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Phosphorylated α-Synuclein in Parkinson's Disease

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Abstract

Phosphorylated α-synuclein (PS-129), a protein implicated in the pathogenesis of Parkinson's disease (PD), was identified by mass spectrometry in human cerebrospinal fluid (CSF). A highly sensitive and specific assay was established and used to measure PS-129, along withtotal α-synuclein, in the CSF of patients with PD, other parkinsonian disorders such as multiple system atrophy (MSA) and progressive supranuclear palsy (PSP), and healthy individuals (a total of ~600 samples). PS-129 CSF concentrations correlated weakly with PD severity and, when combined with total α-synuclein CSF concentrations, contributed to distinguishing PD from MSA and PSP. Further rigorous validation in independent cohorts of patients, especially those where samples have been collected longitudinally, will determine whether PS-129 CSF concentrations will be useful for diagnosing PD and for monitoring PD severity and progression.

Introduction

An essential step in clinical trials is differentiating patients with Parkinson's disease (PD) from those with other parkinsonian disorders that have overlapping symptoms such as multiple system atrophy (MSA) and progressive supranuclear palsy (PSP). Such differential diagnosis can be extremely difficult in the early stages of these diseases (1). Additionally, assessing the efficacy of disease-modifying PD therapies using clinical measures is challenging because symptoms of PD may fluctuate, many clinical assessments are subjective, and the use of dopaminergic drugs can affect clinical symptoms. Thus, much interest surrounds developing markers that will help to improve diagnostic specificity and to more objectively track disease progression.

α-Synuclein is an attractive candidate for a potential marker of PD because it is strongly linked to the pathogenesis of both familial and sporadic forms of this disease (2, 3). Many current studies seek to find new drugs that lower the concentration of α-synuclein in the brains of PD patients to prevent the aggregation of α-synuclein and its sequestration in Lewy

bodies, a pathological hallmark of PD (4-6). Unfortunately, although the total concentration of α-synuclein in cerebrospinal fluid (CSF) provides reasonable diagnostic sensitivity and specificity in differentiating PD patients from healthy controls, it does not readily differentiate PD from other parkinsonian disorders such as MSA or PSP, nor does it correlate with the severity or progression of PD (7-9).

Several posttranslational modifications to α-synuclein are known to occur in PD. Among them is phosphorylation at Ser^{129} [phosphorylated α-synuclein (PS-129)], a modification that may be critical in PD pathogenesis (10, 11). PS-129 has been reported to enhance α-synuclein toxicity both *in vivo* and *in vitro*, possibly by increasing the formation of α-synuclein aggregates (11, 12). On the other hand, a protective role for PS-129 has also been proposed (13, 14). Although the exact role of PS-129 in PD pathogenesis remains to be determined, we sought to discover whether PS-129 is present in human CSF and, if so, whether it could be used alone or in combination with total α-synuclein to help in diagnosing PD and for monitoring disease severity/progression.

Results

Identification of PS-129 in CSF

To investigate whether PS-129 is present in human CSF, we performed immunoprecipitation (IP) to enrich PS-129 from samples collected from five healthy control individuals. Intact PS-129 protein was then detected in CSF using linear mass spectrometry (MS) (Fig. 1, A to C). After trypsin and Asp-N endoproteinasedigestion of the enriched proteins, two unique α-synuclein peptides containing phosphorylated Ser^{129} were identified by tandem MS (Fig. 1D), providing experimental evidence that PS-129 is present in human CSF.

An assay for detecting PS-129 in CSF

To translate MS-based technology into a more robust and practical method for monitoring PS-129 in CSF, we developed an assay using a bead-based multi-analyte profiling technology (Luminex). The sensitivity of the established Luminex assay was about 9 pg/ml, with a signal-to-background ratio of close to 9. The assay specificity was confirmed by several methods, including (i) loss of the signal after predepletion of PS-129 with an anti-PS-129 antibody that was not used in the Luminex assay, (ii) loss of the signal after de-phosphorylation of CSF proteins by calf intestinal alkaline phosphatase, and (iii) absence of the signal using native α-synuclein or the phosphorylated protein β-casein (Fig. 1E). The concentrations of PS-129 in CSF reference samples from healthy controls were estimated to be 81.6 ± 20.4 pg/ml. The assay's accuracy was measured by spiking human recombinant PS-129 protein into CSF; the average recovery was about 82%.

Major variables affecting PS-129 concentrations in CSF

Our previous studies demonstrated that blood contamination of CSF, which occasionally occurs during lumbar puncture, can drastically affect the CSF concentrations of certain proteins including α-synuclein, DJ-1 (another protein implicated in PD), complement 3, and factor H (8, 15). Additionally, α-synuclein, DJ-1, complement 3 and factor H all demonstrated age dependence (8); complement 3, and factor H concentrations were also influenced by sex (15). However, when PS-129 concentrations were stratified against these variables, no significant association with CSF hemoglobin concentration (an index of red blood cell contamination) was observed (see fig. S1, A and B, for discovery and validation set, respectively). With our current Luminex assay, PS-129 could not be detected in human blood further indicating that PS-129 concentrations are unlikely to be affected by blood contamination, a highly desirable characteristic of a marker in CSF. As is the case for total α-synuclein concentrations in CSF (8), PS-129 concentrations in CSF did not significantly

associate with sex in any of the groups (P>0.05). On the other hand, linear regression analysis revealed an age-dependent increase in PS-129 concentrations in CSF from healthy control subjects (P<0.01) (fig. S2A). However, as is the case with total α-synuclein (8), the age dependence observed with PS-129 in CSF disappeared when subjects younger than 50 years of age were excluded (fig. S2B).

Testing the diagnostic use of PS-129 in CSF

The use of PS-129 as a potential diagnostic marker either alone or in combination with total α-synuclein was next evaluated. For this purpose, cases studied were restricted to those ≥ 50 years of age because of the age dependence of PS-129 concentrations in CSF (fig. S2). When total α-synuclein was also analyzed, samples with hemoglobin concentrations of ≥ 200 ng/ml were excluded as previously described (8). Subjects were randomly split into a discovery set and a validation set. The discovery set included a total of 246 subjects: 78 healthy controls, 93 PD patients, 16 MSA patients, 33 PSP patients, and 26 patients with Alzheimer's disease (AD). As shown in Fig. 2A and table S1, PS-129 concentrations were significantly higher in PD patients compared to healthy controls in the discovery set (PD: 79.23 ± 23.22 pg/ml, control: 68.61 ± 17.25 pg/ml, P<0.05), and were lower in patients with MSA (58.12 ± 20.24 pg/ml, P<0.01) and PSP (55.54 ± 16.87 pg/ml, P<0.001) compared to PD patients. PSP patients also had significantly lower PS-129 concentrations in CSF than did controls (P<0.05). The results with total α-synuclein concentrations in CSF (Fig. 2B) largely confirmed earlier observations in PD and other related diseases, that is, total α-synuclein concentrations are significantly lower in patients with PD and MSA compared to healthy controls (7, 8). Although PS-129 concentrations were similar in MSA and PSP patients, the ratio of PS-129 to α-synuclein differentiated these two groups (Fig. 2C; MSA: 0.24 ± 0.12, PSP: 0.15 ± 0.07, P<0.05). Furthermore, there was a significant increase in the ratio of PS-129 to α-synuclein in PD (0.22 ± 0.07) compared to healthy controls (0.15 ± 0.04, P<0.01) and PSP patients (P<0.01). Note that some P-values need to be interpreted cautiously in light of the multiple comparisons that were conducted.

Analysis of receiver operating characteristic (ROC) was performed to further evaluate the diagnostic accuracy of PS-129 alone and in combination with total α-synuclein. Because accurate classification is important in clinical applications, such as patient selection for clinical trials testing disease-modifying treatments, specificities were anchored to be ≥80%. At a specificity of ≥80%, a combination of PS-129 and total α-synuclein performed better than PS-129 alone (table S2) with sensitivities for detecting PD, MSA, and PSP patients versus healthy controls of 61%, 75%, and 67%, respectively. The sensitivities among the three different parkinsonian disease groups were as follows: PD versus MSA patients, 40%; PD versus PSP patients, 72%; and MSA versus PSP patients, 63% (Fig. 3, A to C, and table S2). The diagnostic values (specificity) when sensitivities were anchored to be 80% or above were: PD versus controls, 64%; MSA versus controls, 73%; PSP versus controls, 55%; PD versus MSA, 63%; PD versus PSP, 63%; and MSA versus PSP, 67% (table S3).

The major findings observed in the discovery set were replicated in a validation set that included a total of 344 subjects (126 normal controls, 116 PD, 25 MSA, 27 PSP, and 50 AD patients). As shown in Fig. 2, D to F, and table S1, compared to healthy controls (73.03 ± 17.20 pg/ml), PS-129 concentrations in CSF were significantly lower in the PSP group (58.24 ± 24.93 pg/ml, P<0.05). Compared to PD patients (77.73 ± 20.45 pg/ml), PS-129 CSF concentrations were lower in MSA (61.97 ± 14.19 pg/ml, P<0.01) and PSP (P<0.001) patients. When the ratio of PS-129 to α-synuclein was compared, there was a significant increase in the ratio for PD patients (0.22 ± 0.09) compared to controls (0.17 ± 0.06, P<0.01) and PSP patients (0.15 ± 0.06, P<0.05). When logistic regression models and cutoff values (anchored to ≥80% specificity or sensitivity) determined from the discovery set were applied to the validation set, PS-129 combined with total α-synuclein gave area under the

curve (AUC), sensitivity and specificity estimates similar to those in the discovery set. For example, the specificity and sensitivity for differential diagnosis in the validation set (when anchoring ≥80% specificity in the discovery set) were as follows: PD versus MSA, 94% and 47%; PD versus PSP, 82% and 63%; and MSA versus PSP, 82% and 50% (Fig. 3, D to F, and table S4). Some diagnostic values in the validation set deteriorated compared to those in the discovery set. This is likely due to the fact that the models and cutoff values were optimized for the discovery set and also may reflect the relatively small sample sizes and differences between the two sample sets. Actual distribution of PS-129 in relation to total α-synuclein for all compared groups is shown in fig. S3.

Correlating CSF PS-129 with PD Severity

The correlation between PS-129 concentrations in CSF and the approximated severity of PD as determined by the Unified Parkinson's Disease Rating Scale (UPDRS) part III on-state motor scores was determined in both discovery (n=93) and validation (n=116) cohorts of PD patients (cases <50 years were not excluded in this analysis because no age dependence of CSF PS-129 was noted in PD patients). As shown in Fig. 4, A and B, there was a weak but statistically significant association ($P<0.05$) between CSF PS-129 concentrations and the severity of PD symptoms. Combining the two cohorts increased the statistical significance ($P<0.01$). Consistent with our previous results (8), total α-synuclein CSF concentrations did not correlate with PD severity in either cohort. Furthermore, including total α-synuclein or the ratio of PS-129 to total α-synuclein in the calculation did not improve the performance of PS-129 as a marker of PD severity. No statistical significance was detected when PS-129 concentrations in CSF were correlated with Hoehn-Yahr stages (another measure of clinical PD severity) or disease duration ($p>0.05$). This is not entirely surprising because the Hoehn-Yahr scale is a categorical measure in which a wide range of impairment severities is collapsed together into five stages, and disease duration is only partially correlated with disease severity and progression. In addition, the substantial variability in PS-129 values of PD patients at a given stage may also be a contributory factor. A better test of a potential progression marker will be to study within-person changes over time when longitudinal data are analyzed.

Discussion

The concentrations of total α-synuclein in CSF are reported to be reduced in patients with PD compared to healthy controls in several recent studies (7-9, 16, 17). The effects were more marked in studies that controlled for blood contamination and that had a large cohort of PD patients (7-9). However, comparable decreases in total α-synuclein in CSF in PD versus MSA patients have also been reported (7-9). Thus, total α-synuclein in CSF is unlikely to be useful in differentiating clinically overlapping parkinsonian disorders, such as MSA and PSP, from PD regardless of whether these disorders are caused by synucleinopathy (PD, dementia with Lewy bodies, and MSA) or tauopathy (PSP) (18, 19). Here, we have shown decreases in CSF PS-129, but no significant changes in CSF total α-synuclein in PSP patients (Fig. 2 and table S1), which may be helpful for the differential diagnosis of PSP from PD (Fig. 3 and tables S2 to S4). Neither PS-129 nor total α-synuclein concentrations in CSF were altered in AD relative to healthy controls (AD is also a tauopathy). Assessing both CSF PS-129 and total α-synuclein may enhance the accuracy of differential diagnosis among overlapping parkinsonian disorders, thereby improving the diagnostic classification, especially at early stages of disease. Similar results based on random split samples were also obtained when analyzed using only the samples from the Oregon/Veterans Affairs Puget Sound–University of Washington or the German cohort that has control and diseased subjects across multiple classes (that is, site-specific discovery/validation). However, because most sensitivity values are modest due to the overlap of CSF

PS-129 or total α-synuclein values between PD and healthy control subjects or between PD and other parkinsonian disorders, combining CSF PS-129 or CSF total α-synuclein with other CSF markers identified recently such as Flt3 ligand and tau (9), may be needed to enhance their diagnostic value. It should also be mentioned that the diagnostic accuracy results presented for the two-marker combination on the discovery set might be optimistically biased due to the fact that the linear combination of the marker values was obtained by fitting a logistic regression to the same data. Thus, greater weight should be placed on the validation data that were obtained when the model (derived completely from the discovery set) was applied to the validation set.

We also show that CSF total α-synuclein did not correlate with PD severity (8), probably due to a "floor" effect, that is, CSF α-synuclein concentrations decrease to a certain level due to disease, but do not decrease further during disease progression. In contrast, we report that CSF PS-129, a modified form of α-synuclein that is intimately associated with PD pathogenesis, weakly correlates with UPDRS scores of PD severity (Fig. 4). We recently reported that fractalkine, complement 3, and factor H, when combined with amyloid β (Aβ), correlate with PD severity and progression (9), but it is not clear how these markers are involved with PD pathogenesis. Consequently, the most meaningful applications of these inflammatory markers might be for monitoring PD drugs that modulate neuroinflammation or complement activation/regulation. On the other hand, PS-129 has been implicated in α-synuclein aggregation, a hallmark of PD pathogenesis. Thus, the ability to monitor PS-129 concentrations in a clinically accessible body fluid such as CSF from a given subject may provide an objective way to assess mechanisms of action of new drugs that influence this pathway.

The correlation between PS-129 concentrations in the CSF of PD patients and UPDRS scores, although statistically significant, is weak and may not be robust enough for clinical application. However, as we discussed in a previous study (9), there are several reasons why the correlation between the clinical evaluation (UPDRS) and a CSF marker may be weak. First, it is unknown whether a strong linear relationship exists between worsening UPDRS scores and progressive degeneration of the nigrostriatal system; compensation for loss of nigrostriatal dopaminergic neurons by increasing the levels of dopaminergic terminals or D-2 receptors per cell in the basal ganglia, which may complicate the relationship between α-synuclein pathology and the severity of PD motor symptoms. Certain biochemical markers, on the other hand, may more accurately reflect nigrostriatal degeneration. Also, as PD advances, a number of brain regions other than the nigrostriatal system are affected (1). Unlike the UPDRS, CSF biomarkers usually reflect pathology in the whole brain instead of specifically in the nigrostriatal dopaminergic system. Finally, in a treated patient, the clinical examination is often confounded by the masking effects of dopaminergic therapy; such drugs may not affect CSF protein concentrations (8, 9). It should be emphasized that despite our rigorous control for potential sources of bias and confounding factors, our results need to be validated in a prospectively planned, independent cohort, particularly in samples collected longitudinally.

In summary, we have identified and developed an assay to measure PS-129 in human CSF. When combined with total α-synuclein concentrations in CSF, PS-129 in CSF may be helpful for the differential diagnosis of clinically overlapping parkinsonian disorders. We also showed that CSF PS-129 concentrations significantly, although weakly, correlated with PD severity. These results require replication, ideally in independent cohorts of PD patients where disease has been longitudinally assessed and pathologically confirmed.

Materials and Methods

Demographics of Subjects

This study was approved by the Institutional Review Boards of all participating sites. One hundred and twenty samples (60 PD, 19 AD, and 41 controls) were collected at the Oregon Health and Science University; 194 samples (46 PD, 55 AD, 2 PSP, and 91 controls) were collected at the Veterans Affairs Puget Sound Health Care System and University of Washington School of Medicine; 31 PD samples were collected at the Baylor College of Medicine; 39 control samples were collected at the University of California at San Diego; 38 samples (5 PD, 32 MSA, and 1 control) were collected at the Clinical Neurocardiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health; 55 PD samples were collected at the University of Pennsylvania School of Medicine; 26 samples (1 PD, 9 MSA, 1 PSP, 2 AD, and 13 controls) were collected at the University of Texas Health Science Center at Houston; 11 PSP samples were collected at the University of Louisville School of Medicine; and 76 samples (11 PD, 46 PSP, and 19 controls) were collected at the University of Tuebingen, Germany (table S5). Similar CSF collecting protocols and quality control procedures were followed at all nine participating centers, in particular, use of polypropylene collection and storage tubes, rapid separation into single use aliquots, and freezing of CSF samples, to minimize potential site variations. No significant difference of CSF PS-129 levels in PD or control was identified among different sites, and no significant correlation was identified between PS-129 levels and sample storage time (fig. S7).

The subjects were randomly split into a discovery set, including a total of 246 subjects with a wide range of neurological disorders (93 PD, 26 AD, 16 MSA, and 33 PSP cases) and 78 normal controls, and a validation set with a total of 344 subjects (126 normal controls, 116 PD, 50 AD, 25 MSA, and 27 PSP cases). All CSF samples were obtained after informed consent from patients, and all patients underwent evaluations consisting of medical history, physical and neurological examinations, laboratory tests, and neuropsychological assessments. The inclusion and exclusion criteria for normal controls and patients with PD, AD, and MSA were described previously (8, 15). Briefly, all PD patients met UK PD Society Brain Bank clinical diagnostic criteria for PD (20), and the patients were staged by the UPDRS (21). Patients with AD and MSA were also diagnosed according to generally accepted clinical criteria (22-24). Control subjects were community volunteers in good health (many of them are the patients' spouses). They had no signs or symptoms suggesting cognitive impairment or neurological disease; all subjects had a Mini Mental Status Examination (MMSE) score between 28 and 30, a Clinical Dementia Rating (CDR) score of 0, and New York University paragraph recall scores (immediate and delayed) of >6. Demographic information is listed in table S1 and S5 for all subjects/patients.

For diagnosing patients with possible or probable PSP, the criteria described by Litvan *et al* (25) were followed. PSP subtypes such as PSP-parkinsonism (PSP-P) and pure akinesia with gait freezing (PAGF) were also included; for these subjects, we did not apply the criterion "in the first year of the disease" because these patients often present with classical PSP symptoms at later disease stages (26, 27). A similar inclusion procedure has recently been used by others (28). All included patients provided medical history for at least 2 years from the first manifestation of symptoms.

Collection of CSF sample and quality control

All CSF samples were obtained by lumbar puncture in the morning. Up to 25 ml of CSF was taken from each subject, with every 5 ml pooled into one fraction. Samples were divided into single use aliquots and immediately frozen on dry ice and then stored at -80°C. Before

analysis, all CSF samples were thawed only once when 10% (10 μl/100 μl of CSF) protease inhibitor cocktail (Sigma, prepared in 10 ml of H_2O) was added and samples were further divided into single use aliquots. The details are described in our previous article (8). The hemoglobin concentrations in CSF samples were chosen as an index of the degree of red blood cell contamination and were measured as previously described (8).

Protein enrichment and MS

IP was performed to enrich PS-129 from 40 ml of pooled reference CSF with a rabbit anti-PS-129 antibody (Abcam) and with an ExactaCruz IP kit (Santa Cruz Biotechnology) according to the manufacturer's instructions. The bound proteins were eluted from the IP matrix with 0.1% trifluoroacetic acid (TFA), and then dried down and re-dissolved with 15 μl of 0.5% TFA. The efficiency of the enrichment was about 75% (Fig. 1E) and the procedure was replicated three times. Samples were centrifuged and 1 μl of supernatant was mixed with 1 μl of CHCA (α-cyano-4-hydroxycinnamic acid, 7 mg/ml) matrix. PS-129 was revealed with linear model MS on a 4800 matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometer (Applied Biosystems). For further protein identification, the enriched proteins were digested sequentially with trypsin and endoproteinase Asp-N for 16 hours each. The digest was desalted with an Oasis MCX cartridge (1 cm^3/30mg, 30μm; Waters) and run with the 4800 mass spectrometer as described (29). A list of identified peptides can be found in table S6.

Preparation of recombinant PS-129 protein

Human recombinant phosphorylated α-synuclein protein was prepared as described previously (30). Briefly, recombinant α-synuclein was phosphorylated by casein kinase 2 (New England Biolabs) and unphosphorylated proteins were separated by ion-exchange chromatography (Amersham Biosciences/GE Healthcare). Fractionated samples were analyzed by immunoblotting with an anti-PS-129 antibody and MS (fig. S4). The PS-129-positive fractions were concentrated by ammonium sulfate precipitation.

Bead-based Luminex assays

CSF PS-129 concentrations were measured with a modified Luminex assay based on the total α-synuclein assay (8) (see fig. S6 for a calibration curve). In brief, 75 μl CSF was diluted with 25 μl assay diluent (0.1% bovine serum albumin/phosphate buffered saline) and then incubated with capturing antibody ASY-1 [an antibody recognizing epitopes within the last 45 amino acids of α-synuclein (31)]-coupled beads overnight (~18 hours, 4 °C) at 600 rpm on a plate shaker. After the sample solution was removed, the biotinylated anti-human PS-129 antibody (2 ng/μl) (32) was added at 50 μl (100 ng)/well and incubated for 3 hours on a plate shaker (600 rpm) at room temperature. Fifty microliters (100 ng) per well of diluted streptavidin-R-phycoerythrin (Prozyme) in assay diluent was then added and incubated for 0.5 hour on a plate shaker (600 rpm) at room temperature. All samples were analyzed with a LiquiChip Luminex 200™ Workstation (Qiagen). The intra-assay variation (determined by measuring replicates of multiple samples in the same run/plate) was <10.2% and the inter-assay precision (run to run or plate to plate, determined with an internal standard – an aliquot of a pooled reference CSF sample with known PS-129 concentration – in each plate) was <19.4%. Measurement of total α-synuclein was performed as previously described (8).

Statistical analysis

All analyses were performed with Prism 4.0 (Graphpad) and PASW 18.0 (SPSS/IBM). Linear regression analysis was used to determine the relationships between age, hemoglobin, and PS-129. One-way analysis of variance (ANOVA) followed by the Tukey test was used

to compare differences between groups. Additionally, relationships between the analytes and hemoglobin, age, sex, and UPDRS motor score were analyzed with bivariate correlation using Pearson's correlation coefficients. Values with $p<0.05$ were regarded as significant. ROC curves were used to calculate the relationship between sensitivity and specificity for each disease group (PD, MSA, or PSP) versus each of the other groups (healthy control or other disease groups; the analyses involving AD were performed but the results are not shown), and hence to evaluate the diagnostic and differential diagnostic performance of the analytes. The "optimum" cutoff value from the ROC curve is determined by anchoring the specificity (or sensitivity) to be ≥80%. This was done for both PS-129 and total α-synuclein, as well as the ratio of PS-129/total α-synuclein. Logistic regression was used to determine the best linear combination of PS-129 and total α-synuclein levels for predicting disease status (versus healthy or diseased controls), followed by ROC analysis on the linear combination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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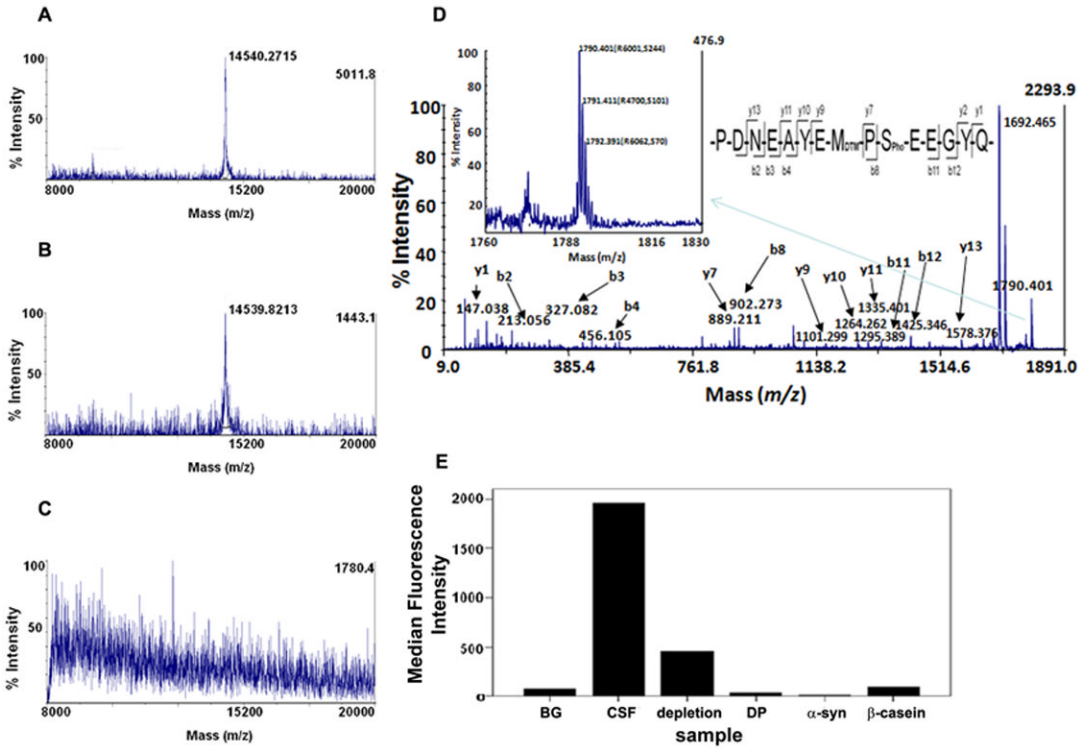

Fig 1. Identification and quantification of PS-129 in human CSF

Phosphorylated α-synuclein at Ser^{129} (PS-129) was detected in human CSF using linear and tandem MS. (**A** to **C**) Linear mode spectra of intact proteins are shown for (A) PS-129 standard (0.5 ng) at a mass of 14,540; (B) candidate PS-129 in CSF after IP with a rabbit anti-PS-129 antibody; and (C) absence of PS-129 after IP with normal rabbit immunoglobulin G. (**D**) The digestion of the eluate from IP (with anti-PS-129 antibody) revealed two peptides with PS-129 phosphorylation (see also fig. S5). Phosphorylation of Ser^{129} was also validated by manual mass match. The dethiomethyl modification of Met^{127} is likely a sample processing artifact, and the loss of N-terminal Asp in the peptide shown is probably due to the acid lability of the Asp-Pro bond, both of which are known artifacts in proteomics analyses. (**E**) The Luminex assay specificity was confirmed by several methods including using pre-depletion of PS-129 from CSF with an anti-PS-129 antibody, de-phosphorylation (DP) of CSF proteins using calf intestine alkaline phosphatase, non-phosphorylated α-synuclein standard (α-Syn), and a non-specific phosphorylated protein β-casein as negative controls. BG, background.

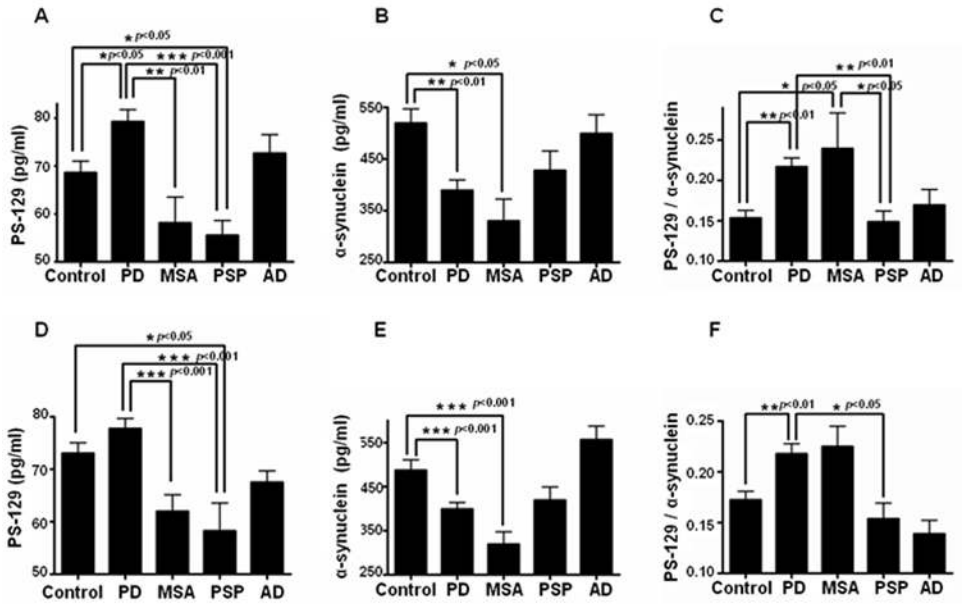

Fig 2. PS-129 in the CSF of parkinsonian patients

(**A** to **F**) PS-129 concentrations in CSF were measured in the discovery set [(A) PS-129, (B) total α-synuclein, (C) ratio of PS-129 to α-synuclein] and validation set [(D) PS-129, (E) total α-synuclein, (F) ratio of PS-129 to α-synuclein). Subjects younger than 50 years were excluded. Comparisons of the average concentration of PS-129 in the CSF of healthy controls, PD patients, MSA patients, PSP patients, and AD patients were performed using one-way ANOVA. Case numbers included in each group can be found in table S1. Data are mean ± S.E. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (Tukey/ANOVA). Only the most relevant statistical significance is indicated; see table S1 for a complete report of all group comparisons.

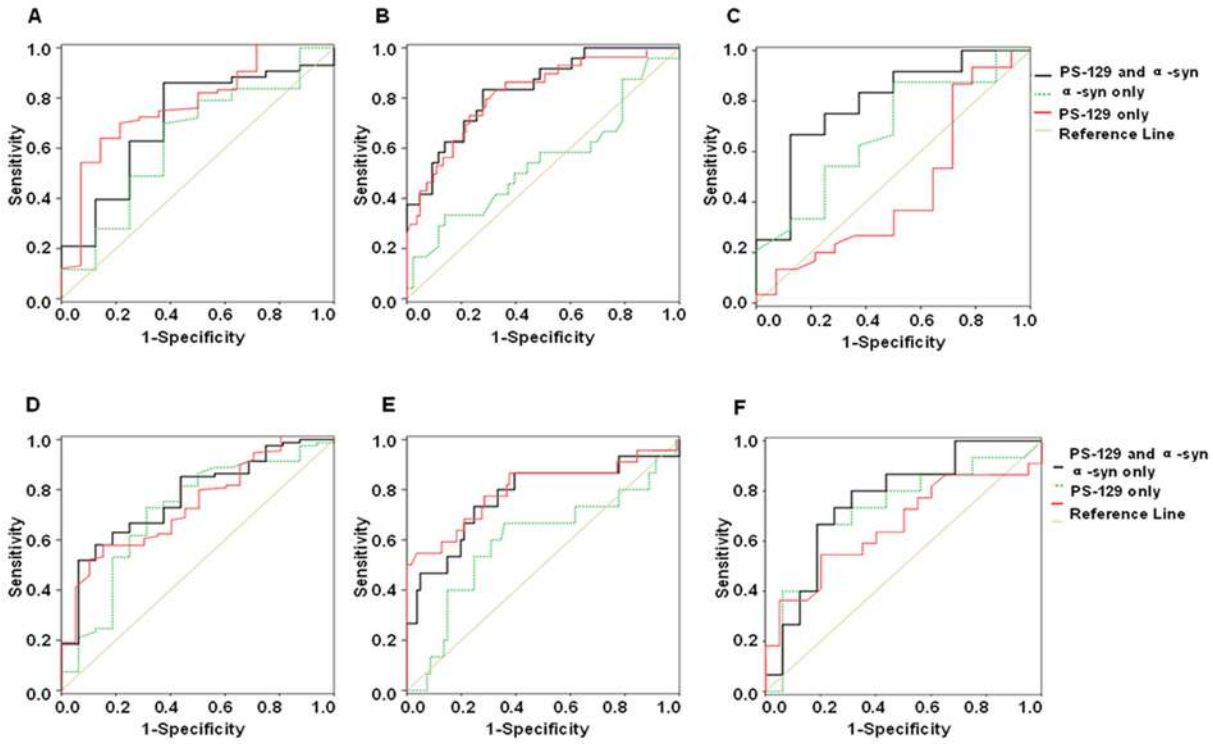

Fig 3. ROC analysis of CSF PS-129, total α-synuclein and their combination

PS-129 and total α-synuclein concentrations in CSF were measured by Luminex assays in the discovery and validation patient cohorts. ROC analysis was performed to evaluate the diagnostic values (sensitivity and specificity) of PS-129 alone, total α-synuclein alone, and the combination of total α-synuclein and PS-129 (in a logistic regression model) for PD diagnosis (not shown) and differential diagnosis between PD and other parkinsonian disorders (MSA and PSP). (**A** to **C**) Discovery set: (A) PD versus MSA, (B) PD versus PSP, (C) MSA versus PSP. (**D** to **F**) Validation set: (D) PD versus MSA, (E) PD versus PSP, (F) MSA versus PSP. Subjects younger than 50 were excluded from all analyses and samples with high blood contamination (hemoglobin ≥200 ng/ml) were excluded when total α-synuclein was measured. The diagnostic values in the discovery set when specificities or sensitivities were anchored to be ≥80% can be found in tables S2 and S3, respectively. The diagnostic values for the combination in the validation set when the models and cutoff values generated from the discovery set were applied into the validation set can be found in table S4.

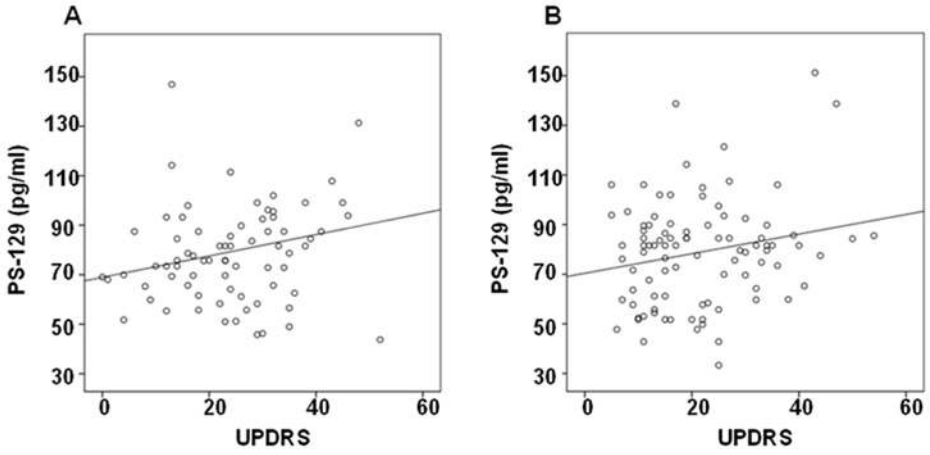

Fig 4. Correlation of PS-129 with UPDRS

PS-129 concentrations in the CSF of patients with PD were measured by a Luminex assay and plotted against PD severity measured by UPDRS motor scores. (**A** and **B**) The correlation between CSF PS-129 concentrations and UPDRS scores was evaluated using linear regression analysis with Pearson's correlation in the discovery set [(A) r=0.23, $P<0.05$], the validation set [(B) r=0.21, $p<0.05$], and the two sets combined (not shown; r=0.22, $P<0.01$).