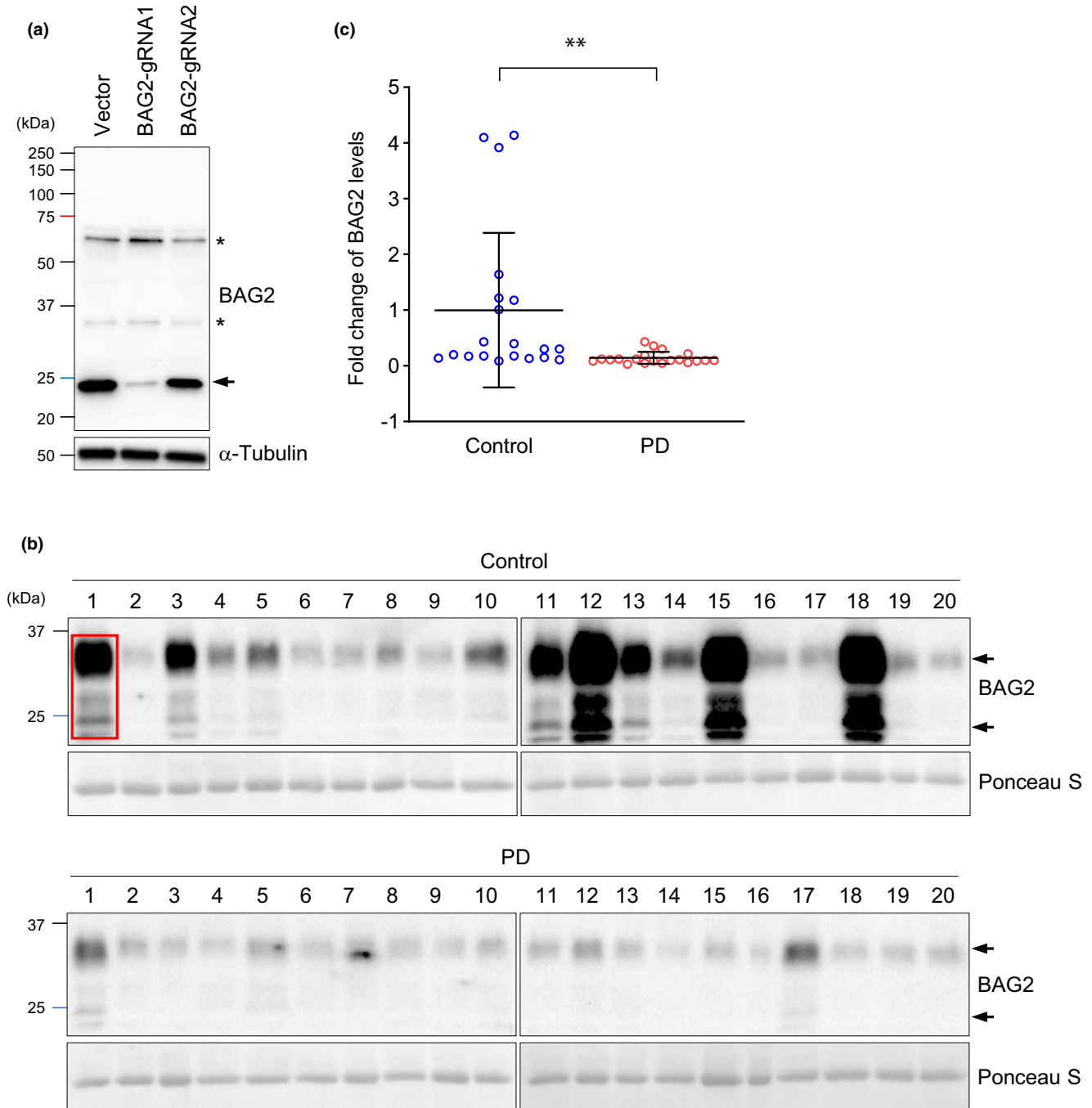
Figure 2 BAG2 levels are decreased in the plasma of patients with PD. (**a**) Validation of the BAG2 antibody for immunoblot analysis. HeLa cells were transfected with an empty vector (Vector) or vectors with gRNAs specific to BAG2 gene (BAG2-gRNA1, BAG2-gRNA2). Cell lysates were subjected to immunoblot with the indicated antibodies. (**b**, **c**) Analysis of the BAG2 level in the plasma of control and patients with PD. (**b**) Albumin/IgG-depleted plasma samples were immunoblotted with a BAG2 antibody. Ponceau S staining was performed after SDS-PAGE to show that equal amounts of plasma proteins were loaded in each lane. (**c**) Intensities of the specific bands (**b**, red box) of each lane were quantitatively analyzed, and the level of fold changes in the PD group was compared with that of the control group. Each blue and red open circle indicates the BAG2 level in the plasma from each control and patient with PD, respectively. Mean ± SD is displayed as a horizontal line in each group. Arrows and asterisks indicate specific and cross-reacting bands, respectively. Molecular weight standards (in kDa) are shown to the left. **$P < 0.01$ (Student t-test). BAG, BCL2-associated athanogene; IgG, immunoglobulin G; PD, Parkinson's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

of those in the control group. This result indicates that the amount of plasma BAG2 proteins is possibly related to the onset or progression of PD.

BAG2 levels are decreased in patients with PD with hyposmia
To specify the relation between BAG2 level and clinical characteristics, we analyzed the correlation between BAG2
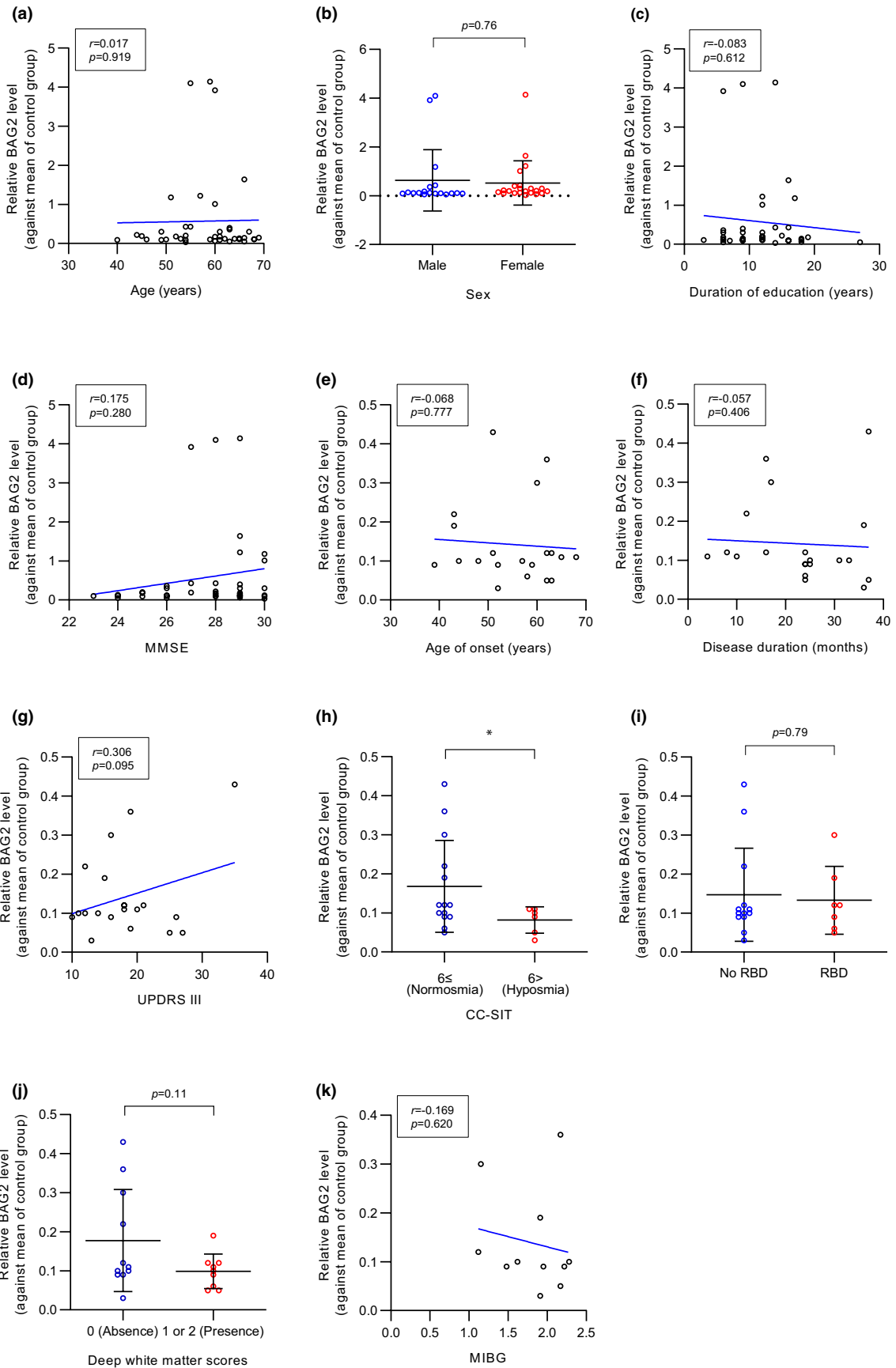
Figure 3 Plasma BAG2 levels are decreased in patients with PD with hyposmia. Correlation of plasma BAG2 levels with age (**a**), sex (**b**), duration of education (**c**), and MMSE scores (**d**). Correlation of plasma BAG2 levels with the age of onset (**e**), disease duration (**f**), UPDRS-III scores (**g**), presence of hyposmia (**h**), history of RBD (**i**), presence of abnormal deep white matter (**j**), and MIBG scores (**k**). Each open circle indicates the BAG2 level in plasma from each patient. **a, c–g, k** Pearson correlation coefficient (*r*) and *P* value (*P*, Student *t*-test) of linear regression are shown in each panel. **b, h–j** Mean ± SD is displayed as a horizontal line in each group. The number of analyzed control and PD subjects are 20 and 20, respectively **a–d**, 20 patients with PD **e–j**, and 11 patients with PD (**k**). *P < 0.05 (Student *t*-test). BAG, BCL2-associated athanogene; CC-SIT, Cross-Culture Smell Identification Test; MIBG, metaiodobenzylguanidine; MMSE, Mini-Mental State Examination; RBD, rapid eye movement sleep behavior disorder; UPDRS, Unified PD Rating Scale Part III.

level and demographic characteristics of the study participants. As shown in **Figure 3a–d**, levels of BAG2 in plasma from patients with PD did not correlate with age, sex, duration of education, and Mini-Mental State Examination scores. In addition, it did not have any correlation with the other clinical characteristics; age of onset, disease duration, UPDRS-III scores, history of RBD, presence of abnormal deep white matter, and MIBG scores (**Figure 3e–g,i–k**). Cross-Culture Smell Identification Test scores indicate the impairment of olfactory function that is a critical non-motor symptom of the prodromal stages of PD. Based on the original study by Doty *et al.*, we classified patients with PD into hyposmia if the Cross-Culture Smell Identification Test score was 5 or less, and into normosmia if the score was 6 or higher.[21] Interestingly, the plasma BAG2 level of patients with PD with hyposmia was significantly lower (*P* = 0.045) than that of patients with PD with normosmia (**Figure 3h**).

Cathepsin D levels are decreased in the plasma of patients with PD
As shown in **Figure 1i,k,** the level of CTSD in plasma was also lower than that of the control group. CTSD is a soluble lysosomal aspartic endopeptidase, which is processed to mature form within endolysosomal compartments and implicated in neurodegenerative diseases.[22] Pro-CTSD with molecular weight 52 kDa undergoes proteolysis that leads to the generation of an active intermediate pro-CTSD and pre-CTSD (~ 42 kDa). Active intermediate CTSD further processes into mature form.[23,24]

To investigate the level of CTSD in individual plasma samples, we first validated the antibody specific to CTSD by CRISPR-Cas9 system. The levels of pro-CTSD and pre-CTSD and processed CTSD heavy chain were decreased by transfection of CTSD-gRNAs (**Figure 4a**, upper arrow, pro-CTSD; middle arrow, pre-CTSD; bottom arrow, CTSD heavy chain). However, given that CTSD that we detected in plasma sample was the secreted form, we needed to confirm whether the antibody could also detect secreted CTSD. We performed immunoblot analysis using HeLa cells or HEK293T cells conditioned media and found that the secreted CTSD had slightly high molecular weight, around 50 kDa, in comparison (**Figure 4b**). These results indicate that the antibody is also available for secreted CTSD and that the detected band around 50 kDa in plasma samples is secreted form of CTSD (**Figure 1e** and **Figure 4c**).

Next, we analyzed the individual plasma samples by immunoblot analysis. The level of CTSD was significantly lower in the plasma samples of patients with PD than the control group (**Figure 4c,d**). The level of plasma CTSD was not correlated with age, sex, duration of education, or Mini-Mental State Examination scores (**Figure S1a-d**). In

addition, there were no significant correlations between the level of plasma CTSD and analyzed clinical characteristics, such as age of onset, disease duration, UPDRS-III scores, presence of hyposmia, history of RBD, presence of abnormal deep white matter, and MIBG scores (**Figure S1e–k**). These results might indicate that decreased level of CTSD in patients with PD would be related to the general pathogenesis of PD rather than the clinically specific type of PD.

ROC curve analysis identified plasma BAG2 and CTSD levels as potential biomarkers of PD
Next, we evaluated the possibility of our biomarker candidates; BAG2 and CTSD, using a statistical prediction model, ROC curve. The ROC curve represents the sensitivity as a function of the specificity, and the area under the curve (AUC) indicates the accuracy of a model to discriminate between the two groups. The AUC of plasma BAG2 levels was 0.840 with 85% sensitivity and 55% specificity (*P* = 0.0002) and that of plasma CTSD levels was 0.760 with 65% sensitivity and 60% specificity (*P* = 0.0049; **Figure 5** and **Table 2**). The integrated multivariate model using BAG2 and CTSD levels also showed high diagnostic accuracy (AUC = 0.875, *P* < 0.0001; **Figure 5** and **Table 2**). These results indicate that the combination of plasma levels of BAG2 and CTSD shows high performance in distinguishing PD from the healthy subjects. Taken together, the above results suggest that plasma levels of BAG2 and CTSD could be novel diagnostic biomarkers for PD.

DISCUSSION

In this study, we suggest the possibility of two autophagy-related proteins, BAG2 and CTSD, as biomarkers for PD using plasma samples. Concerning that neurodegenerative diseases have characteristic protein aggregation as a part of pathogenesis, maintaining and regulating autophagy to promote the degradation of the aggregates is important to prevent diseases and to delay the progression.[25] In addition, several studies suggest the implication of proteins genetically linked to PD, such as LRRK2 and α-synuclein in the autophagy pathway.[26–28] The degradation of α-synuclein is dependent on macro-autophagy and chaperon-mediated autophagy.[29–31] Accordingly, impaired lysosomal function results in the accumulation of α-synuclein, which has cytotoxicity.[32] A study showed that the downregulation of ATG5, which is important for autophagy initiation, led to the increase of PD-associated proteins and the loss of DA neurons in zebrafish.[33] In addition, a mutation in the recessive PD gene, DJ-1, is implicated in the decreased autophagy activity.[34] LRRK2 is known to be degraded by chaperon-mediated autophagy and the G2019S mutation in LRRK2 causes the accumulation of autophagic
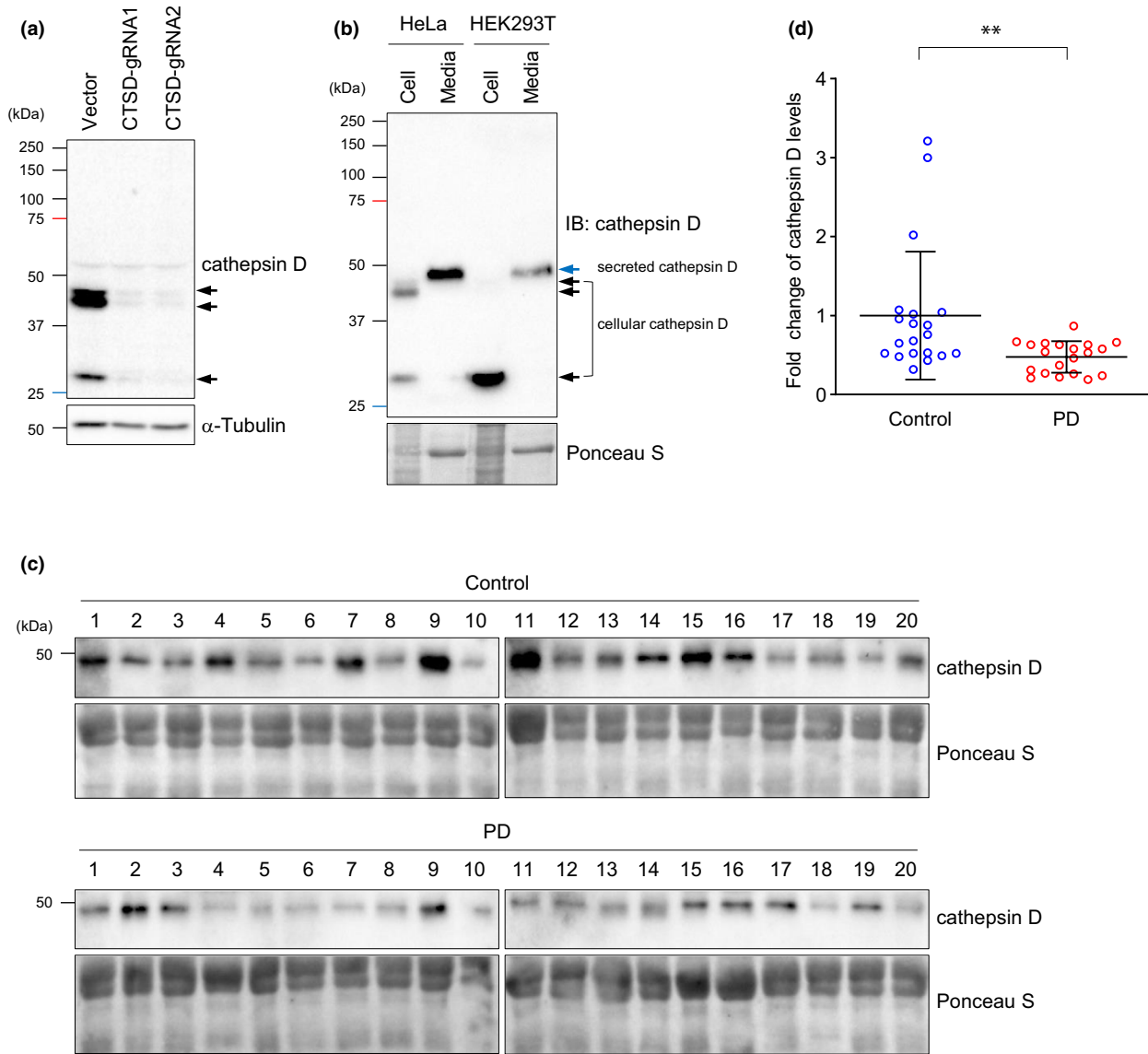
Figure 4 Cathepsin D levels are decreased in the plasma of patients with PD. Validation of the cathepsin D antibody for immunoblot analysis. (**a**) HeLa cells were transfected with an empty vector (Vector) or vectors with gRNAs specific to CTSD gene (CTSD-gRNA1, CTSD-gRNA2). Cell lysates were subjected to immunoblot with the indicated antibodies. Arrows indicate specific cathepsin D bands. (**b**) Intracellular and secreted proteins were prepared from cell lysates or the culture media of the indicated cell lines, respectively. Samples were immunoblotted with a cathepsin D antibody. Ponceau S staining was performed after SDS-PAGE. Blue arrow (~ 50 kDa) indicates a secreted cathepsin D band and black arrows indicate the cathepsin D present in various forms within the cell. Analysis of the cathepsin D level in the plasma of control and patients with PD. (**c**) Albumin/IgG-depleted plasma samples were analyzed by immunoblot with a cathepsin D antibody. Ponceau S staining was performed after SDS-PAGE to demonstrate that equal amounts of plasma proteins were loaded in each lane. (**d**) Intensities of the cathepsin D bands of **c** were quantitatively analyzed, and the level of fold changes in the PD group was compared with that in the control group. Each blue and red open circle indicates the cathepsin D level in the plasma of each control and patient with PD, respectively. Mean ± SD is displayed as a horizontal line in each group. Molecular weight standards (in kDa) are shown to the left. **$P < 0.01$ (Student *t*-test). BAG, BCL2-associated athanogene; IgG, immunoglobulin G; PD, Parkinson's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

vacuoles in the cell.[35] Taken together, these studies suggest the importance of autophagic regulation in PD pathogenesis and even suggest the possibility of the development of drugs for PD prevention and management.

BAG2, a member of the BAG family, is an Hsp70/Hsc70 molecular chaperone-interacting protein.[36,37] Although no direct association between BAG2 and PD has been revealed,

BAG2 has been reported to stabilize PINK1 by decreasing its ubiquitination.[38] In addition, BAG2 inhibits CHIP E3 ligase activity by interacting with Hsp70.[39,40] Intriguingly, the CHIP E3 ligase is known to ubiquitinate PINK1 and promote degradation.[41] Another study showed that BAG2 plays a role as an upstream regulator of the PINK/parkin pathway.[42] These results indicate that BAG2 might play an important role in
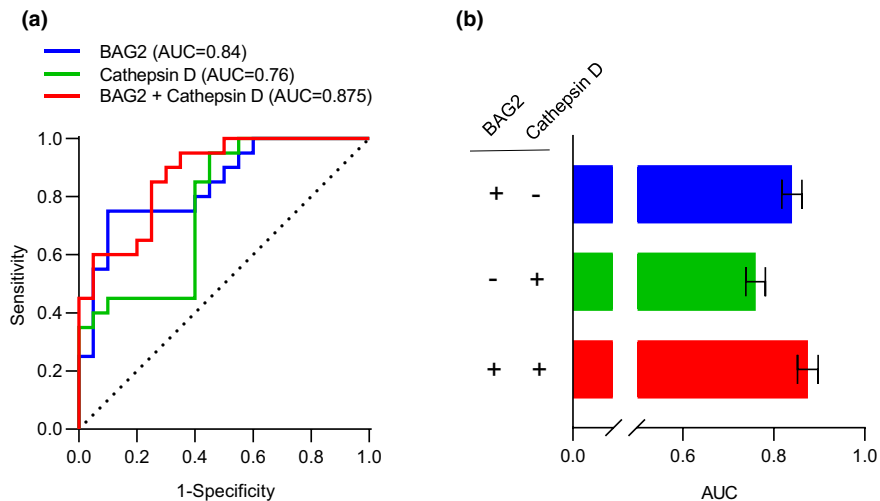
Figure 5 Analysis of plasma biomarker performance in distinguishing PD from control subjects. (**a**) AUC was calculated for PD and control groups to determine the significance of plasma levels of BAG2 and cathepsin D in predicting PD. (**b**) Comparison of ROC curve among combinations of biomarkers. Details are described in **Table 2**. AUC, area under the curve; BAG, BCL2-associated athanogene; PD, Parkinson's disease; ROC, receiver operating characteristic.

the mitochondria-related pathogenesis of PD. As shown in **Figure 2b**, we observed a significant decrease in plasma BAG2 level in the PD group; however, we could also detect seven control subjects, especially three subjects, having relatively high levels of plasma BAG2 compared with the other control subjects. The BAG2 level decrease in the PD group was still statistically significant (30.8% of control, $P = 0.0068$) in the analysis excluding the top three control subjects, suggesting that our findings might not be a false positive result due to a small number of patients. The reduction in BAG2 levels in the plasma of patients with PD that we found in this study suggests that the decrease in the level of intracellular BAG2 of patients with PD might be systematically reflected.

Olfactory dysfunction is one of the clinical features of PD and it may precede motor symptoms for several years. Olfactory dysfunction is, however, limited as an independent predictive diagnostic feature for PD because it can also occur in other diseases, such as Alzheimer's disease and idiopathic RBD.[43,44] In the present study, we revealed that the levels of plasma BAG2 in patients with PD with olfactory dysfunction are significantly lower than those in patients with normosmia, suggesting that plasma BAG2 levels could be associated with olfactory impairment (**Figure 3h**). Overall, these results suggest that an olfactory examination accompanied by plasma BAG2 measurement is worthy of further development for the early diagnosis of PD.

CTSD is a principle lysosomal protease involved in the intracellular degradation of unfolded or nonfunctional proteins, including α-synuclein.[45,46] CTSD deficiency exacerbates α-synuclein accumulation whereas its overexpression reduces α-synuclein aggregation and is neuroprotective against α-synuclein toxicity.[47,48] It is uncertain whether the reduced level of plasma CTSD in patients with PD reflects a reduction in lysosomal CTSD (**Figure 4d**). Because CTSD is known to be secreted by secretory granules,[49] it still needs to be further examined whether the cellular level and activity of CTSD in patients with PD are also altered as secreted CTSD decreased in patients with PD (**Figure 4**).

Although we suggest the possibility of BAG2 and CTSD in plasma as PD biomarkers, there are two limitations in our study. First, the number of subjects was not enough. If there were more subjects, we could have got more significant changes in the other proteins. Nonetheless, we could still suggest two proteins with significant changes that should be further tested with more patients. Thus, investigating more patients with PD is necessary to find more significance in our results and to even understand the underlying mechanism of PD. Second, as previously mentioned, it should be examined that changes in levels of BAG2 and CTSD, which are involved in autophagy, actually do affect the autophagic activity in patients with PD.

In a clinical perspective, however, our study has several strengths. First, all patients with PD were subjected to a

Table 2 ROC curve analysis of plasma BAG2 and cathepsin D

Control vs. PD	AUC	Sensitivity %	Specificity %	*P* value[a]	95% CI of AUC
BAG2	0.840	85.00	55.00	0.0002	0.717 to 0.963
Cathepsin D	0.760	65.00	60.00	0.0049	0.609 to 0.911
BAG2 + cathepsin D	0.875	85.00	75.00	< 0.0001	0.763 to 0.987

Control, $n = 20$; PD, $n = 20$.
AUC, area under the curve; BAG, BCL2-associated athanogene; CI, confidence interval; ROC, receiver operating characteristic.
[a]*P* value by Pearson's χ^2 test.

DaTscan and brain MRI to clearly distinguish this disorder from other neurodegenerative diseases. Second, the disease duration was relatively short (23.2 ± 10.0 months). It means that the patients are relatively homogeneous in the progression of PD, which can be an advantage that helps overcome limitations of a small number of patients. Third, most patients with PD were drug naïve. This means that our results correspond to the purpose of our study, the discovery of early diagnosis biomarkers, and that the biochemical response by taking drugs was not reflected in the results. Finally, the quality of all plasma samples used in this study was consistent and reliable as they were prepared within 3 hours after blood collection.

Nonetheless, further study should be followed to validate our results and demonstrate whether decreased levels of BAG2 and CTSD are causal factors or results of PD progression. We hope that our study provides an insight into further integrative research to develop clinically applicable biomarkers for PD.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Conflicts of Interest. The authors declared no competing interests for this work.

Author Contributions. J.K., J.W.K., J.H.Y., and J.C. wrote the manuscript; J.H.Y. and J.C. designed the research; J.K., J.W.K., H.H., J.L., and K.Y.P. performed the research and analyzed the data.

1. Poewe, W. *et al.* Parkinson disease. *Nat. Rev. Dis. Primers* **3**, 17013 (2017).
2. Surmeier, D.J. Determinants of dopaminergic neuron loss in Parkinson's disease. *FEBS J.* **258**, 3657–3668 (2018).
3. Pakkenberg, B., Moller, A., Gundersen, H.j., Mouritzen Dam, A. & Pakkenberg, H. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. *J. Neurol. Neurosur.* **54**, 30–33 (1991).
4. Hughes, A.J., Daniel, S.E. & Lees, A.J. Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* **57**, 1497–1499 (2001).
5. Boerger, M., Funke, S., Bähr, M., Grus, F. & Lingor, P. Biomarker sources for Parkinson's disease: time to shed tears? *Basal ganglia* **5**, 63–69 (2015).
6. Chahine, L.M., Stern, M.B. & Chen-Plotkin, A. Blood-based biomarkers for Parkinson's disease. *Parkinsonism Relat. Disord.* **20**(suppl. 1), S99–S103 (2014).
7. Lin, C.H. *et al.* Plasma biomarkers differentiate Parkinson's disease from atypical parkinsonism syndromes. *Front. Aging Neurosci.* **10**, 123 (2018).
8. Hong, Z. *et al.* DJ-1 and α-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease. *Brain* **133**, 713–726 (2010).
9. Xu, L. & Pu, J. Alpha-synuclein in Parkinson's disease: from pathogenetic dysfunction to potential clinical application. *Parkinsons Dis.* **2016**, 1720621 (2016).
10. Jin, S.M. & Youle, R.J. PINK1- and parkin-mediated mitophagy at a glance. *J. Cell Sci.* **125**, 795–799 (2012).
11. Deas, E., Wood, N.W. & Plun-Favreau, H. Mitophagy and Parkinson's disease: the PINK1-parkin link. *Biochim. Biophys. Acta.* **1813**, 623–633 (2011).
12. Miller, S. & Muqit, M.M.K. Therapeutic approaches to enhance PINK1/parkin mediated mitophagy for the treatment of Parkinson's disease. *Neurosci. Lett.* **705**, 7–13 (2019).
13. Ramonet, D. *et al.* Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. *PLoS One* **6**, e18568 (2011).
14. Xu, C.Y. *et al.* DJ-1 inhibits alpha-synuclein aggregation by regulating chaperone-mediated autophagy. *Front. Aging Neurosci.* **9**, 308 (2017).
15. Laperle, A.H. *et al.* iPSC modeling of young-onset Parkinson's disease reveals a molecular signature of disease and novel therapeutic candidates. *Nat. Med.* **26**, 289–299 (2020).
16. Hughes, A.J., Daniel, S.E., Kilford, L. & Lees, A.J. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J. Neurol. Neurosurg. Psychiatry.* **55**, 181–184 (1992).
17. Bhatia, K.P. *et al.* Consensus statement on the classification of tremors. from the task force on tremor of the international Parkinson and movement disorder society. *Mov. Disord.* **33**, 75–87 (2018).
18. Lee, S., Chang, J. & Blackstone, C. FAM21 directs SNX27–retromer cargoes to the plasma membrane by preventing transport to the Golgi apparatus. *Nat. Commun.* **7**, 10939 (2016).
19. Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. *Nat. Meth.* **11**, 122–123 (2014).
20. Chung, E.J. & Kim, S.J. (123)I-Metaiodobenzylguanidine myocardial scintigraphy in Lewy body-related disorders: a literature review. *J. Mov. Disord.* **8**, 55–66 (2015).
21. Doty, R.L., Marcus, A. & Lee, W.W. Development of the 12-item cross-cultural smell identification test (CC-SIT). *Laryngoscope* **106**, 353–356 (1996).
22. Zaidi, N., Maurer, A., Nieke, S. & Kalbacher, H. Cathepsin D: a cellular roadmap. *Biochem. Biophys. Res. Commun.* **376**, 5–9 (2008).
23. Woessner, J.F. Jr. Specificity and biological role of cathepsin D. *Adv. Exp. Med. Biol.* **95**, 313–327 (1977).
24. Sapolsky, A.I. & Woessner, J.F. Jr. Multiple forms of cathepsin D from bovine uterus. *J. Biol. Chem.* **247**, 2069–2076 (1972).
25. Banerjee, R., Beal, M.F. & Thomas, B. Autophagy in neurodegenerative disorders: pathogenic roles and therapeutic implications. *Trends Neurosci.* **33**, 541–549 (2010).
26. Bandyopadhyay, U. & Cuervo, A.M. Chaperone-mediated autophagy in aging and neurodegeneration: lessons from alpha-synuclein. *Exp. Gerontol.* **42**, 120–128 (2007).
27. Alegre-Abarrategui, J. *et al.* LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. *Hum. Mol. Genet.* **18**, 4022–4034 (2009).
28. Alegre-Abarrategui, J. & Wade-Martins, R. Parkinson disease, LRRK2 and the endocytic-autophagic pathway. *Autophagy* **5**, 1208–1210 (2009).
29. Friedman, L.G. *et al.* Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of α-synuclein and LRRK2 in the brain. *J. Neurosi.* **32**, 7585–7593 (2012).
30. Vogiatzi, T., Xilouri, M., Vekrellis, K. & Stefanis, L. Wild type α-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J. Biol. Chem.* **283**, 23542–23556 (2008).
31. Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N. & Rubinsztein, D.C. α-synuclein is degraded by both autophagy and the proteasome. *J. Biol. Chem.* **278**, 25009–25013 (2003).
32. Xilouri, M., Brekk, O.R. & Stefanis, L. Autophagy and alpha-synuclein: relevance to Parkinson's disease and related synucleopathies. *Mov. Disord.* **31**, 178–192 (2016).
33. Hu, Z.Y., Chen, B., Zhang, J.P. & Ma, Y.Y. Up-regulation of autophagy-related gene 5 (ATG5) protects dopaminergic neurons in a zebrafish model of Parkinson's disease. *J. Biol. Chem.* **292**, 18062–18074 (2017).
34. Mccoy, M.K. & Cookson, M.R. DJ-1 regulation of mitochondrial function and autophagy. *Autophagy* **7**, 531–532 (2011).
35. Gómez-Suaga, P. *et al.* Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. *Hum. Mol. Genet.* **21**, 511–525 (2012).
36. Kabbage, M. & Dickman, M.B. The BAG proteins: a ubiquitous family of chaperone regulators. *Cell. Mol. Life Sci.* **65**, 1390–1402 (2008).
37. Takayama, S., Xie, Z. & Reed, J.C. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J. Biol. Chem.* **274**, 781–786 (1999).
38. Bonifati, V. *et al.* Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. *Neurology* **65**, 87–95 (2005).
39. Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S. & Höhfeld, J. BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. *Mol. Biol. Cell.* **17**, 2996–3008 (2006).
40. Dai, Q. *et al.* Regulation of the cytoplasmic quality control protein degradation pathway by BAG2. *J. Biol. Chem.* **280**, 38673–38681 (2005).
41. Yoo, L. & Chung, K.C. The ubiquitin E3 ligase CHIP promotes proteasomal degradation of the serine/threonine protein kinase PINK1 during staurosporine-induced cell death. *J. Biol. Chem.* **293**, 1286–1297 (2018).

42. Qu, D. *et al.* BAG2 gene-mediated regulation of PINK1 protein is critical for mitochondrial translocation of PARKIN and neuronal survival. *J. Biol. Chem.* **290**, 30441–30452 (2015).

43. Huang, S.F. *et al.* Odor identification test in idiopathic REM-behavior disorder and Parkinson's disease in China. *PLoS One* **11**, 1–13 (2016).

44. Krismer, F. *et al.* Sniffing the diagnosis: olfactory testing in neurodegenerative parkinsonism. *Parkinsonism Relat. Disord.* **35**, 36–41 (2017).

45. Benes, P., Vetvicka, V. & Fusek, M. Cathepsin D-many functions of one aspartic protease. *Crit. Rev. Oncol. Hematol.* **68**, 12–28 (2008).

46. Hossain, S. *et al.* Limited proteolysis of NACP/α-synuclein. *J. Alzheimers Dis.* **3**, 577–584 (2001).

47. Cullen, V. *et al.* Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo. *Mol. Brain* **2**, 1–17 (2009).

48. Qiao, L. *et al.* Lysosomal enzyme cathepsin D protects against alpha-synuclein aggregation and toxicity. *Mol. Brain* **1**, 17 (2008).

49. Dragonetti, A. *et al.* The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL. *J. Cell Sci.* **113**, 3289–3298 (2000).