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Mini-review

Proteomics for Cerebrospinal Fluid Biomarker Identification in Parkinson's Disease: Methods and Critical Aspects

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Abstract: Parkinson's disease (PD), similar with other neurodegenerative disorders, would benefit from the identification of early biomarkers for differential diagnosis and prognosis to address prompt clinical treatments. Together with hypothesis driven approaches, PD has been investigated by high-throughput differential proteomic analysis of cerebrospinal fluid (CSF) protein content. The principal methodologies and techniques utilized in the proteomics field for PD biomarker discovery from CSF are presented in this mini review. The positive aspects and challenges in proteome-based biomarker research are also discussed.

Keywords: cerebrospinal fluid; Parkinson's disease; biomarker; quantitative proteomics; post translational modifications; mass spectrometry; two-dimensional electrophoresis

1. Background

Various methodological approaches can be used for the analysis of the protein content in the cerebrospinal fluid (CSF). The most commonly used techniques are addressed to carry out differential protein expression analysis, juxtaposing group of samples referring to the investigated disease with other similar diseases, healthy control and possibly experimental conditions produced on CSF samples *ex vivo*. The so call quantitative proteomics measure the relative and/or absolute amount of proteins, or the abundance of specific post-translational modifications, fostered by the pathologic conditions with the aim to use these differences as putative biomarkers [1,2].

At the present time, the quantitative differential proteomics approach is principally based on mass spectrometry, though classical gel-based analyses, namely 2-dimensional electrophoresis (2DE), are still largely used. The limitation of gel-based analyses resides in the restricted number of proteins that can be resolved in a gel and in the specific type of proteins that can be determined. On the

contrary, mass spectrometry approaches coupled with liquid chromatography (LC-MS/MS) are able to determine large number of proteins without restrictions regarding the type of proteins [3,4]. Fold change of protein expression is generally reported for the protein relative quantitation and can be obtained by label-based or label-free methods, while the addition of internal stable-isotopic standards in the samples is used to determine the absolute quantitation of a certain protein expression (see [2] and [5] for a detailed methodological review).

2. CSF-based proteomic biomarkers in PD

Even though some promising biomarker candidates have been found in the CSF of Parkinson's disease (PD) patients (e.g. alpha-synuclein, neurofilament light chain protein, total tau protein, amyloid-β42, DJ-1), none of these have been discovered by classical differential proteomics approach (see [6] and [7] for review). With diverse proteomics approaches, some studies which aimed to find CSF biomarkers have been performed and resulted in non-homogeneous or inconsistent findings. For example, by using iTRAQ (isobaric Tagging for Relative and Absolute protein Quantitation) in one study, apolipoprotein H and ceruloplasmin have been proposed to be able to discriminate PD patients from healthy subjects and from some others non-PD neurodegenerations [8], while in a different study, tyrosine-kinase-non-receptor-type 13 and Netrin G1 have been proposed as early biomarkers for PD development of dementia [9]. A study using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) found a panel of four proteins (ubiquitin, β2-microglobulin and two secretogranin 1 fragments) to discriminate PD patients from atypical-PD patients but not from healthy controls [10]. By 2DE differential analysis, altered levels of albumin, transferrin, haemoglobin, prolin rich repeat 14, and globin proteins were found to distinguish PD patients from controls [11], while serpin A1 sialylation isoform has been reported to characterize PD patients showing dementia development [12].

In the light of these results, primarily due to lack of reproducibility by independent laboratories, robust and validated CSF biomarkers have not yet established in PD. The complex and evolving interaction of different pathogenic mechanisms in PD requests to consider a panel of biomarkers instead of a single one, to investigate a very large cohort of patients to improve statistical outcomes and to collect longitudinal samples in the same patients to monitor disease progression.

3. Potential of post-translational modifications as biomarkers

A useful source of putative biomarker discovery, which should also be taken into consideration, is the analysis of protein post-translational modifications that may occur in neurological diseases as consequence of the pathological conditions. For example, similar with other neurodegenerative diseases, PD is characterized by the presence of oxidative stress conditions in the central nervous system. The oxidative conditions promote several CSF protein modifications that can be investigated by applying conventional biochemical analyses or large-scale proteomics approaches. Modifications of the CSF protein have been investigated as proteins acidification following modification of their isoelectric point [13], or as increase in protein carbonylation [13,14], protein nitration [15], or protein thiol-group modifications (S-cysteinylation, S-glutathionylation, S-nitrosylation) [16,17]. In PD in particular, the presence of ferroxidase ceruloplasmin isoforms with more acidic isoelectric point due to oxidative modifications has been reported to be able to discriminate PD patients from Alzheimer's

disease patients and also from healthy subjects and patients with peripheral neuropathies [13]. In PD patients, the oxidation rate of ceruloplasmin correlated with Unified Parkinson's Disease Rating Scale clinical grading, suggesting a putative use for patients stratification. Interestingly, the ceruloplasmin oxidation resulted in the enzymatic ferroxidase loss of function that promotes intracellular iron accumulation in neurons and astrocytes might represent a contribution to the pathological mechanism underlined by brain iron deposition [13]. Furthermore, protein phosphorylation, the most common post-translational modification for the reversible regulation of protein function, has been investigated in normal CSF and may be also evaluated in its pathological conditions [18].

Another interesting protein modification that can be investigated is the deamidation of asparagine residues that spontaneously occurs during protein aging [19] which can be accelerated by the oxidative environment [20]. Therefore, protein deamidation might reflect the accelerated protein aging that can occur under pathological conditions in neurodegenerative disease characterized by the presence of the oxidative stress. Protein deamidation, if occurs at specific protein motif targets, can change protein properties. For example, deamidation of Asparagine-Glycine-Arginine (NGR) motifs in the sequence of ceruloplasmin in the CSF of Alzheimer's patients can induce protein structural changes and gain of new integrin binding functions [20].

4. Challenges

Proteomic experimental approaches have holds an enormous potential as sources of biomarker discoveries for neurological disorders. However, the biological and chemical features of CSF rise some problems for accurate and useful analyses [21]. Among these problems are 1) CSF is a difficult specimen to obtain, 2) very few longitudinal studies are allowed with CSF collection, 3) CSF is difficult to obtain from proper control groups, and 4) clinical validation of CSF biomarkers needs large cohorts that are difficult to put together in single, small medical centers. In addition, the inter-individual variation of the specific protein concentration [22–24], the age-related changes in protein relative abundance [25], and the risk of CSF contamination by blood proteins that may occur during sample collection, might dramatically affect the outcome of the analyses. In particular the analysis of CSF protein profile changes in age-related neurodegeneration requires the use of appropriated age-control population in order to discriminate pathological- from normal aging changes both at quantitative and qualitative (structural and post translational modifications) levels [25, 26].

Furthermore, after collection of CSF, additional pre-processing steps are usually needed to improve the quality of the sample. For example, relative low protein concentration of CSF (0.2–0.7 mg/ml) requires the introduction of a protein enrichment step that can be performed by ultrafiltration or protein precipitation [3]. Another technical problem for CSF protein analysis is the disequilibrium of protein abundance; the presence of some specific proteins in large amount can cause difficulties in detection of low-abundant proteins. Hence, abundant proteins such as albumin, immunoglobulin, transthyretin, etc. should be removed by affinity-chromatography or pre-fractionation [3]. Proteins pre-fractionation can be achieved by standard biochemical methods that exploit different biochemical features of the protein like hydrophobicity, size, charge etc. [3,25]. For CSF protein fractionation, frequently used techniques include reversed-phase solid-phase extraction [27] and liquid- or semi-solid-phase isoelectric focusing that allow protein separation on a

solid pH-gradient and a protein recovery in liquid phase [28].

On the other hand, the analysis of specific post-translational modifications may be affected by "artificial modifications" induced by the experimental and/or sample storage conditions as, for example, the induction of ex-vivo protein oxidation [13,16]. These experimental oxidation pitfalls can be avoided by storing the samples and performing the experiments under nitrogen-conditioned atmosphere [13,20]. In some cases, the normal experimental protocols used must be modified. For example during protein deamidation analysis, it is mandatory to set up a different protocol for tryptic digestion necessary for mass spectrometry analysis, because the standard conditions used (ammonium bicarbonate buffer at pH 8.5, at 37 °C) are the ideal environment to promote very strong in vitro protein deamidation [20]. Thus, different types of analysis require different development of simple, robust, and reproducible methods to inhibit artificial modifications and eliminate false positive results.

Another challenge is the needs of complex statistical and bioinformatics analyses to process the enormous amount of data generated by proteomics investigations. Furthermore, in the case of multifactorial diseases like PD, no changes would be expected in a single biomarker, but complex changes in a set of proteins and redox compounds will represent the more useful biomarker. Therefore, new approaches such as neural networks and machine learning have been increasingly used for data mining [13,29–31].

In conclusion, proteomics approaches may offer very interesting contribution for CSF biomarker discovery in PD. However, the biological implications and its relevance related to proposed biomarkers remain to be clearly established. Standardization of procedures from sample collection, sample processing to analytical methods is an important aspect for validation studies of proteome-based PD biomarkers.

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Conflict of Interest

The authors declare no competing financial interests.

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