SPANDIDOS PUBLICATIONS

Novel cerebrospinal fluid biomarkers of axonal degeneration in frontotemporal dementia

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Abstract. Frontotemporal dementia (FTD) is a heterogeneous disease with substantial interpersonal variance in aggressiveness. Novel biomarkers for rapidly progressive FTD could improve diagnosis and provide clues regarding its pathogenesis. In this study, surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) was used to analyze peptide profiles in cerebrospinal fluid (CSF) from 24 FTD patients. Thirteen patients had rapidly progressive FTD with distinct pathology in a brain MRI after less than 3 years of disease duration. Eleven patients had slowly progressive FTD with a normal brain MRI, but had abnormal findings in SPECT/PET after more than 5 years of disease duration. The axonal damage marker CSF neurofilament light-chain (NF-L) was measured in all subjects to evaluate the amount of axonal degeneration. A CSF NF-L level of 150 ng/l was used as a cut-off point for high NF-L expression. SELDI-TOF analysis of peptides in the range of 2000-20000 m/z revealed one peak with m/z of 6378 that was expressed at a significantly different level ($p<0.01$) when rapidly versus slowly progressive cases of FTD were compared. Eleven peaks were expressed at different levels when high versus low CSF NF-L were compared. Using chromatographic purification followed by tandem mass spectrometric analysis, five of these peaks were identified as follows: C-terminal fragment of neuroendocrine protein 7B2 (3512.84 Da), C-terminal fragment of osteopontin (7658.19 Da) as well as its mono- and diphosphorylated forms (7738.16 Da and 7818.13 Da, respectively) and pancreatic ribonuclease (14566.33 Da). The peak intensity of pancreatic ribonuclease was higher in patients with low NF-L expression, while the other peptides had a lower peak intensity in this group. Altered levels of these peptides have also been described in other neurodegenerative diseases. Taken together, these data suggest that differentially-expressed peptides are general markers of axonal degeneration. Further studies are needed to verify their prognostic value in FTD.

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Introduction

Frontotemporal lobar degeneration (FTLD) is characterized by focal atrophy of the frontal and anterior temporal brain regions, which may present different clinical conditions. Its behavioural variant, frontotemporal dementia (FTD), is characterized by disorders of the personality and social functions such as disinhibition, loss of empathy and stereotyped behaviour (1). Other manifestations of FTLD include the language disorders progressive aphasia (2) and semantic dementia (3).

The clinical course of FTD is highly variable, with disease duration ranging from 1 to 20 years (4). Although it has been suggested that FTD is an aggressive disease with a significantly shorter survival time than Alzheimer's disease (AD) (15), a more benign variant with a lack of gross atrophy on structural imaging or post-mortem macroscopy has been identified (5,6).

Apart from its clinical presentation, pathological findings in FTD are heterogeneous. Neuronal loss with a variety of histological changes is seen in the disease, including tau-positive, ubiquitin-positive and TDP-43-positive intracellular inclusions (7). Independently of these changes, the amount of brain atrophy correlates to clinical severity, disease duration and astrocytic apoptosis. It has been suggested that neurodegeneration in FTD is linked to a loss of astrocytic support (8,9).

In the light of its clinical and pathological variability, it is relevant to find prognostic markers associated with the progression rate of FTD. Cerebrospinal fluid (CSF) neurofilament light chain protein (NF-L) is a specific marker for

Table I. Demographic data, MMSE and CDR scores and CSF NF-L and GFAP concentrations in patients with rapidly and slowly progressive FTD[a].

Group	No.	Sex (M/F)	Age at first symptom (years)	Disease duration at brain imaging (years)	Disease duration at CSF sampling (years)	MMSE	CDR	NF-L (ng/l)	GFAP (ng/l)
Rapidly	13	10/3	58 (48-80)	2 (1-3)	3 (0-6)	26 (12-30)	2.0 (1.0-3.0)	410 (125-2480)	810 (330-1490)
Slowly	11	9/2	53 (34-71)	6 (3-10)	6 (3-10)	27 (24-30)	1.0 (0.5-2.0)	125 (125-920)	580 (240-1430)

[a]Data are presented as median (range); MMSE, Mini-Mental State Examination; CDR, Clinical Dementia Rating; CSF, cerebrospinal fluid; NF-L, neurofilament light-chain; GFAP, glial fibrilliary acidic protein; FTD, frontotemporal dementia.

axonal damage. High NF-L levels are correlated with severe cognitive impairment in FTLD and late-onset AD (10). CSF levels of the astrocyte-specific glial fibrilliary acidic protein (GFAP) are elevated in AD, probably reflecting gliosis (11-13). Using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) followed by tandem MS-based identification of purified proteins, our group has previously discovered biomarkers that differentiate FTD from healthy controls (14). Here, we tested the hypothesis that CSF levels of NF-L and GFAP differentiate rapidly progressive cases of FTD from less aggressive varieties. SELDI-TOF MS was further employed to identify novel biomarkers distinguishing the two groups.

Materials and methods

Patients. The study population included 24 patients with FTD from the VU University Hospital memory clinic (Table I). Diagnosis was made according to the International Consensus Criteria (1). All subjects underwent a standard battery of examinations including medical history, physical and neurological examination, screening laboratory tests, psychometric tests and brain MRI. In patients with normal or non-conclusive MRI findings, 99mTc-hexamethyl propyleneamine oxide (HMPAO) SPECT was performed. Dementia severity was rated using the Clinical Dementia Rating (CDR) (16). Thirteen patients were diagnosed with rapidly progressive FTD, which was defined as distinct pathological signs in brain MRI after a maximum 3 years of disease duration. Eleven patients were diagnosed with slowly progressive FTD. These had normal or non-conclusive brain MRIs, but abnormal findings on SPECT/PET after at least 5 years of disease duration. Post-mortem examination of one patient with a normal MRI and HMPAO-SPECT after 3 years of disease duration revealed spongiosis and gliosis of temporal cortices, hippocampi and amygdalae with tau-negative and ubiquitin-positive inclusions. The study was approved by the local ethics committee.

Sample collection and biochemical analyses. CSF samples were obtained from all subjects using lumbar puncture. In all but one patient, lumbar puncture was performed within one year of MRI/SPECT. The samples were aliquoted and stored at -80˚C pending analysis, without being thawed and refrozen. CSF concentrations of NF-L and GFAP were analyzed using previously described ELISA methods (13,17). The detection limit for the NF-L ELISA was 125 ng/l.

SELDI analysis. Each CSF sample was analyzed on three different array surfaces: cation-exchange (CM10), anion-exchange (Q10) and metal binding (IMAC-Cu). Sinapinic acid (SPA) was used as the energy-absorbing molecule on all surfaces. The samples were subjected to duplicate measurements on each surface. Binding of proteins to the array surfaces was performed in a 96-well format bioprocessor (Ciphergen Biosystems). IMAC-Cu arrays were pre-treated twice with 50 μl 100 mM $CuSO_4$ on a shaker for 5 min, followed by two washes with 100 μl water. Subsequently, all ProteinChip arrays were equilibrated twice with 100 μl binding buffer on a shaker for 5 min. Binding buffers used for the arrays were 100 mM NaAc pH 4.0 for CM10, 100 mM Tris-HCl pH 9.0 for Q10, and 100 mM phosphate buffer with 0.5 M NaCl pH 7.0 for IMAC-Cu. CSF samples were thawed, mixed and centrifuged for 10 min. Binding buffer (50 μl) was added to the surface spots together with 5 μl of each sample. The arrays were incubated for 30 min on a shaker, then washed three times with 100 μl binding buffer, followed by a final water wash. Arrays were removed from the bioprocessor and allowed to air dry, then 0.8 μl of SPA (5 mg dissolved in 400 μl 50% acetonitrile, 0.5% TFA) was applied twice to the spots. A PBSIIc ProteinChip reader (Ciphergen Biosystems) was used to analyze the arrays. Data were averaged over 200 transients for each spot. Arrays were analyzed in the mass range of 2.000-20.000 m/z. To minimize experimental variation, all CSF samples were analyzed concurrently and sample positions were randomized.

Peak analysis. Data handling, including peak identification and clustering of peaks across multiple spectra, was performed using Ciphergen Express Software 3.0.6. Mass spectra representing individual array surfaces were normalized to the same total ion current, and the baseline was subtracted. Settings for cluster formation were first pass S/N 3 in 15% of all spectra and second pass S/N 2. The cluster mass window was one times the peak width.

SELDI-TOF mass spectrometry signal intensities of peaks differing in frontotemporal dementia in patients with high (>150 ng/l) versus low (<150 ng/l) cerebrospinal fluid NF-L concentrations.

Identity	Calculated MW, Da	Array type	Direction of change in high NF-L	AUC ROC high NF-L vs. low NF-L	p-value high NF-L vs. low NF-L	Correlation to N-FL[a]
Neuroendocrine protein 7B2, C-terminal fragment	3512.84	IMAC30	Up	0.794	0.01	rho=0.582 p=0.003
Osteopontin, C-terminal fragment	7658.19	CM10	Up	0.878	0.0024	rho=0.638 p=0.001
Osteopontin C-terminal fragment, monophosphorylated	7738.16	CM10	Up	0.934	<0.001	rho=0.683 p<0.001
Osteopontin, C-terminal fragment diphosphorylated	7818.13	CM10	Up	0.822	0.0034	rho=0.565 p=0.004
Pancreatic ribonuclease	14566.33	IMAC30	Down	0.178	0.0034	rho=-0.614 p=0.001

SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight; NF-L, neurofilament light-chain; [a]Spearman correlation, mean SELDI-TOF MS peak intensities versus cerebrospinal fluid NF-L levels.

Biomarker purification and identification. Protein biomarkers were purified from CSF by liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then identified by tandem MS essentially as described previously (14,18).

Statistical analyses. Single marker statistics were calculated with Ciphergen Express Software 3.0.6 using the non-parametric Mann-Whitney U test for 2-group analyses. Quantitative variables are presented as median (range). SPSS 13.0 (SPSS Inc., Chicago, USA) was used for all other statistical analyses. The Spearman correlation coefficient was used for analyses of correlations between continuous variables. A p-value ≤0.01 was considered significant.

Results

GFAP and NF-L in rapidly and slowly progressive FTD. No significant differences were seen in NF-L (p=0.186) or GFAP (p=0.392) CSF levels when rapidly versus slowly progressive FTD were compared. NF-L correlated to GFAP (Spearman rho=0.713, p<0.0001), but neither NF-L nor GFAP correlated to the clinical scores of the CDR or the Mini-Mental State Examination (MMSE), or to age at symptom debut. Rapidly progressive patients had a higher CDR score (median 2.0, range 1.0-3.0) than did the slowly progressive patients (median 1.0, range 0.5-2.0, p=0.006), but no differences were observed in MMSE or age at symptom onset in the two groups.

Peptide peaks detected with SELDI-TOF MS differentiating rapidly versus slowly progressive FTD. A total of 170 peptide/protein peaks (mass/charge ratios) were detected in the samples. Peaks with low signal-to-noise ratio and/or poor resolution, as well as peaks representing multiply charged proteins, were excluded. After data processing, one peak differed significantly between rapidly and slowly progressive FTD (on the CM10 ProteinChip Array). A 6378 Da biomarker of unknown identity, it had a lower mean peak intensity in rapidly progressive FTD (p=0.008).

Peptide peaks detected with SELDI-TOF MS differentiating high and low CSF NF-L. When examining peak intensity in relation to CSF NF-L concentration, eleven peaks (six on the CM10 ProteinChip Array, four on the IMAC-Cu ProteinChip Array and one on the Q10 ProteinChip Array) differed significantly between high (>150 ng/l) and low (<150 ng/l) NF-L concentrations. Five of these were purified further and identified by tandem MS (Table II). CSF NF-L levels correlated highly to the mean SELDI peak intensities of these detected biomarkers, as seen in Table II. Similar correlations were seen with GFAP (data not shown).

Discussion

Though CSF NF-L and GFAP concentrations failed to distinguish rapidly from slowly progressive FTD, there was a non-significant tendency towards higher NF-L levels in the rapid group. The lack of correlation might reflect weaknesses in diagnostic definitions, or could be due to the relatively small sample number. Earlier studies of FTD survival show a median survival time of three years from clinical presentation (15). In this study, rapid progression was defined as distinct frontal and/or temporal atrophy in a brain MRI after a maximum three years of disease duration, and survival time was not included in the definition. Only one novel biomarker detected by SELDI

analysis differed significantly between rapidly and slowly progressive FTD, and the identity of this biomarker remains unknown. Defining rapid progression using a short survival time might reveal additional relevant biomarkers. However, since clinical classifications remain somewhat arbitrary, grouping by CSF NF-L concentration is likely to reveal biomarkers more specifically related to rapidly progressive neurodegeneration. A weakness of this approach is that the total amount of cerebral neurodegeneration could be subordinate to the degree of engagement of vital neural structures. SELDI analysis of the study population grouped in terms of high versus low NF-L revealed several possible biomarkers.

The peptides correlating to NF-L levels in this study have been linked to neurodegeneration in other surveys as well, particularly to FTD and to amyotrophic lateral sclerosis (ALS) (19,31,32,33). One study found increased concentrations of a carboxy-terminal fragment of neuroendocrine protein 7B2 in the CSF of ALS patients (20). Normally, neuroendocrine protein 7B2 aids in the maturation of proprotein convertase 2, catalyzing the conversion of hormone and neuropeptide precursors into active forms (21,22). Neuroendocrine protein 7B2 also functions as a chaperone in the maturation of growth factors (23). Its possible role in ALS is unclear.

The correlation between a high CSF concentration of osteopontin fragments and extensive axonal damage adds to the accumulating evidence that supports a role for osteopontin in neurodegeneration. Osteopontin is an integrin-binding ligand influencing apoptosis, inflammation, oxidative stress, cytokine regulation and cell migration, all of which could be important in dementia (24). Brain osteopontin expression is increased in animal experimental models of multiple sclerosis and stroke (25,26). In AD, osteopontin correlates to expression of amyloid-ß peptide (Aß) (27). In Parkinson's disease, CSF osteopontin levels are increased in patients with dementia and reduced in patients on dopaminergic treatment, perhaps reflecting a partial recovery of dopaminergic cells (28). Osteopontin is also associated with HIV-dementia, where it might support the survival and accumulation of macrophages within the brain (29).

Using SELDI-TOF MS defining proteomic profiles differentiating AD and FTD, Simonsen *et al* found lower expression of a 14560 Da biomarker identified as pancreatic ribonuclease (30). This is the same protein as the 14566.33 Da biomarker described in this study. Pancreatic ribonuclease is a member of the ribonuclease superfamily, including the angiogenic protein Angiogenin (ANG). Loss-of-function mutations in the ANG gene are probable causative factors in familial as well as sporadic ALS (31-33).

In summary, the biomarkers described in this study are not specific to FTD, but appear to be general markers of axonal degeneration. Their altered levels in FTD are in accord with previous findings in other neurodegenerative diseases, and their value as prognostic biomarkers in FTD and other neurodegenerative disorders should be evaluated in prospective studies.

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