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Decreased α-synuclein in cerebrospinal fluid of aged individuals and subjects with Parkinson's disease

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Abstract

There is ample biochemical, pathological, and genetic evidence that the metabolism of α-synuclein (α-syn) plays a crucial role in the pathogenesis of Parkinson disease (PD). To examine whether quantification of α-syn in cerebrospinal fluid (CSF) is potentially informative in the diagnosis of PD, we developed a specific ELISA system and measured the concentration of α-syn in CSF from 33 patients with PD (diagnosed according to UK PD Society Brain Bank criteria) and 38 control subjects including 9 neurologically healthy individuals. We found that PD patients had significantly lower α-syn levels in their CSF than the control groups ($p < 0.0001$) even after adjusting for gender and age. Age was independently associated with lower α-syn levels. Logistic regression analysis showed that reduction in CSF α-syn served as a significant predictor of PD beyond age and gender alone (area under ROC curve, $c = 0.882$). Furthermore, we observed a close inverse correlation between α-syn levels in CSF and assigned Hoehn and Yahr score in this cohort of 71 living subjects ($p < 0.0001$), even after adjusting for age. These findings identify in the quantification of α-syn from CSF a potential laboratory marker to aid the clinical diagnosis of PD.

Keywords: Parkinson disease; Neurodegenerative diseases; α-Synuclein; Cerebrospinal fluid; ELISA; Biomarker; Diagnosis

Idiopathic Parkinson disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. It is characterized pathologically by selective cell loss, astrocytic gliosis and the presence of Lewy neurites as well as Lewy bodies in surviving cells. These pathognomonic changes represent intraneuronal inclusions found in affected brain regions. The major constituent of Lewy bodies has been identified as α-synuclein (α-syn), a lipophilic phosphoprotein [1]. Missense mutations in the gene encoding α-syn (*SNCA*) have been reported to be associated with rare familial PD [2–4]. Furthermore, genetic studies have revealed that rare triplication events of the *SNCA* gene can be associated with severe forms of young-onset, familial PD that also feature dementia with Lewy body-type changes [5]; in turn, duplication mutations cause a familial PD phenotype that resembles more late-onset, idiopathic PD [6,7]. These collective studies indicate that an increased expression rate of wild-type α-syn enhances the risk of developing PD [8], and suggests that the protein level of wild-type α-syn might be an important determinant of the severity of the parkinsonian phenotype. These results also suggested that α-syn could serve as a marker candidate

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for idiopathic PD, in which insoluble, wild-type α-syn accumulates in the brain.

α-Syn is mainly expressed by neuronal cells, and is generally considered to exist as a cytoplasmic and lipid vesicle-associated protein. However, recently we have shown that neural cells also release α-syn into their conditioned culture medium, and that α-syn can be found in CSF and peripheral plasma [9]. Our findings therefore suggested that cells constitutively release α-syn into the extracellular space, both *in vitro* and *in vivo*, by an as of yet unclear mechanism [10]. We hypothesized that the quantification of extracellular α-syn in body fluids may offer an opportunity for the development of diagnostic tests for PD and related diseases [9]. However, our previously employed WB method was not quantitative and sensitive enough to measure accurate protein concentrations, especially at very low levels, that we have observed in human CSF. Therefore, we developed a sensitive, sandwich-type enzyme-linked immunoabsorbent assay (ELISA), which measures α-syn at lower protein concentrations than can be routinely detected by SDS/PAGE and WB, and then we applied this method to monitor the level of α-syn in CSF from PD patients and control cases.

Experimental procedure

CSF samples. CSF samples were obtained from 33 patients with idiopathic PD (ages 43–79, mean ± SD = 63.4 ± 11.1) and 38 control patients (ages 15–82, mean ± SD = 47.4 ± 17.9). The control subjects comprised neurologically normal individuals ($n = 9$) and cases with mild cognitive impairment ($n = 5$), myopathy ($n = 4$), cranial nerve dysfunction ($n = 4$), peripheral neuropathy ($n = 8$), motor neuron disease ($n = 1$) and cerebellar ataxia ($n = 2$), psychogenic movement disorder ($n = 3$), and myelopathy ($n = 2$). All study subjects provided written informed consent to participate, which was approved by the University Ethics Committee (Kyoto Prefectural University, Kyoto, Japan). The study procedures were designed and performed in accordance with the Declaration of Helsinki. Fresh CSF samples were collected from living PD and control cases, and a cocktail of protease inhibitors (Calbiochem-Novabiochem Corporation, San Diego, USA) was added to the samples. The CSF was cleared by centrifugation at 3000*g* for 10 min at 4 °C. The supernatant was collected and then stored at −80 °C until used for the ELISA.

An ELISA to measure α-syn levels in CSF. α-Syn in CSF was measured using a sandwich ELISA system. There an anti-human α-syn monoclonal antibody 211 (Santa Cruz Biotechnology, USA), which recognizes amino acid 121–125 of human α-syn, was used for capturing and anti-human α-syn polyclonal antibody FL-140 (Santa Cruz Biotechnology, USA), raised against recombinant full-length human α-syn, was used for antigen detection through a horseradish peroxidase (HRP)-linked spectrometric assay [11]. To eliminate inter-assay variability as a confounding factor, all samples were run in duplicate on the same day with the same lot of standards. Each ELISA plate (Nunc Maxisorb, NUNC, Denmark) was coated during 8 h of incubation with 1 μg/ml of 211 (100 μl/well), in 200 mM $NaHCO_3$ (Sigma–Aldrich), pH 9.6, containing 0.02% (w/v) sodium azide at 4 °C, washed four times with PBST (PBS containing 0.05% Tween 20) and incubated with 200 μl/well of blocking buffer (PBS containing 2.5% gelatin and 0.05% Tween 20) for 2 h at 37 °C. The plate was washed four times with PBST, and 100 μl of the CSF samples (concentrated from 500 μl CSF by vacuum centrifugation) were added to each well and the plate was incubated at 37 °C for 3 h. After washing four times with PBST, 100 μl FL-140 antibody (0.2 mg/ml) diluted to 1:1000 in blocking buffer was added, and incubated at 37 °C for 2 h. The wells were washed four times with PBST and incubated with 100 μl/well of horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulins (DAKO, Denmark) and incubated for 1 h at 37 °C. Bound HRP activities were assayed by color development using the TMB microwell peroxidase system (KPL, USA). The reaction of HRP was stopped with 0.3 M sulfuric acid (100 μl/well), and absorbance at 450 nm was measured with a microplate reader (SpectraMax Plus384, Molecular Devices, Tokyo). The standard curve for the ELISA was carried out using 100 μl/well of recombinant human α-syn (Calbiochem, USA) solution at different concentrations (0.002, 0.005, 0.01, 0.025, 0.05, 0.1, 0.3, 0.6, 1.0, 1.5, and 2.0 μg/ml) of the protein in PBS (Fig. 1). Also, different concentrations of recombinant human β-syn and γ-syn (Alpha Diagnostic International) were included as negative controls for the assay. The relative concentration estimates of CSF α-syn were calculated according to each standard curve and the dilution factor. The intra-assay and inter-assay precision was <9%.

Statistical analysis. Statistical analyses were performed using Pearson correlation and partial correlation coefficients, *T*-tests, multiple linear, and logistic regression analyses, analysis of covariance and ROC plots. All analyses were carried out using SAS statistical software (SAS Institute, Inc., Cary, NC, Version 9.1.3, 2006).

Results

Fig. 1 shows the standard curves obtained with our ELISA system, demonstrating that this method detected human α-syn with high specificity and sensitivity (14 fmol/well) without any cross-reactivity to human β-syn or γ-syn. The levels of α-syn were log transformed in order to better meet assumptions in significance tests (i.e., homogeneity of group variances, normally distributed residuals with homogeneous variance, and homogeneity of regression line slopes for analysis of covariance). An analysis of covariance test was also performed including diagnostic groups (PD versus controls) and gender as factors, their interaction, and age as a covariate, with Log CSF α-syn as the dependent variable.

The analysis of covariance indicated that levels of log α-syn declined significantly with age within the CSF from both the diagnostic and gender groups ($p = 0.0076$; Fig. 2A). This decline was linear and was observed to have a similar slope in the control and PD groups, as determined by the regression lines of log α-syn on age (Fig. 2B). Note, no significant interactions of age with diagnosis or gender

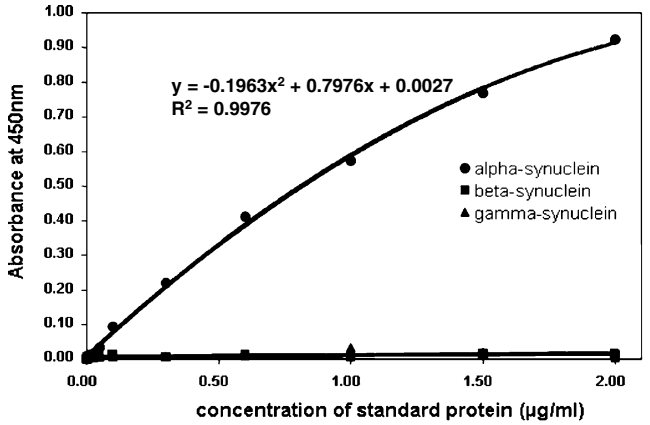

Fig. 1. Standard curves of the sandwich-ELISA. Specificities and sensitivities for human α- (●), β- (■) and γ-syn (▲) in the sandwich-ELISA we used.

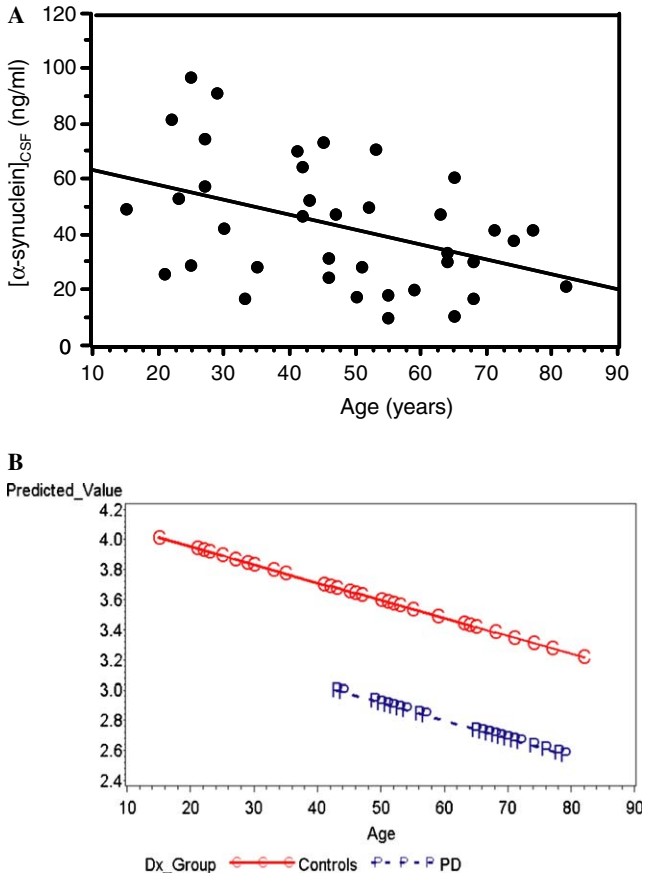

Fig. 2. (A) Scatter plot of age versus CSF α-syn in the control patient groups, as measured by sandwich ELISA. The concentration of α-syn was significantly reduced with aging. (B) Predicted values of log CSF α-syn (ng/ml) versus age, by diagnostic group (44 percent of variance accounted for; see text for details).

were observed in the analysis of covariance test; however, the correlation of log α-syn levels with age within the PD group was not significant, because of the more restricted age range of the PD group (Fig. 2B). The analysis of covariance test further indicated that neither gender nor the interaction between gender and PD were significantly associated with log α-syn levels, but the diagnosis of PD was ($p < 0.0001$). Means adjusted for the gender imbalances and for age were 3.55 (control groups) and 2.88 (PD group; Fig. 2B). When the non-significant gender effects were removed, diagnostic category and age each remained significant, holding each other constant ($p < 0.0001$ and $p = 0.007$, respectively) with 44% of the variance of log α-syn accounted for by the model (18% uniquely by diagnostic group, 6% by age, and 20% unseparable; data not shown). With mean α-syn in the PD group being 18.16 ng/ml, the corresponding mean CSF levels in the control patients was almost twofold higher (Fig. 3A).

Multiple logistic regression analysis of diagnosis on age, gender, and log α-syn levels showed gender not to be a significant predictor. Removing gender showed significant prediction by the model as a whole ($p < 0.0001$) with log α-syn levels and age each contributing significant prediction beyond that of the other ($p = 0.0003/\beta = -2.38$ and $p = 0.045/\beta = 0.047$, respectively) (Fig. 3B). The area under the ROC curve (AUC) indicated high sensitivity and specificity for reduced log CSF levels of α-syn in clinically diagnosed PD cases (AUC=0.874). When residuals of log α-syn levels (regressed on age, thereby holding age constant) were entered alone as a predictor in the logistic regression equation, it provided significant prediction ($p < 0.0001$) and an AUC of 0.815 (Fig. 3C). Since normality is not a required assumption for logistic regression, the above analyses were rerun with untransformed CSF α-syn values; the results were virtually identical (not shown).

Intriguingly, the clinically assigned Hoehn Yahr (HY) stages in our PD cohort were also related to CSF α-syn levels. The partial correlation of HY with log α-syn levels within the PD group was $r = -0.62$ ($p < 0.0002$). Age and HY together accounted for 41% of the variance of log CSF α-syn levels among PD subjects, but HY alone accounted for 37% of the variance; the variance of age was not significant. When subjects in the control cohort were assigned HY scores of "0" (consistent with the absence of any parkinsonian signs clinically) and pooled with the PD group, multiple regression analyses revealed significantly negative relations to log CSF α-syn levels holding each other constant. HY was more significant than age ($p < 0.0001$ versus $p = 0.034$) and accounted for more unique variance (26% versus 3%); 52% accounted by both (Fig. 4).

Discussion

To our knowledge, this is the first study to report significantly decreased concentrations of CSF α-syn in both aged individuals and in patients with PD compared with those of age-matched controls. To date, there have been very few studies investigating the protein level of α-syn in human CSF [9,12,13] because until recently α-syn has been considered to be a purely intracellular protein [14,15]. However, we and others have recently detected α-syn in human CSF by WB analysis of proteins enriched by immunoprecipitation [9,13]. In these studies, the immunoreactivities of α-syn did not differ significantly in PD and normal cases (in a small cohort comprising $n = 5$ subjects), but these methods were semi-quantitative. Here, we processed CSF samples with specific attention to protein stability, including the immediate addition of protease inhibitors to prevent degradation by CSF proteases, such as by neurosin (human kallikrein-6) [16] that can degrade α-syn [17], and employed a human isoform-specific and quantitative ELISA system to monitor α-syn levels.

Quantifiable levels of select proteins found in human CSF will reflect the concentration in the interstitial, extracellular space of the human brain, if these polypeptides are of principally neural origin. Another possible source for α-syn reactivity in the CSF represents normal diffusion through the brain parenchyma as a result of arterial blood

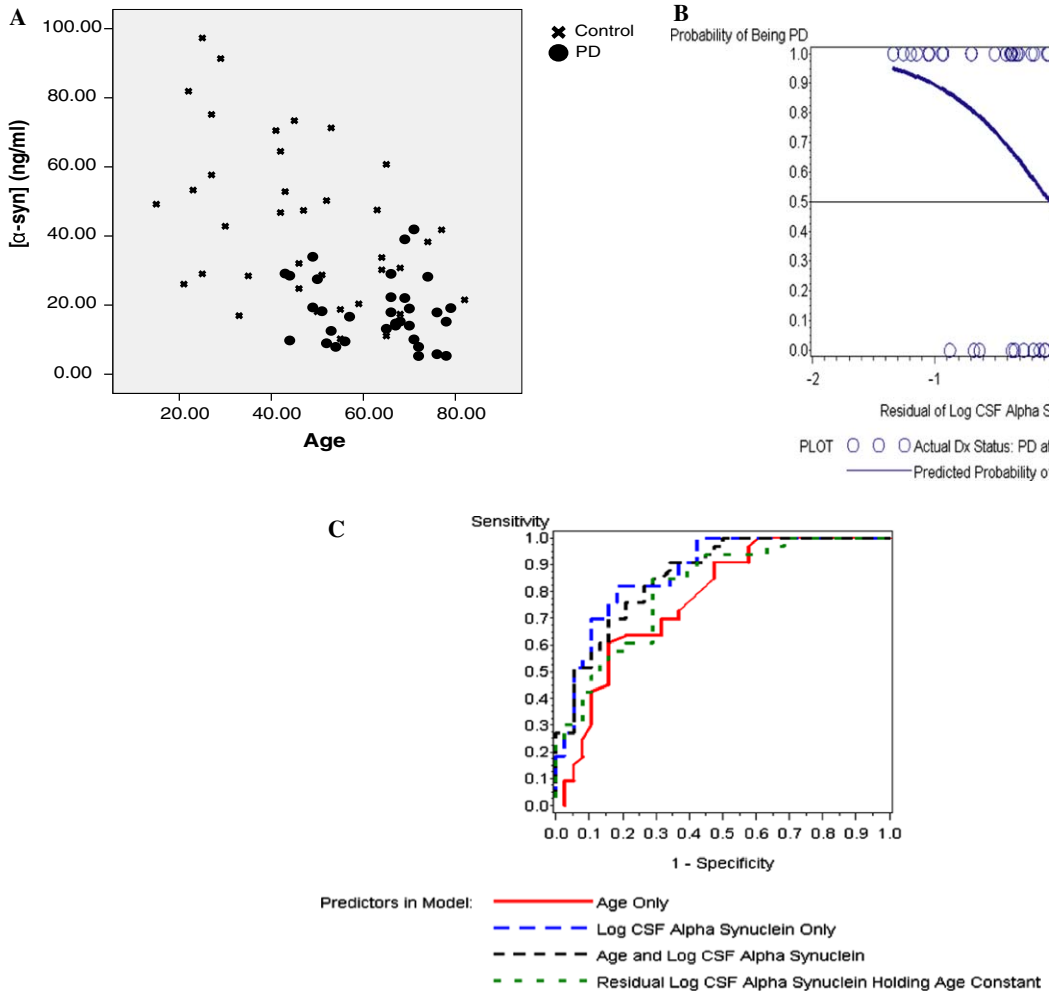

Fig. 3. (A) Scatter plot of the concentrations of α-syn in CSF in the PD (●) and control (*x*) groups, as measured by sandwich ELISA. (B) Plot of predicted probability of being a PD patient as opposed to control subject (for selected subpopulation) versus residuals of log transformed CSF α-syn (holding age constant) based on logistic regression of probability assessment. (C) ROC curves based on logistic regression analyses for the classification of Parkinson disease patients versus control subjects based on various predictors and combination of predictors.

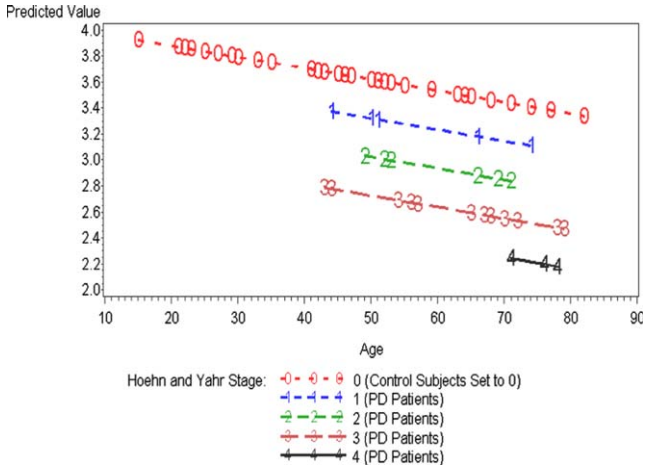

Fig. 4. Predicted values of log CSF α-syn (ng/ml) versus age, by Hoehn and Yahr score (52 percent of variance accounted for; see text for details).

flow to the brain, or in the case of a blood–brain barrier breakdown, extravasation of blood products. If extracellular α-syn in CSF arises from the central nervous system (rather than a peripheral source) and if future reports confirm our findings, the observed reduction in CSF α-syn in PD subjects could be due to its intracellular aggregation (or sequestration) and subsequent accumulation within affected neurons [1].

Alternate explanations for decreased CSF levels of α-syn in sporadic PD are: one, a pathological reduction in the functional activity of those neurons that normally release α-syn, (i.e., a decline beyond that normally associated with the ageing process; see Fig. 2b); and two, a reduced rate of *SNCA* gene expression in still healthy cells in response to a PD-linked signaling event that leads to a subsequent decline in α-syn exocytosis. Our finding that CSF α-syn levels are decreased in clinically diagnosed probable PD subjects shows parallels with the growing literature of reduced concentrations of amyloid β-peptide ($A\beta_{1-42}$) in the CSF of patients with moderate to severe Alzheimer's disease [18] and the dysregulation of tau protein in CSF from subjects with Alzheimer's disease and frontotemporal dementia when compared with control subjects [18–20].

We recently found that the levels of α-syn oligomers which represent a fraction of the total pool of detectable α-syn reactivity in peripheral blood and human brain [11] were elevated in both plasma and post-mortem CSF specimens from PD patients when compared with control samples [11]. In contrast, in this study, we have used an assay to monitor the total pool of detectable α-syn in CSF from living patients. Future studies will address whether CSF steady-state levels reflect more the pool of brain α-syn (as would be expected), or are in equilibrium with the peripheral pool of α-syn proteins. In the case of plasma α-syn reactivity, it is likely that the bulk of the protein originates from a peripheral source(s) (e.g., corpuscular elements of whole blood) [21,22] or from peripheral organs (e.g., liver), as the overall detectable level of α-syn is markedly higher in human blood than in CSF (El-Agnaf, et al., submitted for publication).

Taken together, all of these findings suggest that quantification of total and oligomeric forms of α-syn in CSF and peripheral plasma (or both) has potential value as a diagnostic laboratory tool for patients with suspected PD. Large-scale, prospective, and well-controlled studies, especially those that include subjects with neuroimaging-supported, definite PD and autopsy-confirmed synucleinopathy, are necessary to validate the use of α-syn quantification as an urgently needed surrogate (or biomarker) [9,11 23,24].

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References

[1] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, α-Synuclein in Lewy bodies, Nature 388 (1997) 839–840.
[2] R. Kruger, W. Kuhn, T. Muller, et al., Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease, Nat. Genet. 18 (1998) 106–108.
[3] M.H. Polymeropoulos, C. Lavedan, E. Leroy, et al., Mutation in the α-synuclein gene identified in families with Parkinson's disease, Science 276 (1997) 2045–2047.
[4] J.J. Zarranz, J. Alegre, J.C. Gomez-Esteban, et al., The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia, Ann. Neurol. 55 (2004) 153–156.
[5] A.B. Singleton, M. Farrer, J. Johnson, et al., α-Synuclein locus triplication causes Parkinson's disease, Science 302 (2003) 841.
[6] M.C. Chartier-Harlin, J. Kachergus, C. Roumier, et al., α-Synuclein locus duplication as a cause of familial Parkinson's disease, Lancet 364 (2004) 1167–1169.
[7] P. Ibanez, A.M. Bonnet, B. Debarges, et al., Causal relation between α-synuclein gene duplication and familial Parkinson's disease, Lancet 364 (2004) 1169–1171.
[8] P. Pals, S. Lincoln, J. Manning, et al., α-Synuclein promoter confers susceptibility to Parkinson's disease, Ann. Neurol. 56 (2004) 591–595.
[9] O.M. El-Agnaf, S.A. Salem, K.E. Paleologou, et al., α-Synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma, FASEB J. 17 (2003) 1945–1947.
[10] H.J. Lee, S. Patel, S.J. Lee, Intravesicular localization and exocytosis of α-synuclein and its aggregates, J. Neurosci. 25 (2005) 6016–6024.
[11] O.M. El-Agnaf, S.A. Salem, K.E. Paleologou, et al., Detection of oligomeric forms of α-synuclein protein in human plasma as a potential biomarker for Parkinson's disease, FASEB J. 20 (2006) 419–425.
[12] M.W. Jakowec, G.M. Petzinger, S. Sastry, D.M. Donaldson, A. McCormack, J.W. Langston, The native form of α-synuclein is not found in the cerebrospinal fluid of patients with Parkinson's disease or normal controls, Neurosci. Lett. 253 (1998) 13–16.
[13] R. Borghi, R. Marchese, A. Negro, et al., Full length α-synuclein is present in cerebrospinal fluid from Parkinson's disease and normal subjects, Neurosci. Lett. 287 (2000) 65–67.
[14] A. Iwai, E. Masliah, M. Yoshimoto, et al., The precursor protein of non-A β component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system, Neuron 14 (1995) 467–475.
[15] R. Jakes, M.G. Spillantini, M. Goedert, Identification of two distinct synucleins from human brain, FEBS Lett. 345 (1994) 27–32.
[16] A. Okui, K. Kominami, H. Uemura, S. Mitsui, N. Yamaguchi, Characterization of a brain-related serine protease, neurosin (human kaillikrein 6), in human cerebrospinal fluid, Neuroreport 12 (2001) 1345–1350.
[17] A. Iwata, M. Maruyama, T. Akagi, et al., α-Synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies, Hum. Mol. Genet. 12 (2003) 2625–2635.
[18] C.M. Clark, S. Xie, J. Chittams, et al., Cerebrospinal fluid tau and β-amyloid: how well do these biomarkers reflect autopsy-confirmed dementia diagnoses? Arch. Neurol. 60 (2003) 1696–1702.
[19] M. Grossman, J. Farmer, S. Leight, et al., Cerebrospinal fluid profile in frontotemporal dementia and Alzheimer's disease, Ann. Neurol. 57 (2005) 721–729.
[20] M. Kanai, E. Matsubara, K. Isoe, et al., Longitudinal study of cerebrospinal fluid levels of tau, A β1-40, and A β1-42(43) in Alzheimer's disease: a study in Japan, Ann. Neurol. 44 (1998) 17–26.
[21] Q.X. Li, B.C. Campbell, C.A. McLean, et al., Platelet α- and gamma-synucleins in Parkinson's disease and normal control subjects, J. Alzheimers Dis. 4 (2002) 309–315.
[22] D.W. Miller, S.M. Hague, J. Clarimon, et al., α-Synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication, Neurology 62 (2004) 1835–1838.
[23] O.M. El-Agnaf, D.M. Walsh, D. Allsop, Soluble oligomers for the diagnosis of neurodegenerative diseases, Lancet Neurol. 2 (2003) 461–462.
[24] C. Klein, M.G. Schlossmacher, The genetics of Parkinson's disease: implications of neurological care. Nat. Clin. Prac. Neurol. 2006, in press.