Development of Quantitative Methods to Measure alpha-Synuclein and Related Sub-Species in CSF as Candidate Biomarkers for Parkinson’s Disease Progression

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Abstract

Objectives: There is abundant and compelling evidence that alpha-synuclein (SNCA) is familial and sporadic Parkinson’s disease (PD). CSF levels of SNCA have been extensively evaluated in PD, however they have limited utility as a biomarker of disease progression. Splice variants of SNCA have been reported (SNCA 98, 112, 126), and SNCA is subject to several post-translational modifications (PTM), including phosphorylation and truncation. These variant and modified forms of SNCA are impacted in disease pathogenesis, yet most assays of SNCA are agnostic to them. Here we report the development of methodology to fully characterize SNCA variants and PTMs in human CSF, with the objective of using these methods to measure changes in these species as markers of disease progression in PD.

Sub-Subjects in CSF as Candidate Biomarkers for Parkinson’s Disease Progression

Development of Quantitative Methods to Measure alpha-Synuclein and Related Species

Immunoprecipitation, LC-MS/MS

Immunoprecipitation, LC-MS/MS methodology of detecting specific SNCA species may provide a novel biomarker approach for PD. We have used this method to identify splice variants, phosphorylated forms, as well as other post-translationally modified forms of SNCA in CSF, including a variety of C-terminal truncations as well as deamination of asparagine and glutamine residues. This technique is readily applicable to other low abundance CSF analytes and provides sufficient sensitivity for their detection.

Table 2. Truncations and Other Post-translational Modifications Detected in Normal CSF using a Central Epitope Antibody (S52) for Immunoprecipitation from 1 mL CSF

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Modifications</th>
<th>Sequence Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDQLKNEEGAPQELIDEM</td>
<td>Truncation @ 116</td>
<td></td>
</tr>
<tr>
<td>KDQLKNEEGAPQELIDMP</td>
<td>Oxidation(M) Truncation @ 117</td>
<td></td>
</tr>
<tr>
<td>KDQLKNEEGAPQELIDMPV</td>
<td>Deamidated(L) Truncation @ 119</td>
<td></td>
</tr>
<tr>
<td>KDQLKNEEGAPGELIDMPVD</td>
<td>Deamidated(L) Truncation @ 120</td>
<td></td>
</tr>
<tr>
<td>NEEGAPQELIDMPVDPENE</td>
<td>Deamidated(N) Truncation @ 123</td>
<td></td>
</tr>
<tr>
<td>KDQLKNEEGAPQELIDMPVDPEA *</td>
<td>Splice Variant SNCA 126</td>
<td></td>
</tr>
<tr>
<td>KDQDYDPEA*</td>
<td>Splice Variant SNCA 112</td>
<td></td>
</tr>
</tbody>
</table>

*Immunoprecipitated using proprietary central epitope antibody

Methods

Antibody Validation

Full length, N-terminal (AA 1-95) and C-terminal (AA 96-140) SNCA peptides were phosphorylated using casein kinase-1 (CK1). Phosphorylated and non-phosphorylated peptides were immunoprecipitated and subjected to LC-MS/MS (see below), or western blot methods using antibodies to unmodified SNCA and phosphorylated SNCA. Western blots were imaged using a Licor Odyssey imager.

Immunoprecipitation, LC-MS/MS

The phosphorylated species of SNCA (at S87, Y125, S129 and Y133) were immunoprecipitated from non-PD human CSF and EDTA plasma with phospho-specific antibodies conjugated to magnetic beads. Full-length SNCA and splice variants were immunoprecipitated using a central epitope antibody. We also used Epitomics antibody to full length SNCA to immunoprecipitate full length protein. The immunoprecipitated proteins were then subjected to tryptic digestion and analyzed by Shumadzu Prominance UFLC system (LC) coupled to an ABSCIEX Triple TOP 5600 mass spectrometer (MS) using an Information Dependent Acquisition (IDA) method, and an UFLC coupled to an ABSCIEX Triple Quadrupole 4000 (MS) using a targeted SRM to measure signature alpha synuclein peptides.

Results

Antibody Validation

Identification of SNCA was confirmed by LC-MS/MS using a proteomic IDA method. The highlighted peptide fragment from tryptic-digested full-length alpha-synuclein was measured by mass-to-charge ratio (upper right spectrum) at 530.29 m/z. The peptide was then fragmented (lower right spectrum) and all fragments were measured to confirm peptide ID as SNCA peptide TKQGVAEAAGK.

LC-MS/MS Analysis

Table 2. Phosphopeptides (ser129) relative to total α-syn from human CSF

<table>
<thead>
<tr>
<th>Phosphopeptide</th>
<th>Relative to total α-syn from human CSF</th>
</tr>
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<tbody>
<tr>
<td>Y10+2</td>
<td>100%</td>
</tr>
<tr>
<td>Y5+1</td>
<td>100%</td>
</tr>
<tr>
<td>y8+1</td>
<td>100%</td>
</tr>
<tr>
<td>y10</td>
<td>100%</td>
</tr>
</tbody>
</table>

Note: Full length and C-terminal phospho-proteins were IP’d by pS129 antibodies. Unphosphorylated full-length SNCA was not IP’d by pS129 antibodies.

IP using anti-α-syn MAb to capture phosho (ser129)- α-syn from human CSF

IP using anti-α-syn MAb to capture total α-syn from human plasma

IP using anti-α-syn MAb to capture phosho (ser129)- α-syn from human plasma

Figure 1. α-syn plays a central role in PD

Methods

Antibody Validation

Full length, N-terminal (AA 1-95) and C-terminal (AA 96-140) SNCA peptides were phosphorylated using casein kinase-1 (CK1). Phosphorylated and non-phosphorylated peptides were immunoprecipitated and subjected to LC-MS/MS (see below), or western blot methods using antibodies to unmodified SNCA and phosphorylated SNCA. Western blots were imaged using a Licor Odyssey imager.

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Figure 2. Full Length SNCA immuno-precipitated with anti-p129 SNCA (CVD, Clone 81A)

Identification of SNCA was confirmed by LC-MS/MS using a proteomic IDA method. The highlighted peptide fragment from tryptic-digested full-length alpha-synuclein was measured by mass-to-charge ratio (upper right spectrum) at 530.29 m/z. The peptide was then fragmented (lower right spectrum) and all fragments were measured to confirm peptide ID as SNCA peptide TKQGVAEAAGK.

Figure 3. Western blots of SNCA Peptides

A. Western blot with anti-SNCA (Epitomics). B. Western blot with anti-p129 SNCA (Covance). C. High resolution of C-terminal SNCA from gel in B. Lanes 2.4 full-length SNCA; Lanes 2.6 N-terminal SNCA; Lanes 3.8 C-terminal SNCA. Lanes 1,3, non-phosphorylated peptides, lanes 4-6 phosphorylated peptides.

Note specific staining of full length SNCA, weak staining of C-terminal SNCA peptide and no staining of N-terminal SNCA peptide using p129 phospho-SNCA antibody.

Figure 4. IP-LC-MS/MS of CSF and plasma samples.

This IP-LC-MS/MS method was successfully applied to the measurement of alpha-synuclein and phosphorylated alpha-synuclein in human CSF and plasma from non-PD donors. Phosphorylation at S87, Y125, S129, and Y133 were immunoprecipitated and analyzed using a signature alpha-synuclein peptide via LC-MS/MS. All four phosphorylation sites were measurable.

Summary

Phosphopeptides (ser129) relative to total α-syn from human CSF

Note: Full length and C-terminal phospho-proteins were IP’d by pS129 antibodies. Unphosphorylated full-length SNCA was not IP’d by pS129 antibodies.

Note specific staining of full length SNCA, weak staining of C-terminal SNCA peptide and no staining of N-terminal SNCA peptide using p129 phospho-SNCA antibody.