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Proteomic profile of cerebrospinal fluid in patients with multiple sclerosis using two dimensional gel electrophoresis

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Multiple sclerosis (MS) is a highly heterogeneous disease. Its pathology is characterized by a combination of factors such as inflammation, demyelination and axonal damage.[1] The median age of the disease onset is 29.2 years with a higher prevalence in women as compared to men. [2] Although the etiology remains largely unknown, MS is generally believed to be of autoimmune origin. [3,4] Current diagnosis depends on clinical assessment supported by paraclinical tests including magnetic resonance imaging to visualize lesions and cerebrospinal fluid (CSF) biochemistry assessment that includes oligoclonal bands and CSF indices.[5] However, the diagnosis of MS is not straightforward because of absence of reliable serological or CSF tests.

Proteomics technology has proven to be a powerful tool to provide a comprehensive protein expression of biological samples.[6] There has been an intensive scientific interest directed towards CSF biomarkers because CSF is the closest sample next to the disease process.[7] The absence of an active clotting system in CSF is a further advantage for most analysis techniques. In addition, complicated pre-treatment procedures used in serum peptide profiling are not needed in CSF proteome profiling due to the lower CSF protein load.[8] It is likely that by studying several proteins with proteomic techniques, a better description of the disease state is obtained rather than with a single protein.[9]

Using two dimensional gel electrophoresis (2-DE) and mass spectrometry, the present study aimed to analyze CSF proteome changes in MS patients in order to identify candidate biomarkers with potential clinical utility based on the ability of CSF protein profiles to discriminate healthy persons from MS patients.

CSF samples were collected from seven remitting-relapsing MS patients and seven controls. The approval of Medical Ethics Committee and consents of subjects were obtained. A detailed history was taken from subjects enrolled in the study. Samples were collected by lumbar puncture under strict aseptic technique in polypropylene tubes. The samples were centrifuged 10 min at 4 °C 1000*g* without break. The supernatant was kept at −80 °C.

First dimensional electrophoresis was conducted on IPGphor isoelectric focusing system (Amersham Pharmacia Biotech). Protein quantitation was done using the bicinchoninic acid assay (Sigma Aldrich BCA-1). Twenty µg of the CSF proteins were added to a rehydrating solution (9.8 M urea, 4% CHAPS and 50 mM DTT) and 1 µl of IPG buffer (pH 3–10). For a 13 cm strip, the total volume of the rehydration solution with the sample included was 250 µl. After being covered with paraffin oil, the IPG dry strips (GE health care, Immobiline Drystrip, pH 3–10, 13 cm) were rehydrated by low voltage (30–50 V) applied for 7 h. They were then subjected to gradual increase in focusing voltage reaching 8000 V. The strips were equilibrated for 30 min in equilibrating buffer I (72.07 g urea, 6.7 ml tris/trisHCl 1.5 M pH 8.8, 60 ml glycerol 100%, 50 ml distilled water, 20 ml SDS 10%) with dithiotreitol (0.0125 g/10 ml equilibrating buffer per strip) and then for another 30 min in a second equilibrating buffer with iodoacetamide (0.225 g/10 ml equilibrating buffer per strip). The IPG strips were placed on the top of the SDS–PAGE 12% gels prepared according to Lammeli.[10] The gels were set up in the migrating bugger and run for 17 h at 70 V and 200 mA using an Amersham Pharmacia Biotech power supply. Visualization of the proteins in gels was performed with silver stain as described by Shevchenko et al. [11]. The gels were scanned using ImageMaster Labscan V3.01 (Amersham Biosciences, UK) (Figure 1). Analysis was

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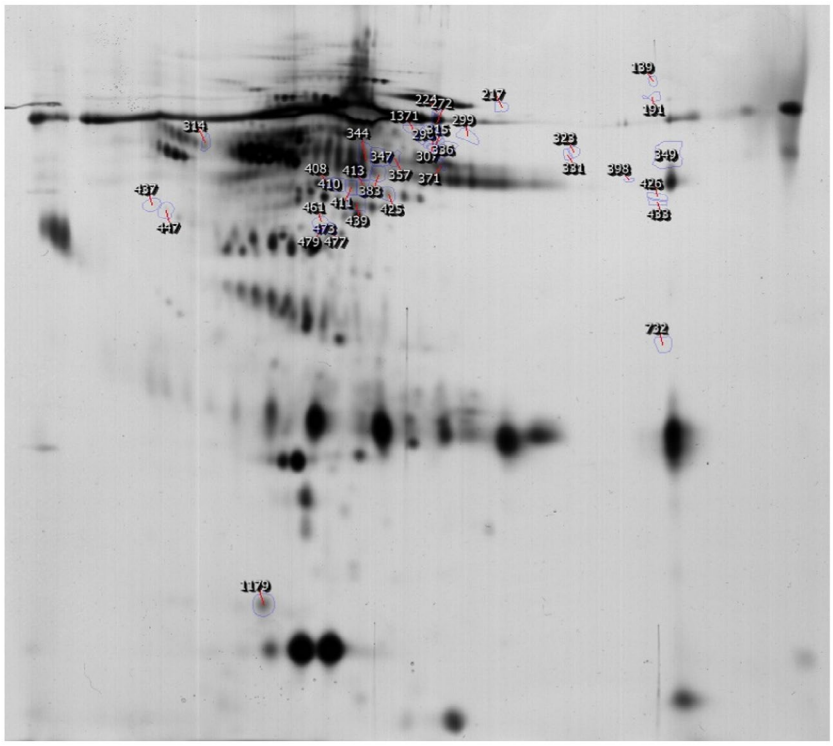

Figure 1. 2-DE reference gel of CSF individual analysis. CSF proteins were separated in a dry strip pH 3–10 for the first dimension, 12% SDS–PAGE for the second dimension, and silver stained.

done using Progenesis SameSpots software which used one-way ANOVA test and maximum fold change to find spots showing expression difference.

In order to decrease the inter-individual variations and to confirm the spots which seemed to be different, two pools of CSF were prepared; the first was composed of the patients' samples and the other was composed of the controls samples. 2-DE was performed as described above.

Based on the results of the individual and the pool analyses, some spots were picked and trypsin-digested manually. The trypsin digested peptide mixture of each spot was analyzed by Ultraflex II MALDI-TOF/TOF (BrukerDaltonics, Germany). Mass spectra were acquired automatically using the AutoXecute™ module of Flexcontrol™ (Bruker Daltonics). The spectrum peaks were analyzed using FlexAnalysis™ software (Bruker Daltonics) and calibrated internally with the autoproteolysis peptides of trypsin (*m/z* 842.51, 1045.56, 2211.10). Protein samples that could not be identified by MALDI-TOF/TOF analysis were sent for identification using LC–MS/MS. The machine is composed of U3000 nano-LC system (Dionex, Amsterdam, Netherlands) coupled to a Linear Trap Quadrupole LTQ Orbitrap XL with electron-transfer dissociation (ETD) (Thermo Fischer Scientific). The Spectra were loaded into Proteome Discoverer 1.3 software (Thermo Fisher Scientific). Peptide identification of proteins was performed by searching against the human proteome entries (CSF_human) of the Mascot v2.2 algorithm (www.matrixscience.com) with trypsin enzyme specificity and one trypsin miscleavage. Carbamidomethyl of cysteine was set as fixed modification and oxidation of methionine was set as variable modification for searches.

Subjects of this study were six men and one woman cases vs. five men and two women controls (P = 1.0, Fisher's exact test). Mean age for the cases was 37.9 ± 13.2 years while in the control group it was 30.4 ± 11.4 years (t test P = 0.318). The mean protein value for cases was 0.65 ± 0.26 µg/µl while the mean protein value for the controls was 0.58 ± 0.11 µg/µl (P = 0.710). Protein concentration showed no statistical significant difference among the studied groups. This finding is in accordance with the view that MS patients may have normal CSF protein level even during acute episodes.[12]

In individual analysis of gels, we detected 372 spots in all gels. Spots of interest were defined as having ANOVA P-value test ≤0.05 and maximum fold change ≥2. Fifteen spots fulfiled the first condition, 30 spots the second condition, and 4 spots were able to fulfil both conditions. These four spots were up-regulated in the gels of MS patients in comparison to controls. This supports the presence of CSF proteome changes in MS patients. In comparing CSF proteome of 10 relapsing remitting MS patients with 11 patients with clinically isolated syndrome and 10 control subjects without neurological or systemic diseases, Chiasserini et al. detected a difference in the expression of six polypeptide spots in the relapsing–remitting MS group.[9] Plubinx et al. found that the intensity of seven protein spots was found to be significantly altered in the CSF of remitting relapsing MS patients compared to controls.[13]

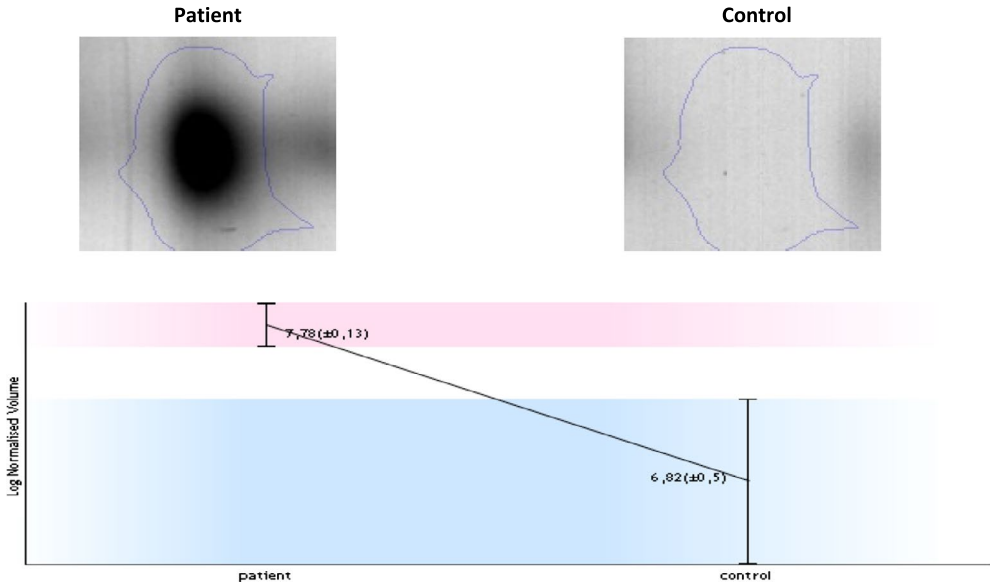

Figure 2. Zoomed shots of one statistically significant spot in the pool analysis. By analyzing the normalized volumes of this spot across the gels, significant increase was noticed in the patient pool with ANOVA *P*-value test 0.005 and maximum fold change 7.9. Data mean and standard deviation.

In the pool analysis, 36 spots had an ANOVA *P*-value ≤0.05, 99 spots had a maximum fold threshold ≥2, and 16 spots had both conditions. Of the 16 spots, 4 spots were up-regulated in the control pool while the remaining 12 spots were up-regulated in the patient pool. The pooling of samples allows decreasing inter-subject variability whilst minimizing sampling variations. It could also be advantageous because unavoidable mistakes introduced when dealing with parallel gels can be reduced.[14] Hammack et al. used pooled CSF from three patients with MS and from three patients with non-MS inflammatory central nervous system disorders. The MS gels revealed 103 protein spots that were not seen on the other gels.[15]

Fourteen spots were selected to be analyzed by the mass spectrometry. Some spots were chosen because they had significantly differential expression based on the ANOVA *P*-value in both the individual analysis and the pool analysis. Other spots were selected as they showed a significant rise in either their maximum fold change or their ANOVA *P*-value from the individual analysis to the pool analysis (Figure 2). Using MALDI-TOF/TOF, the peaks of peptides of interest emerged with difficulty from the back- ground noise and so could not be identified. This could be explained by the low protein quantity in silver-stained spots and losses that occur during the preparation of peptide digests. Using LC–MS/MS analysis, we were able to identify 4 proteins which were over-expressed in the patients in relation to the controls: Alpha-1-antichymotrypsin, Prostaglandin-H2 D-isomerase, Desmoplakin and Hornerin (Table 1)**.** It should be noted that some spots were statistically significant but it was impossible to identify them by mass spectrometry due to their inaccessibility for excision.

Alpha-1-antichymotrypsin, which was over-expressed in patients in relation to controls, is an acute phase reactant protein. It is also over-expressed in Alzheimer's disease brain, mainly in astrocytes located in the plaques and the adjacent surrounding tissue. Ottervald et al. detected increases in the CSF levels of alpha-1-antichymotrypsin, alpha-1 macroglobulin and fibulin1 in remitting relapsing MS patients.[16] Dumont et al. constructed a protein database of 2-DE separated CSF proteins samples from five MS patients. By means of liquid chromatography tandem mass spectrometry, 65 proteins including alpha-1-antichymotrypsin have been identified.[17]

Prostaglandin-H2 D-isomerase, which was over-expressed in the patient group in the present study, is anti-apoptotic in oligodendrocytes, and a neuromodulator and trophic factor. Füvesi et al. detected seventy-eight proteins including prostaglandin-H2 D-isomerase in a fulminant case of MS using isobaric tag labeling and nanoflow liquid chromatography in conjunction with MALDI-TOF/TOF.[6]

Desmoplakin, a major high molecular weight protein of desmosomes, was found to be up-regulated in our patient group. Gawinecka et al. detected an elevated CSF level of desmoplakin in 78% of the Creutzfeldt-Jakob disease patients when compared to controls.[18] Hornerin, found in keratohyalin granules in the granular cells of the epidermis, was up-regulated in our patient group. According to Dumont et al., it is a potential skin contaminant introduced during sample collection and preparation.[17]

The study supports the hypothesis that CSF protein profiles can discriminate healthy persons from MS patients helping to search for biomarkers with potential clinical utility. The detected profiles should be validated in larger studies including different types of patients. This

Table 1. Proteins identified in CSF from multiple sclerosis patients.

Spot ID in the pool analysis	Accession number	Protein	Gene	Matched peptides	Cellular component	Molecular Function	MW [kDa]
432	P01011	Alpha-1-antichymotrypsin	Serpina3	4	Secreted	Protease inhibitor	47.6
1192	P41222	Prostaglandin-H2 D-isomerase	PTGDS	3	Cytoplasm, endoplasmic reticulum, golgi apparatus, membrane, nucleus and secreted	Isomerase	21
504	P15924	Desmoplakin	DSP	4	Cytoskeleton	Structural constituent of cytoskeleton and protein binding, bridging	331.6
531	Q86YZ3	Hornerin	HRNR	3	Cornified envelope	Developmental protein	282.2

work represents an advance in biomedicine because it promotes the identification of potential CSF biomarkers that could help in the diagnosis of MS.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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