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ORIGINAL PAPER

Proteome Analysis of Cerebrospinal Fluid in Amyotrophic Lateral Sclerosis (ALS)

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Abstract Cerebrospinal fluid (CSF) is a promising source of biomarkers in amyotrophic lateral sclerosis (ALS). Using the two-dimensional difference in gel electrophoresis (2-D-DIGE), we compared CSF samples from patients with ALS (n = 14) with those from normal controls (n = 14). Protein spots that showed significant differences between patients and controls were selected for further analysis by MALDI-TOF mass spectrometry. For validation of identified spots western blot analysis and ELISA was performed. We identified 2 proteins that were upregulated and 3 proteins that were down-regulated in CSF in ALS. Of these, two proteins (Zn-alpha-2-glycoprotein and ceruloplasmin precursor protein) have not been reported in CSF of patients with ALS so far. In contrast, several other proteins (transferrin, alpha-1-antitrypsin precursor and beta-2-microglobulin) seem to be unspecifically affected in different neurological diseases and may therefore be of limited value as disease-related biochemical markers in ALS. Further evaluation of the candidate proteins identified here is necessary.

Keywords Amyotrophic lateral sclerosis · ALS · Motor neuron disease · MND · Cerebrospinal fluid · Proteome · Difference in gel electrophoresis

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease characterized by progressive degeneration of spinal and bulbar innervating motor neurons as well as the pyramidal motor neurons [1]. While several mutations underlying rare cases of familial ALS have been identified, the pathogenesis of sporadic ALS remains poorly understood [2]. Accordingly, there is an ongoing search for biomarkers to advance the understanding of disease pathology as well as to support clinical diagnosis and identification of different subtypes of disease. Cerebrospinal fluid (CSF) is a promising source of biomarkers for neurodegenerative diseases like ALS, since the CSF compartment is in close contact with the brain interstitial fluid, where biochemical changes related to the disease may be reflected. Accordingly, alterations in protein expression, post-translational modification or turnover within the tissue of the central nervous system may be mirrored in corresponding changes in CSF protein content [3].

To date there are only few studies on CSF biomarkers in ALS using proteomic analysis. Two previous studies using the proteomic approach surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF-MS) identified few candidate proteins in a lower molecular range (<15 kDa) that were found to be upregulated or downregulated in CSF of patients with ALS [4, 5]. However, changes in the identified proteins were found to be unspecific for ALS and did not yet satisfy the criteria required for an accurate diagnostic test [4, 5].

In the present study we intended to use a proteomic detection method capable to identify a much larger number of proteins in a much higher molecular weight range in CSF samples from ALS patients. For this purpose, we used the two-dimensional fluorescence differential in gel electrophoresis (2-D-DIGE) known for its high sensitivity and high reproducibility as compared with classical 2-D electrophoresis techniques. We thereby aimed to identify

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proteins which may be specifically up- or downregulated in patients with ALS. A characteristic proteome fingerprint would provide disease-related biomarkers for clinical practice and could in the long run allow new insight into the pathomechanisms underlying ALS.

Experimental Procedure

CSF samples were collected in a prospective study by the Department of Neurology, University of Ulm (Germany) from 14 patients with probable or definite sporadic ALS according to revised EL Escorial criteria (Table 1) [6]. The disease presented as classical ("Charcot") ALS in 10 patients and as bulbar-onset in 4 patients. Disability was rated using Medical Research Council sumscore (MRCS) [7] and Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS) [8]. At time of lumbar puncture, 4 patients (28.6 %) were being treated with Riluzole (50 mg twice a day). The control group consisted of 14 age- and sex-matched patients who presented with tension-type headache and showed no evidence of a structural, haemorrhagic or inflammatory lesion. Informed consent was obtained from all patients. Aliquots of 1 ml CSF were stored in polypropylen tubes at −80°C until analysis.

Details on handling and storage of CSF samples, sample preparation, fluorescence labeling, isoelectric focusing and electrophoretic procedure, MALDI-TOF mass spectroscopy, protein identification criteria and data analysis have been published before by our group [9].

We used two-dimensional difference in gel electrophoresis technology allowing a simultaneous co-separation of multiple samples. For each of the three 2-D-DIGE experiments we labeled 50 μg protein of each sample pool (ALS, control, internal standard) with 400 pmol of the appropriate CyDye. Gel images were analyzed with commercial available DeCyder software (version 5.0, Amersham Biosciences) using DIA (Difference In Gel Analysis) module and BVA (Biological Variation Analysis) module. Protein spots that showed a significant difference between patients and controls over three independent 2-D-DIGE gels were selected for further analysis with MALDI-TOF mass spectrometry. Protein spot digestion and mass spectrometry were performed by TOPLAB GmbH (Martinsried, Germany). Differentielly expressed protein spots of interest were manually picked and trypsinated. For MALDI-TOF MS analysis, trypsin peptide solutions were spotted on stainless-steel MALDI sample plates, mixed with matrix solution and analyzed with a Voyager DE-STR (Applied Biosystems) MALDI-TOF mass spectrometer. Each spectrum was internally calibrated using the monoisotopic protonated masses of trypsin autolysis peptides. The observed m/z values between 700 and 4,200 were submitted to ProFound (Genomic solutions Inc., UK, version 2004.01.26) for peptide mass fingerprint searching and spectra were analyzed by searching the non-redundant protein database National Centre for Biotechnology Information (NCBI). For database search, a mass range of 5–200 kDa and a pI range of 2–14 was applied. The chosen taxonomy was homo sapiens and a mass tolerance of 100 ppm was used.

For validation experiments we used a western blot technique as previously described [10]. Native CSF-samples (8 μg protein/lane, denaturated in reduced Lämmli sample buffer) were electrophoresed on a precast 12% SDS polyacrylamid gel (Pierce, Rockford, USA) at 150 V. After electrophoresis, proteins were transferred to Polyvinylidendifluorid membrane (Invitrogen, Carlsbad, USA) at 30 V. After blocking in 2% ECL advanced blocking reagent (GE Healthcare Biosciences, Europe) in TBS for 1,5 h at RT and 5-times rinsing in TBS/Tween (0.05%), blots were co-incubated with rabbit anti human ZAG (BioVendor, Heidelberg, Germany) 1:5,000 and mouse human anti albumin (1:7,500) antibodies over night at 4 C. After consecutive washes, blots were co-incubated with horse radish peroxidase (HRP)-conjugated goat anti rabbit and goat anti mouse secondary antibodies (both 1:7,500) for 1.5 h at RT. The HRP complex was detected by Enhanced Chemiluminescence Plus System (GE Healthcare Biosciences, Europe). Immunoreactive images of the blots were scanned with Ettan Dige Imager (GE Healthcare Biosciences) and densities of immunoreactive bands were analyzed using Image Quant TL software version 2005 (GE Healthcare Biosciences). Furthermore, we used MagicMark XP Western Protein Standard marker (range: 20–200 kDa) and as control 10 ng/lane of recombinant ZAG protein (BioVendor, Heidelberg, Germany).

Zinc-alpha-2-glycoprotein was determined using the Human Zinc-Alpha-2-Glycoprotein ELISA (BioVendor, Laboratory Medicine, Inc.) according to the instructions as supplied by the manufacturer. Differences between groups

Table 1 Demographic data and CSF/serum albumin quotient (Q_{alb}) of the subjects included in this study

	n (female/male)	Median (range)				Q_{alb}
		Age (years)	Disease duration (months)	MRCS	ALSFRS	
ALS	14 (8/6)	63.5 (46–80)	16.5 (3–48)	54 (38–60)	34.5 (26–37)	7.3 (4.9–10.5)
Controls	14 (8/6)	63.5 (48–84)				7.3 (2.9–18.3)

were compared using the two-sided Wilcoxon two-sample test. *P*-values <0.05 were considered significant.

Results

We observed no significant difference regarding routine CSF analysis (cell count, total protein content, albumin CSF/serum quotient Q_{alb}, lactate concentration) between patients and controls.

As a result of three independent 2-D-DIGE gels 2312–2488 total spots could be detected (difference due to three independent 2-D-DIGE runs) and 185–254 of them were different in spot volume (Fig. 1). Analysis of only those spots with a significant difference over all three gels between ALS and controls revealed 6 spots, corresponding to 6 different proteins. We identified 2 proteins that were upregulated (alpha-1-antitrypsin precursor, Zn-alpha-2-glycoprotein (ZAG)) and 3 proteins (ceruloplasmin precursor (CPP), transferrin, beta-2-microglobulin, an additional protein that could not be identified due to a very low CSF concentration) that were down-regulated in CSF in ALS (Table 2).

For one of the candidate proteins (ZAG), western-blot analysis was performed for the 14 ALS patients and controls included in the 2-D-DIGE analysis (results are presented in Table 3, Fig. 2).

ELISA on the same patients and controls showed CSF ZAG to be elevated in ALS as compared to controls ($P = 0.042$, Fig. 3). We observed no significant difference between ALS and controls with regard to ZAG serum concentrations ($P = 0.68$).

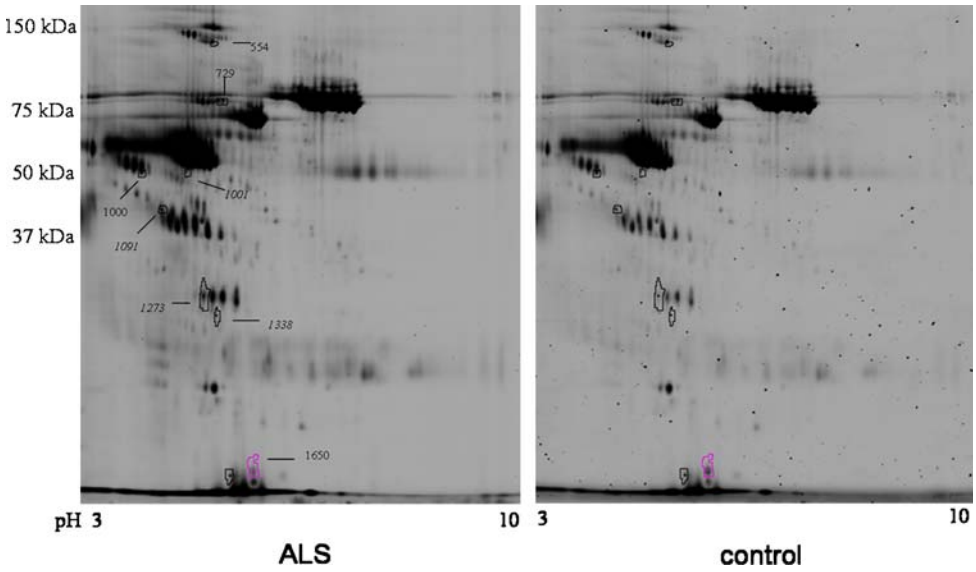

Fig. 1 Difference in-gel analysis of CSF from patients with ALS versus controls analyzed with 2-D DIGE and DeCyder Difference Analysis Software. Left side: ALS sample pool, right side: control sample pool. Marked spots showed a significant difference between patients and controls over three independent 2-D-DIGE gels. Scripture in italics = up-regulated spots; other spots are down-regulated. Spot 1050 is coincidentally selected in the analysis program

Table 2 Proteins in CSF in ALS identified by 2-D DIGE and mass fingerprinting analysis, with a significant difference in CSF protein concentration

Spot ID	*T*-test	Factor of regulation	Protein name	Accession number	Mw/Pi[a] (kDa/pH)	Sequence coverage[a]
1001	0.045	1.58	Alpha-1-antitrypsin precursor	177836	46.9/5.5	57
1091	0.031	1.56	Zn-alpha2-glycoprotein	38026	35.0/5.7	56
554	0.035	−1.29	Ceruloplasmin precursor	4557485	123.0/5.4	35
729	0.036	−1.52	Transferrin	4557871	79.3/6.9	47
1000	0.022	−1.79	No identification possible			
1650	0.0017	−2.04	Beta-2 microglobulin	34616	12.9/5.7	[b]

Factor of regulation describes the relation of the spot volumes to another (ALS vs. CTRL)

[a] As obtained by ProFound (Genomic Solutions Inc., UK, Version 2004.01.26) searching the National Centre for Biotechnology Information (NCBI) nonredundant protein database

[b] Not available due to very low protein concentration

Table 3 Results of Western Blot analysis of ALS samples and their age-and sex-matched controls (CTRL)

Patient Nr.	Diagnosis	Volume albumin[a]	Albumin relative protein content (%)	Volume ZAG[a]	ZAG % relative protein content (%)
1	CTRL	17298649	100	618763	100
1	ALS	17876741	103	759601	123
2	CTRL	6274791	100	5201756	100
2	ALS	6248155	100	6794537	131
3	CTRL	18757887	100	371998	100
3	ALS	17787096	95	1621514	436
4	CTRL	18313763	100	2629907	100
4	ALS	19697073	108	2532308	96
5	CTRL	5668076	100	4637471	100
5	ALS	5563825	98	4982510	107
6	CTRL	14512538	100	2222936	100
6	ALS	16583345	114	11811151	531
7	CTRL	15936210	100	6206042	100
7	ALS	16470113	103	9192557	148
8	CTRL	17226792	100	11305198	100
8	ALS	18864985	110	12358722	109
9	CTRL	11677213	100	5140326	100
9	ALS	11941044	102	9234692	180
10	CTRL	5852891	100	4208419	96
10	ALS	6018068	103	4388388	100
11	CTRL	13019193	100	9541377	100
11	ALS	12872458	99	10614231	111
12	CTRL	6803740	100	4145780	100
12	ALS	6844751	101	5521355	133
13	CTRL	8740592	100	2790075	100
13	ALS	8577114	98	2702080	97
14	CTRL	8159943	100	3709555	100
14	ALS	7307264	90	2096368	57

Albumin was used as loading control

[a] As measured by densitometric analysis of Western Blot using Ettan Dige Imager and Image Quant TL software version 2005 (Amersham Biosciences)

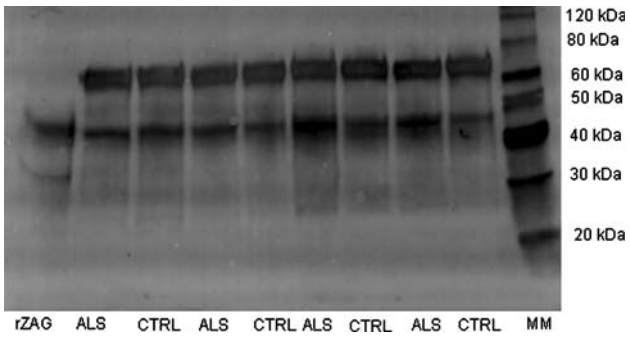

Fig. 2 Western blot analysis of CSF Zn-alpha-2-glycoprotein (ZAG) in ALS and matched controls (CTRL), including patients and controls 2, 5, 10, and 12 as shown in Table 3. MM = marker of molecular mass, rZAG = human Zn-alpha-2-glycoprotein, recombinant protein (BioVendor Laboratory Medicine, Inc.), thick bands = albumin (loading control), slight bands = ZAG

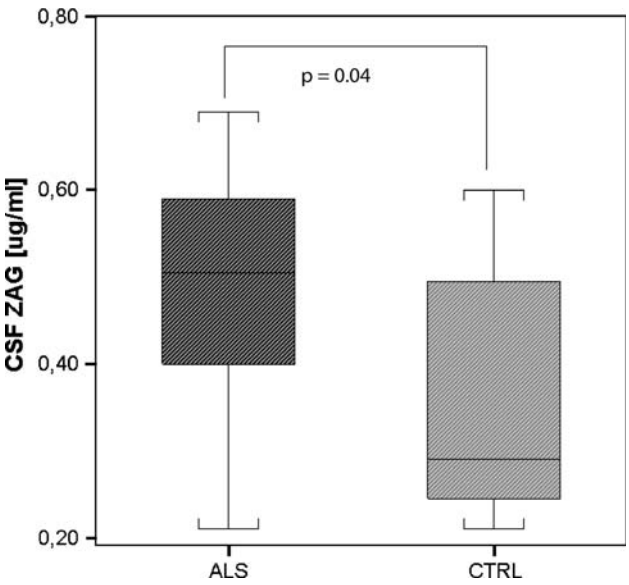

Fig. 3 Box plots show CSF Zn-alpha-2-glycoprotein (ZAG) concentrations in ALS and matched controls (CTRL). The box represents the 25th to 75th quartile, the whiskers represent the range, and the horizontal line in the box represents the median

Discussion

Methodological Considerations

To our knowledge this is the first report on analysis of CSF from ALS patients using the 2D-gel electrophoresis as a detection method. A basic problem in 2D proteome analysis of CSF is that concentrations of the targeted brain specific proteins are rather low as compared to an abundance of extra-cerebral proteins like albumin and immunoglobulins. Accordingly, CSF has to be pre-processed by extracting the bulk of extra-cerebral proteins followed by concentration of the remaining fluid. To allow for these pre-processing steps, large quantities of CSF are necessary, a demand which in the present study was met by an approach using pooled CSF samples. Another rationale for pooling of CSF was to minimize potential inter-individual differences of CSF protein content between single patients which may be due to a high degree of disease heterogeneity in ALS [11] as well as to non-disease related external influences. To our opinion, such differences of individual CSF protein content may contribute to controversial results of previous CSF proteome studies in ALS [4, 5]. However, pre-processing of CSF may also limit the validity of the present approach due to several potential drawbacks: Removal of the abundant extra-cerebral proteins may also nonspecifically remove other,

potentially interesting proteins of low concentration, or proteins bound to the discarded proteins of high concentration. Furthermore, pooling of CSF may hinder the detection of markers of different ALS subtypes linked with potentially different pathomechanisms.

The sensitivity of the present approach was also limited by the use of colloidal brilliant blue staining of proteins after electrophoresis. This technique is well compatible with mass spectrometric protein identification and commonly used, but its disadvantage is a detection limit of about 200 ng protein/spot. Therefore, detection of low abundant CSF-proteins was a priori limited in the present study.

Discussion of the Proteins Identified

To our best knowledge, ZAG has not been linked with ALS pathology so far. Though ZAG is widely distributed in different body fluids and epithelia [12], studies investigating ZAG in CSF are scarce. Interestingly, ZAG was described to be similarly affected in CSF by a study investigating the proteome of patients with frontotemporal dementia (FTD) [13]. Though none of the ALS patients included in our study showed clinical symptoms of FTD, the concurrence of FTD and ALS is well known [14]. Therefore, it seems intriguing to speculate on a common mechanism underlying elevation of ZAG in CSF in both FTD and ALS. Western blot analysis and ELISA confirmed our finding of elevated CSF ZAG in ALS (Figures 2 and 3, Table 3). However, analysis by ELISA showed a considerable overlap between the groups. The capacities of ZAG as a diagnostic marker in ALS may therefore be limited and need further investigation on a larger cohort of patients.

As in the case of ZAG, the mechanisms leading to low CPP concentrations in ALS (Table 2) remain a matter of speculation. While defects of the copper-system were discussed in various neurodegenerative diseases [15] and gain-of-function mutations in the cytosolic copper enzyme superoxide dismutase have been associated with motor neuron degeneration in familiar forms of the disease [11, 16], there is no current evidence linking CPP with sporadic ALS.

Some proteins identified here (transferrin, alpha-1-antitrypsin precursor) have been described by previous CSF proteome studies investigating various neurological diseases [5, 9, 10, 13, 17, 18].

CSF transferrin was decreased in ALS, which is in accordance with our own findings in patients with multiple sclerosis and Guillain-Barré syndrome [9, 10]. Similarly, alpha-1-antitrypsin precursor was elevated in ALS, which is consistent with our observations in GBS [9]. Proteins like transferrin or alpha-1-antitrypsin precursor seem to be unspecifically affected by different neurological diseases and may in turn be of limited value as disease-related biochemical markers in ALS. This may also be the case for beta-2-microglobulin, which has been described to be differently affected by various neurological diseases [19, 20].

Conclusion

Our study provides new CSF candidate markers of disease in ALS. The 2D-proteomic results could be confirmed for one candidate protein (ZAG) using western-blot analysis and ELISA. However, due to limitations regarding the methodological approach as well as the numbers of patients included, this study should be regarded as a pilot study that makes further evaluation of the identified candidate markers necessary. The elevation of ZAG in ALS will have to be confirmed on a larger cohort of patients and will have to be analyzed with regard to clinical parameters including subtypes of ALS and prognostic relevance.

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