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Targeted human cerebrospinal fluid proteomics for the validation of multiple Alzheimer's disease biomarker candidates

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ABSTRACT

There is significant interest in the development of methods to validate novel biomarkers for Alzheimer's disease (AD) diagnosis. Previously, a proteomic panel of cerebrospinal fluid (CSF) biomarker candidates that differentiated AD and non-AD CSF with accuracy higher than 90% was found; information about these CSF proteins can be used to develop multiple reaction monitoring (MRM) based analytical assays, which offer the possibility of quantifying protein expression level changes in samples, as well as, validation among multiple laboratories. Here we report an MRM assay that demonstrates good linearity (average $R^2 = 0.969$) and reproducibility (average coefficient of variance of 6.93%) for the proposed AD CSF biomarkers. MRM quantification results of Aβ1-40, Aβ1-42, retinol-binding protein and cystatin C correlated well with those from ELISA (average $R^2 = 0.974$). Analysis shows that 12 out of 16 selected targets exhibit the same trend in protein expression as that in literature.

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1. Introduction

There is a need for the development of quantitative assays to measure levels of sets of molecules present in a relatively complex mixture. This need is driven by an increasing emphasis on personalized medicine and the measurement of multiplexed biomarkers present in biological fluids such as blood, saliva, cerebrospinal fluid (CSF), and urine. Such markers are being discovered for a variety of diseases including Alzheimer's disease (AD). AD is the most common type of dementia in the elderly and its prevalence in the United States was more than 5.4 million in 2011 [1]. There has been significant effort to understand the underlying molecular pathology. Currently, treatment is focused on the delay of the progression of patients' cognitive decline and it is believed that treatments are most effective at the earlier stages of AD [1–3]. As such, it is important to have an accurate diagnosis of AD as early as possible.

Early diagnosis of AD is difficult because of the lack of a suitably accurate antemortem diagnostic method [4] and tests with sensitivity and specificity >80% are of interest [5]. The interest in CSF [6,7] is based on brain proximity and there are many brain-associated CSF diagnostic markers in addition to others that have been proposed after a discovery-proteomics experiment [8,9]. While it is possible to measure the levels of molecules of interest, for example biomarkers, using traditional enzyme-linked immunosorbent assays (ELISA), the development of ELISAs is not well-suited to the confirmation and validation of putative biomarkers for several reasons: ELISAs require the availability of appropriate affinity reagents which are specific to the molecule or epitope of interest; these reagents can be relatively expensive to obtain; and they are not typically multiplexed – each assay is performed individually, which requires more sample to perform analysis of multiple analytes. As a complement to ELISAs, there has been interest in the use of a separation based method to facilitate the confirmation and early validation of biomarker discoveries because such methods are more cost effective to develop and once established, can be easily replicated by other laboratories. Multiple reaction monitoring (MRM) is an approach that combines a liquid phase separation with detection by mass spectrometry to measure the levels of molecules of interest and it can be multiplexed so that a single assay measures the levels of many molecules of interest [10,11]. MRM is a targeted proteomics technique typically performed on triple quadrupole-based mass spectrometers. Generally, in this method, a certain ion of interest (a precursor ion) is transmitted to the second quadrupole and only a selected fragment ion from the precursor ion is sent to

Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; Aβ, amyloid beta; MS, mass spectrometry; 2DE, 2D gel electrophoresis; MALDI-TOF/TOF MS, matrix assisted laser desorption ionization tandem time of flight mass spectrometry; MRM, multiple reaction monitoring; nLC-MRM/MS, nano liquid chromatography multiple reaction monitoring tandem mass spectrometry; RS, regional standard; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; RT, room temperature; CI, confidence interval; RBP, retinol-binding protein; Cys C, cystatin C; CV, coefficient of variance; Sy|x, the residual standard deviation; VDBP, vitamin D-binding protein.

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the detector. MRM assays have high sensitivity and high specificity [12]. Further, MRMs are an alternative to immunoassays for protein/peptide quantification and they demonstrate a wide dynamic range [13]. Finally, MRM assays are an ideal tool for the validation of multiple markers because the analysis time is short and they are easily multiplexed [10,11]. A potential drawback of this approach is the time and effort needed for method development. The use of MRM methods based on MS^3 and centralized databases of MRM transitions offers significant promise (MRM^3, MRMAtlas) [14,15] and methods with labeled standards (SISCAPA) [16,17] can facilitate reproducibility.

One area in which the use of separations-based MRM assays can be particularly important is in the initial validation of putative biomarkers as a follow-on to a discovery-based proteomics experiment [18,19]. Large-scale proteomics experiments are difficult to replicate precisely between laboratories. MRM assays offer an opportunity to confirm findings from proteomics experiments across laboratories. Previously, a panel of AD CSF marker candidates, which showed 93% sensitivity and 90% specificity in differentiating AD CSF and non-AD CSF (including normal, neurologic, and demented controls) using 2D gel electrophoresis and matrix assisted laser desorption ionization time of flight tandem mass spectrometry (MALDI TOF/TOF MS), was reported [20]. Here, we report a CSF MRM assay for the validation of many of the proteins in the proposed panel of CSF AD biomarkers. Twenty-four peptides representing the different AD CSF biomarker candidates were successfully included in the method to monitor non-depleted human CSF using nano liquid chromatography MRM tandem mass spectrometry (nLC-MRM/MS). Good analytical performance of this assay was confirmed and the day to day reproducibility of measurements was improved using regional standards (RSs). RS is our terminology for a set of internal standards (i.e. peptides) different from the analytes of interest, that are spiked into the sample prior to LC separation to facilitate normalization over a set of experiments where the internal standard peptides have an elution profile that is well-distributed over the course of the LC run. Finally, quantitative results from MRMs of select AD biomarker candidates were found to correlate well with those from ELISAs. Therefore, these preliminary results show the potential of the method to be a new AD diagnostic method using multiple AD biomarkers, as well as a robust approach for the fast validation of AD biomarkers previously reported. This is the first report of a nLC-MRM/MS application to comparative human CSF proteomics targeting AD diagnosis.

2. Materials and methods

2.1. CSF sample preparation

This work has been approved by the University of Delaware Institutional Review Board. A pooled-normal CSF sample was purchased from Biochemed Services (Winchester, VA) and an antemortem CSF sample from a definite AD subject (confirmed by autopsy) was provided by the Institute for Brain Aging and Dementia Tissue Repository (University of California, Irvine, CA), these samples are termed non-AD and AD, respectively. Additional antemortem CSF samples from definite AD cases and nonAD samples were provided by the Institute for Brain Aging and Dementia Tissue Repository and the National Neurological Research Specimen Bank at UCLA (samples AD1, AD2, AD3, nonAD1, nonAD2, nonAD3). Samples were shipped on dry ice and stored at −70 °C until needed. Three hundred microliters of a CSF sample was loaded onto a 3000 Da cutoff filter (Microcon YM-3, Millipore, Billercia, MA) for buffer exchange. The volume was increased to 500 μL by adding 0.2 M ammonium bicarbonate and the filter unit was centrifuged at 10,000 rpm for 30 min. This buffer exchange step was repeated twice and the retentate was dried by vacuum centrifugation. The proteins in the retentate were resuspended, denatured and reduced with 15 μL of 6.0 M urea (in 0.1 M ammonium bicarbonate) and 1 μL of 200 mM dithiothreitol (DTT, in 0.1 M ammonium bicarbonate) for 1 h at room temperature (RT). Then, 2 μL of 200 mM iodoacetamide (in 0.1 M ammonium bicarbonate) was added and the mixture was incubated for 1 h at RT in the dark. After quenching the remaining iodoacetamide with 2 μL of 200 mM DTT for 1 h at RT, the solution was mixed with 75 μL of 40 mM ammonium bicarbonate and 5 μL of trypsin (Promega, Madison, WI) dissolved (20 μg in 30 μL) in its dissolution buffer (Promega), for the protein digest. The mixture was incubated for 14 h at 37 °C, then 1 μL of 20% formic acid was added. The digested sample was dried by vacuum centrifugation and then dissolved in 60 μL of 0.1% formic acid. The reconstituted sample was diluted 50× with 0.1% formic acid and (when appropriate) a tryptic digest of yeast alcohol dehydrogenase (1 pmol/μL, Michrom Bioresources, Auburn, CA) was spiked into the diluted sample to 10 fmol/μL as RSs.

2.2. Nano liquid chromatography tandem mass spectrometry and nLC-MRM/MS

A portion (15 μL, if not otherwise specified) of the diluted sample was separated on a Dionex 3000 nLC system (Sunnyvale, CA) with an Acclaim PepMap 100 C18 trap column (300 μm × 5 mm, 5 μm, Dionex) for on-line desalting (2% aqueous acetonitrile solution with 0.1% formic acid, flow rate of 30 μL/min for 5 min) and an Acclaim PepMap 100 C18 analytical column (75 μm × 15 cm, 3 μm, Dionex, flow rate of 250 nL/min). Peptides were eluted over a gradient of 2–50% acetonitrile with 0.1% formic acid for 100 min and the eluent was directly introduced into an Applied Biosystems 4000 QTRAP MS (Foster City, CA) through a Nanospray II source (Applied Biosystems, gas1 of 14 psi and spray voltage of 2.8 kV). The MS was operated in data-dependent scan mode with Analyst v1.5 software (Applied Biosystems) set to acquire MS/MS spectra of the five most intense ions in a survey scan (full MS scan or MRM scan). Data were searched within the NCBInr protein database using ProteinPilot software (Applied Biosystems) with either the Paragon algorithm (Applied Biosystems) or the Mascot search engine (v. 2.2, Matrix Science, Boston, MA) for peptide/protein identification (at confidence interval (CI) ≥ 95%) and also verified manually. In the case of the nLC-MRM/MS study, each sample was analyzed twice by two different MRM acquisition methods ("abundant method" and "non-abundant method") composed of different sets of targets to maximize sensitivity and specificity (abundant targets versus non-abundant targets as indicated in Table 1). All analyses were carried out in triplicate. Target MRM transition candidates, extracted from experimental observations previously reported [20], or generated *in silico*, were tested. Verified transitions were merged into one of the two MRM acquisition methods based on their peak areas and retention times to prevent undersampling of targets. The list of target biomarker candidates (including MRM transitions) and their corresponding RSs is given in Table 1. Note that the transitions are intended to measure changes in levels of specific peptides from a given protein that are believed to be diagnostic [20] rather than changes in the levels of intact proteins. An additional test was carried out in the presence of RSs to study their influence on target quantification (see Supplemental Table 1, "serial CSF addition" test). For nLC-MRM/MS of Aβ1-40, retinol-binding protein (RBP) and cystatin C (Cys C), MRM transitions representing the individual proteins/peptides were monitored under the same conditions as the analyses of CSF digest samples (see Supplemental Table 2).

2.3. LC-MRM/MS of Aβ1-42

10 μg of Aβ1-42 (Covance, Dedham, MA) was dissolved in 400 μL of an aqueous ammonium hydroxide solution (30% as

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