SELDI technology

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Identification of a Panel of Cerebrospinal Fluid Biomarkers for Alzheimer's Disease by SELDI-TOF-MS

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia. In people over 60 years of age, its frequency doubles every 5 years, from 1% at 60–65 years to over 40% at 90–95 years. Today, over 24 million people suffer from AD, with symptoms including impeded memory, language, and ability to care for one's self.

The diagnosis of AD, particularly in the early phases of the disease, is challenging because of the lack of a specific diagnostic marker. Current clinical diagnostic accuracy of AD can range from 65% to 90% in specialized centers and in the later stages of the disease, but AD can only be confirmed upon postmortem examination of the brain to identify amyloid plaques and neurofibrillary tangles in certain brain regions (Waldemar et al. 2007). In some clinical settings, the cerebrospinal fluid (CSF) markers β-amyloid protein, tau, and phosphorylated tau are used to support clinical diagnosis (Blennow and Hampel 2003); however, more biomarkers for the diagnosis of AD are needed to improve diagnostic accuracy and to achieve greater insight into disease-related changes occurring in the brain.

Parallel quantitative analysis of hundreds of proteins from large numbers of samples offers a means of identifying novel AD biomarkers with potential clinical utility. Such large sample sets minimize the effects of individual variability and permit separation of samples into model-building and blinded model test sets. However, many of the methods commonly used in biomarker research, including two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) coupled with mass spectrometry (MS) and many standard liquid chromatography-MS (LC-MS) techniques, do not provide the throughput required to rapidly and reproducibly identify novel panels of biomarkers from large sample sets.

The aim of this study was to discover and identify novel AD-associated protein biomarkers in CSF using the ProteinChip® SELDI system. Surface-enhanced laser desorption/ionization. (SELDI) technology combines the separation power of chromatography with the sensitivity of time-of-flight MS (TOF-MS) for rapid, content-rich proteomic analysis of complex biological samples. The chromatographic nature of the ProteinChip array surface allows direct TOF-MS analysis of samples in a robust and high-throughput manner (up to 1,344 array spots without user intervention). Furthermore, since SELDI analyses require very low sample volumes, samples can be profiled on multiple array surfaces (for example, reverse-phase, anion and cation exchange, metal affinity) to increase the number of detectable species.

In a discovery study, SELDI-TOF-MS analysis of CSF from 95 AD patients and 72 healthy controls yielded a panel of 30 candidate biomarkers that could differentiate between AD and healthy controls. These markers were further evaluated and characterized in a second validation study using CSF samples from 85 AD patients, 32 healthy controls, and 20 frontotemporal dementia (FTD) patients. Fifteen of the 30 candidate markers from the discovery study were confirmed, and of these, seven could also differentiate between AD and FTD. The panel of CSF biomarkers discovered may serve as an adjunct in the clinical diagnosis of AD.

For a more complete description of the clinical studies performed and methods used, see Simonsen et al. (2007a, 2007b).

Methods

Sample Collection

Patients were recruited from specialist dementia centers located at Sahlgrenska University Hospital (Gothenburg), Malmö University Hospital, Piteå River Valley Hospital, Athens Eginition Hospital, and Örebro University Hospital (Table 1). Global cognitive function was assessed using mini-mental state examination (MMSE) scores (Folstein et al. 1975). All individuals (or their primary caregiver) gave informed consent to participate in the studies, which were conducted according to the Declaration of Helsinki. The studies were approved by the local ethics committees. For more details regarding patient selection, see Simonsen et al. (2007a, 2007b).

Since the differential expression of CSF proteins may reflect differences in preanalytical handling or storage of samples rather than disease-related changes in protein or peptide levels, we observed strict standardized operating procedures for sample collection, handling, and storage. CSF samples (10–12 ml) were obtained by lumbar puncture, collected in polypropylene tubes, and gently mixed. The samples were centrifuged at 2,000 x g for 10 min to remove cells and other insoluble material. Supernatants were frozen in aliquots and stored at –80°C pending analysis. No sample contained more than 500 erythrocytes/µl based on blood cell counts. The sampling and storage procedures were the same for all sample groups.

Sample and Array Preparation

Each CSF sample (5 µl) was diluted into 45 µl of the appropriate binding buffer and analyzed on the following ProteinChip array types: anion exchange (CM10), cation exchange (Q10), hydrophobic (H50), and immobilized metal affinity capture (IMAC30) coupled with nickel or copper. The following binding buffers were used for each array type: 10% acetonitrile, 0.1% trifluoroacetic acid (TFA) for ProteinChip H50 arrays; 100 mM sodium phosphate, pH 7.0, 0.5 M NaCl for ProteinChip IMAC30-Cu and IMAC30-Ni arrays; 100 mM sodium acetate, pH 4.0 for ProteinChip CM10 arrays; and 100 mM Tris-HCl, pH 9.0 for ProteinChip Q10 arrays. The samples were allowed to bind for 60 min at room temperature. Each array was then washed three times with the appropriate buffer and rinsed twice with HPLC-grade water prior to addition of ProteinChip SPA (sinapinic acid) matrix in 50% acetonitrile and 0.5% TFA.

To ensure reproducibility of sample preparation and array analysis, a reference CSF standard was randomly distributed in several separate aliquots among the clinical samples and analyzed under the same conditions as the samples. All array preparation was performed using a Biomek 2000 laboratory automation workstation (Beckman Coulter, Inc.).

SELDI Analysis

Each sample was run in duplicate on separate arrays. All arrays were analyzed using the ProteinChip SELDI system, Enterprise Edition. The arrays were read at two different reader settings to focus on lower and higher masses. Protein profiles were generated in which individual proteins were displayed within spectra as unique peaks based on their mass-to-charge ratio (m/z).

Statistical Analysis

Spectral data were collected using ProteinChip data manager software, which was also used for data processing and univariate statistical analysis. All spectra were internally mass calibrated, and peak intensities were normalized using total ion current. Peak clustering was performed in a range that excluded the very low mass region, which is dominated by SPA peaks. *P* values across each group were calculated for individual peak clusters using a Mann-Whitney *U* test for two group comparisons and a Kruskal-Wallis test for multiple group comparisons. The area under the receiver-operator characteristic curve (ROC AUC) value was calculated for each peak cluster. All peak clusters showing a significant difference in the initial univariate analysis were checked manually to exclude any spurious peaks.

Purification and Identification of Candidate Biomarkers

Candidate biomarkers were purified using a combination of chromatographic methods and SDS-PAGE. Appropriate ProteinChip arrays were used to monitor purification. SDS-PAGE gel bands were subjected to tryptic digest and analysis by peptide mapping or by CID sequence analysis on a tandem mass spectrometer. Biomarkers smaller than 4 kD were enriched by a combination of chromatographic techniques and were identified directly by tandem MS (MS/MS) without SDS-PAGE purification or trypsin digestion.

Results

Study I — Discovery

In this study, CSF samples from a total of 95 probable AD cases from two separate sites were compared with samples from 72 clinically normal (healthy) patients from two sites (Table 1). Samples were profiled on ProteinChip CM10, IMAC30-Ni, and Q10 arrays. Profiling on multiple array chemistries was found to increase the number of peptides and proteins detected (Figure 1).

The AD samples from the two different sites were compared separately with the Gothenburg controls to select candidate biomarkers with an ROC AUC greater than 0.70 in both comparisons. Using a model set of randomly selected samples comprising 65 AD cases (30 from Piteå and all 35 from Gothenburg) and the 44 healthy controls from Gothenburg, ranking of performance by partial least squares (PLS) regression using XLSTAT PLS software (Addinsoft) was

Table 1. Samples used in study I (AD vs. healthy controls) and in study II (AD vs. healthy controls and FTD). MMSE, mini-mental state examination. Maximum score is 30. An MMSE score of <23 indicates moderate dementia. SD, standard deviation.

Clinical Diagnosis	Collection Site	Number of Patients	Mean Age (SD)	Mean MMSE (SD)
Study I — Discovery				
Probable AD	Gothenburg	35	69.5 (6.3)	17.7 (6.6)
	Piteå	60	74.8 (5.7)	21.2 (5.0)
Healthy	Gothenburg	44	72.0 (8.4)	28.4 (1.5)
	Malmö	28	74.0 (8.4)	29.0 (1.1)
Study II — Validation				
Probable AD	Athens	55	63.7 (9.7)	16.7 (6.1)
	Örebro	30	74.6 (11.4)	20.0 (3.7)
Healthy	Athens	32	68.3 (12.6)	29.1 (0.9)
FTP	Athens	20	61.5 (9.1)	21.0 (8.2)

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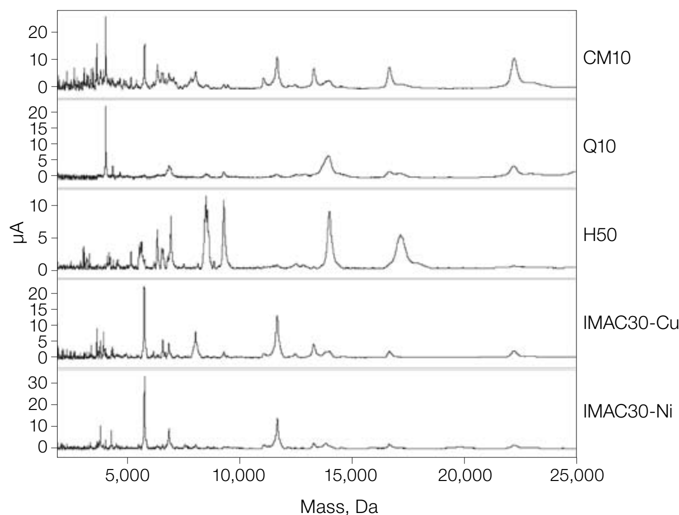

Fig. 1. Representative spectra of a CSF sample analyzed on five different ProteinChip SELDI array types. Profiling on multiple array chemistries increases the number of peptides and proteins detected.

performed to select protein peaks from the reduced list of univariate markers. In this manner, 30 candidate biomarkers were discovered that could differentiate AD from healthy controls; the levels of 12 of these markers were increased and 18 were decreased in AD. Fifteen out of the 30 were also positively identified using MS/MS (not shown).

Study II — Validation

For validation, the 30 candidate biomarkers from study I were analyzed in another independent set of CSF samples that were collected at different sites from 85 AD patients, 32 healthy controls, and 20 FTD patients (Table 1). In this study, the CSF samples were profiled on ProteinChip CM10, IMAC30-Ni, IMAC30-Cu, H50, and Q10 arrays. For further validation and perhaps insight into another form of non-Alzheimer's dementia, group comparisons were also made between AD patients and controls and between AD and FTD patients.

Fifteen of the 30 evaluated biomarkers from study I were confirmed, and seven of these could also differentiate between AD and FTD. Table 2 shows the statistics for all 15 verified biomarkers and the identities of the eight biomarkers that were identified using MS/MS. Figures 2 and 3 show scatterplots of two of the proteins that were able to differentiate between AD and both FTD and healthy controls.

Conclusions

We used ProteinChip SELDI technology to analyze CSF samples from large groups of patients with and without AD and another form of dementia, FTD. Unlike other methods commonly used in biomarker research, including 2-D PAGE coupled with MS or standard LC-MS, SELDI technology can

Table 2. Candidate biomarkers discovered in study I and verified in study II. Results of the univariate statistical analysis of AD patients vs. healthy individuals and AD vs. FTD patients. Candidate biomarkers differentiating between AD and FTD are highlighted in green. NS, not significant.

m/z	Level in AD	ProteinChip Array	*P* Value: AD vs. Healthy	*P* Value: AD vs. FTD	Identity	Function	Proposed Link to AD
2,430.077	Up	CM10	0.039	NS	Integral membrane protein 2B CT	Implicated in rare forms of early dementia (el-Agnaf et al. 2004)	Unknown
3,379.265	Down	IMAC30	0.039	0.01			
3,495.122	Up	IMAC30	0.036	0.007			
3,909.032	Down	Q10	0.011	0.026	Chromogranin A peptide (vasostatin II)	Synaptic protein	Implicated in neuronal death (Taupenot et al. 2003)
4,810.349	Down	Q10	0.002	NS	VGF peptide	Energy expenditure and sympathetic nerve activity (Levi et al. 2004)	Unknown
5,109.393	Down	IMAC30	0.0118	NS			
5,749.135	Down	IMAC30	<0.0001	0.011			
6,437.276	Down	CM10	<0.0001	NS	Apo C-1 (2 aa deleted from N-terminus)	Cholesterol homeostasis	Impaired cholesterol metabolism may increase β-amyloid deposition in the brain (Sehayek and Eisenberg 1991)
7,229.774	Down	IMAC30	0.009	0.007			
8,937.064	Up	IMAC30	0.004	NS	C3a des-Arg	Inflammatory processes	Inflammation is widespread in the AD brain (Akiyama et al. 2000)
12,282.91	Down	Q10	0.023	NS			
12,524.14	Up	CM10	<0.0001	<0.0001	Cystatin C (8 aa deleted from N-terminus)	Cysteine protease inhibitor	Plaque formation (Levy et al. 2001, Sastre et al. 2004)
13,347.95	Down	CM10	<0.0001	<0.0001	Cystatin C	Cysteine protease inhibitor	Plaque formation
14,559.94	Up	IMAC30	<0.0001	NS	Pancreatic ribonuclease	RNA degradation (Gaur et al. 2001)	Unknown
14,944.76	Down	IMAC30	<0.0001	NS			

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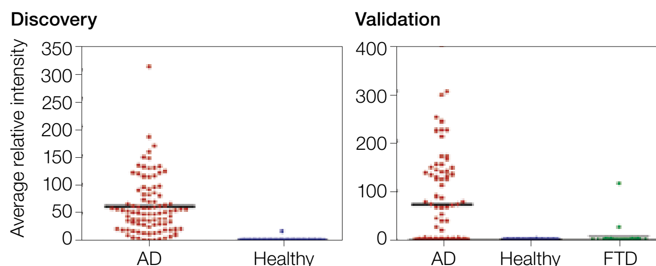

Fig. 2. Scatterplots of truncated cystatin C in study I (left) and study II (right) patients. Cystatin C colocalizes with β-amyloid protein and has been implicated in neurodegenerative processes in the brain (Levy et al. 2001, Sastre et al. 2004). Horizontal bars indicate median peak intensity values.

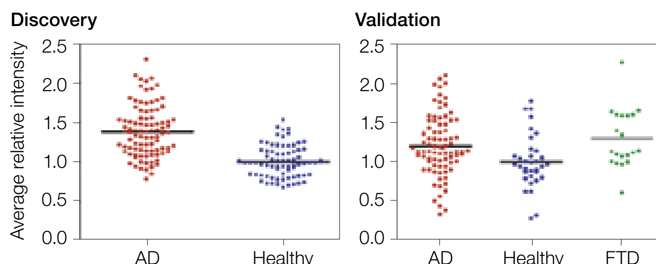

Fig. 3. Scatterplots of complement C3a des-Arg in study I (left) and study II (right) patients. C3a des-Arg is part of the complement system implicated in the inflammatory processes of AD (Akiyama et al. 2000). Horizontal bars indicate median peak intensity values.

rapidly process large numbers of biological samples. The work we present confirms that protein profile changes in the brain of AD patients are reflected in the CSF and that these proteins can be analyzed directly, quickly, and reproducibly with SELDI technology.

AD is a multifactorial disease in which several different pathogenic mechanisms, such as plaque deposition, tangle formation, inflammatory processes, and neurovascular dysfunction or death, occur at the same time. In our studies, we discovered and validated a total of 15 biomarkers capable of distinguishing AD patients from healthy controls. Moreover, seven of these markers are capable of further distinguishing AD patients from FTD patients.

Seven of the 15 biomarkers were identified, and most are proteins with strong links to the AD disease pathophysiology (Table 2), such as cystatin C and ubiquitin (involved in plaque formation; Levy et al. 2001, Sastre et al. 2004), C3a des-Arg (involved in inflammation; Akiyama et al. 2000), and chromogranins (involved in neuronal loss; Taupenot et al. 2003). The other proteins that do not have any known link to the pathogenesis of AD may shed further light into the disease process.

The results presented in Table 2 also demonstrate that the use of multiple array types was instrumental to the discovery and validation process: the 15 validated biomarkers were discovered using three different ProteinChip array chemistries.

The biomarkers have been discovered and corroborated in independent sample sets from different clinical settings, producing a panel of strong biomarkers for further clinical research studies. Larger multicenter prospective studies are needed, as are studies in neuropathologically confirmed AD cases and in studies of other neurodegenerative diseases. Studies to compare the performance of these markers with other potential markers (for example, CSF β-amyloid protein, tau, and phosphorylated tau) analyzed by ELISA, MRI, and PET brain imaging should also be considered. In addition to their use in the diagnosis of AD, the biomarkers reported here may prove useful for the selection of patients for clinical trials and in identifying patients who might benefit from emerging disease-modifying treatments.

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The SELDI process is covered by U.S. patents 5,719,060, 6,225,047, 6,579,719, and 6,818,411 and other issued patents and pending applications in the U.S. and other jurisdictions.

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