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ORIGINAL PAPER

Comparative Proteomics Analysis of Cerebrospinal Fluid of Patients with Guillain–Barré Syndrome

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Abstract To better understand the pathophysiologic mechanisms underlying Guillain–Barré syndrome (GBS), Comparative proteomic analysis of cerebrospinal fluid (CSF) between patients with GBS (the experiment group) and control subjects suffering from other neurological disorders (the control group) was carried out using two-dimensional gel electrophoresis (2-DE) technique, in combination with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and database searching to determine abnormal CSF proteins in GBS patients. Image analysis of 2-DE gels silver stained revealed that 10 protein spots showed significant differential expression between the two groups of CSF samples. The expression of cystatin C, transthyretin, apolipoprotein E and heat shock protein 70 were decreased. However, haptoglobin, α-1-antitrypsin, apolipoprotein A-IV and neurofilaments were elevated. The subsequent ELISA measured the concentration of cystatin C and confirmed the result of the proteomic analysis. These identified proteins may be involved in the pathophysiological process of GBS and call for further studying the role of these proteins in the pathogenesis of the disease.

Keywords Cerebrospinal fluid · Guillain–Barré syndrome · Proteome · Two-dimensional electrophoresis · Mass Spectrometry · ELISA

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Introduction

Guillain–Barré syndrome (GBS) is an immune-mediated inflammatory disease of the peripheral nerves involving mainly the myelin sheath. GBS occurs worldwide and with an often severe course and poor prognosis (Hughes and Cornblath 2005). Even when patients are treated in well-equipped intensive care units, mortality rates are still 3–7%. Moreover, about 7–15% of patients have permanent neurological

sequelae (Ropper 1992), which severely affect the patients daily life. However, the molecular mechanisms underlying the disease remain poorly understood and so far no reliable disease-related markers are available. Increased protein level without pleocytosis in the cerebrospinal fluid (CSF) is a characteristic of patients with GBS, which suggest that CSF is a promising source of proteins that may provide important information about the pathomechanisms in GBS.

Now, the proteomic approach for identification and quantification of the entire protein content (proteome) of the organism is helpful in discovery of biomarkers. Therefore, the study of proteome of CSF will provide new insight into the pathomechanisms underlying GBS. In this study, by means of proteomic analysis with high-resolution two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS) and database searching, the proteins which may be specifically up or down regulated in patients with GBS maybe provided as disease-related proteins to both aid in the diagnosis of GBS and possibly its treatment and prognosis.

Materials and Methods

CSF Samples

In the experimental group, the CSF samples were taken from eight patients with GBS of the typical demyelinating according to Asbury diagnostic criterion (Asbury and Cornblath 1990) including electromyogram (EMG) used to substantiate demyelinating and not axonal variety of GBS. The obvious clinical signs of patients had lasted on average 4 (2–7) days, which means the nadir phase clinically of the disease. Six of them reported a preceding infection (mild diarrhoea or flu-like symptoms before the onset of GBS). At the time of lumbar puncture, none of the patients received an immunomodulatory therapy like IVIg or plasmapheresis. In the control group, CSF samples were taken from 12 patients with other neurological disorders (headache but normal CSF). Lumbar puncture was performed in the L4–L5 intervertebral space, CSF samples that became turbid or mixed with blood were excluded in this study. Each case had 1 ml of CSF sample obtained and centrifuged at 16,000 g for 10 min to eliminate cells and other insoluble materials. The samples were then stored at −80°C until analysis. The study was approved by the local Ethical Committee at Qilu Hospital, Shandong University, China. All the patients were from Qilu Hospital of Shandong University and gave informed consent to participate in the study. The clinical and laboratory features of the patients are summarized in Table. 1.

Chemicals and Materials

Urea, Acrylamide, Bis, DTT and Tris were from BIO BASIC INC. CHAPS, glycine, SDS, agarose, sodium acetate, sodium carbonate and sodium thiosulphate were from Amresco. Glycerol, mineral oil, Coomassie Blue R-350, IPG buffer, Immobiline DryStrips (3–10 NL, 18 cm) and 2D Quant kit were purchased from GE. TEMED, Iodoacetamide, silver nitrate, ammonium bicarbonate and acetic acid (HAc) were from Sigma. Acetonitrile and methanol were from Fisher. TFA was from Merck. 2-DE protein markers were from HyClone, and Trypsin (sequencing grade) was obtained from Boehringer Mannheim. Human cystatin C ELISA kit was from Biovendor. All buffers were prepared with Milli-Q water.

Sample Preparation for Electrophoresis

The solubilization was done according to the protocol described by Sanchez and his co-workers (Sanchez et al. 1997) with our own modifications. Briefly, 600 μL CSF were precipitated with 100% ice-cold acetone in a 4:1 ratio of acetone to CSF and stored overnight at −20°C to remove salt from the sample. Then the mixture was centrifuged at 16,000 g at 4°C for 30 min. The pellet was washed twice with 90% acetone and air-dried. The precipitated proteins were solubilized in a lysis buffer containing 8 M urea, 4% CHAPS, 65 mM DTT and 2% IPGbuffer. The protein concentrations were measured with 2D Quant kit according to the manufacturer's instructions.

2-DE

The first dimension of 2-DE was run on an IPGphor isoelectric focussing electrophoresis (IEF) system (GE Healthcare). A 100 μg of proteins for analytical runs or 1,000 μg for preparative runs were mixed with a rehydration solution containing 8 M urea, 2% CHAPS,

Table 1 The clinical and laboratory features of GBS and controls

Diagnosis	N (male/female)	Age (years)	Qabl	CSF IgG (mg/l)	Disease duration (days)
GBS	8 (4/4)	43 (20–56)	16.5 (4.0–33.5)	46.1 (30.2–99.6)	4 (2–7)
Controls	12 (6/6)	46 (18–61)	5.4 (3.1–15.0)	21.1 (13–42.5)	3 (1–5)

0.5% IPG buffer, 18 mM DTT and a trace of bromophenol blue to a total volume of 340 μl and applied to IPG dry strips (18 cm, 3–10 NL). After active rehydration for 12 h at 30 V and 20°C, IEF was performed for both analytical runs and preparative runs as follows: 200 V for1 h; 500 V for 1 h; 1,000 V for 1 h; gradient to 8,000 V over 2 h; 8,000 V for 60,000 V. After IEF, the gel strips were equilibrated for 2 × 15 min in an equilibration buffer containing 50 mM Tris–HCl (pH 8.0), 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue. Around 1% DTT was added to the first equilibration buffer, and in the second equilibration buffer, DTT was replaced with 2.5% iodoacetamide. After washing with electrophoresis buffer (10 × electrophoresis buffer contains 250 mM Tris, 1,920 mM glycine, 1% SDS, approximately pH 8.3), equilibration strips were placed on 12% SDS-polyacrylamide gels (20 × 20 × 1.0 mm) and overlaid with 0.5% agarose solution containing a trace of bromphenol blue. The run in the second dimension was carried out vertically in an PROTEANIIxi Cell electrophoresis system (BIO-RAD), SDS-PAGE was performed for 30 min at a constant current of 10 mA/gel and then at 30 mA/gel until the bromophenol blue reached the bottom of the gels.

The CSF samples were run individually in the same conditions. After 2-DE, the analytical gels were stained with silver nitrate based on procedure described by Hochstrasser (Hochstrasser et al. 1998), and the preparative gels were stained with Coomassie Blue.

Image Acquisition and Analysis

The silver-stained 2-D gels were scanned at an optical high resolution using a UMAX PowerLook 2100XL imaging densitometer. Spot detection, quantification and matching were performed using Image Master 2D-Elite 5.0 software package (GE Healthcare) and images were checked manually to eliminate artefacts. In the Image Master 2D-Elite 5.0 software, following spot detection, a matchset was built which included all the experimental and control gels. A reference gel was selected from one of the experimental gels and unmatched protein spots of the member gels were automatically added to the reference gel in order to correct for variability due to silver staining and to reflect the quantitative variations of protein spots, The raw quantity of each spot in a member gel was divided by the total quantity of the valid spots in the gel (i.e. all the spots in the gel minus cancelled spots) and expressed as ppm (%vol). The same quantity of protein was in each gel, which was to say the volume of all spots in each gel was equal. Therefore the mean ppm of each matched protein spot in the GBS group was compared with the mean ppm of the same protein spot in control group. The significance of expression differences of protein spots between experimental and control groups were estimated by the Independent-samples *t*-test, $P < 0.05$. The Mr and pI of each protein spot were calibrated with protein markers.

In-gel Digestion of Proteins

The protein spots that were found altering significantly of interest were manually excised from the preparative gels, destained in microtubes with 50 mmol/l NH_4HCO_3, 50% acetonitrile, after being dried completely by centrifugal lyophilization. Trypsin solution (20 ng/μl in 50 mM NH_4HCO_3) was added and the samples were digested at room temperature (RT) overnight. The gel pieces were extracted with 200-μl extraction solution of 60% acetonitrile and 0.1% TFA for 20 min with sonication, the extraction was repeated three times, the supernatant was removed and dried.

MALDI-TOF MS and Database Searching

Peptide extracts were analyzed on a MALDI-TOF MS (ABI) in positive ion reflection mode. The accelerating potential was 20 KV, Trypsin autodigestion peaks were used as external calibration.

Peptide mass fingerprint (PMF) was obtained by being processed with DATA EXPLORER software and peptide mass data were searched against SWISS-PROT protein database with the search engine of Mascot with homo sapiens as the species searched.

ELISA

Another two batches of CSF samples were collected, one contains 30 patients with GBS and the other contains 40 healthy volunteers matched with age. The sample collection criterion was according to 2.1 and all participants gave informed consent prior to enrolment. The ELISA kit for human cystatin C (Biovendor) were used and the assays were run according to manufacture's directions. The data was acquired on the model 680 microplate reader (Bio-RAD) by reading absorbances at 450 nm. The ELISA data were statistical analyzed and difference between experimental group and control group were assessed by the Independent-samples *t*-test. Statistical significance was defined as $P < 0.01$.

Results

Proteome Differential Expression in CSF Between GBS Patients and Control

In this study, the total proteins of the CSF samples taken from both the experiment and control groups was separated by 2-DE. The typical silver stained 2-DE gels of CSF in the GBS patients compared to the control group were shown in Fig. 1. The gels were digitized and analysed by Image Master 2D-Elite 5.0, Totally 387 ± 29 and 398 ± 36 protein spots were detected in the experimental group and control group and average of matching ratio were (90 ± 2.7) % with selecting one gel from the experimental gels as a reference gel. The regulations differential in 10 protein spots was found to differ significantly in the gels between the two groups. Six had expression levels reduced and four were increased in the experimental group in comparison to the control.

Image Master 2D-Elite5.0 analysis software can also provide 3D simulation of the protein spots, which is a more intuitionistic way to find the change. The spot 1, identified as Cystatin C later and the spot 7 as haptoglobin were showed in Fig. 2 in which the amount of protein is proportional to the volume of the protein peak showed by 3D simulation.

Identification of Differentially Expressed Proteins by MALDI-TOF MS

The different expressed protein spots of interested from the Coomassie Blue-stained gels were excised and subjected to in-gel tryptic digestion. The extracted peptides were analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and raw data were used to search database with Masscot search programme in the SWISS-PROT protein database (http://www.us.expasy.org/sprot), in which protein scores greater than 55 are significant ($P < 0.05$). The result showed these spots correspond to 10 distinct proteins or isoforms, Out of these, six protein spots were down-regulated in GBS including cystatin C, transthyretin, apolipoprotein E and heat shock protein 70. Three spots (spot 2, 3, 4) were identified as transthyretin. Whereas, four protein spots were found to be up-regulated in GBS, including haptoglobin, α-1-antitrypsin, apolipoprotein A-IV and neurofilaments. Table 2 presented the identified spots with their summary scores, protein coverage, *P*-values, %volume, etc. A typical MALDI-TOF peptide mass fingerprint spectrum and mascot search result of trypsin-digested spot 1, which was later identified as cystatin C, were showed in Fig. 3.

The Result of ELISA

For one of the candidate protein cystatin C, we measured the CSF concentrations in another two batches of CSF samples. Levels of cystatin C in experimental group was (2.79 ± 0.65) mg/l that was lower than (3.65 ± 0.82) mg/l in control group ($P < 0.01$). The result of expression change of cystatin C between the GBS patients and the controls consistent with 2-DE results (Fig. 4).

Discussion

In the present study, the comparative proteomic method was used to analyze the changes of all proteins in CSF between GBS patients and controls. The CSF samples were analyzed individually, the result can be used to show confidence interval, which

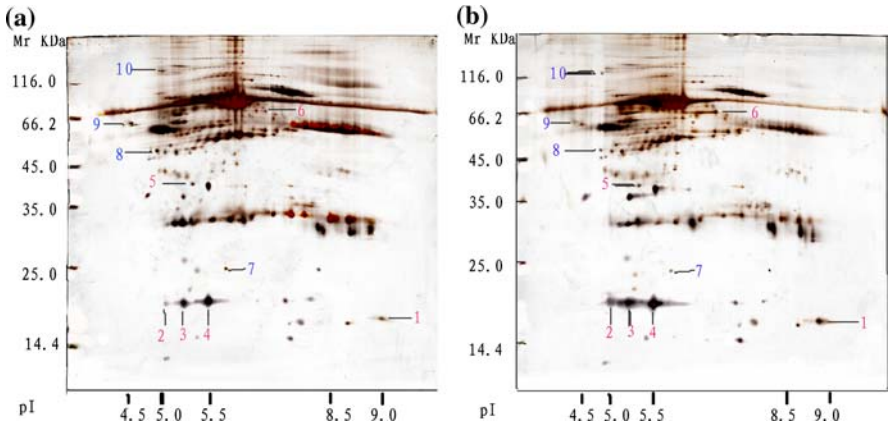

Fig. 1 Two-dimensional gel electrophoresis (2-DE) gel images of cerebrospinal fluid (CSF) proteins from Guillain–Barré syndrome (GBS) (**a**) and control (**b**). Red numbers (1–6) indicate proteins that were down-regulated in CSF of GBS patients and blue Numbers (7–10) indicate proteins that were up-regulated

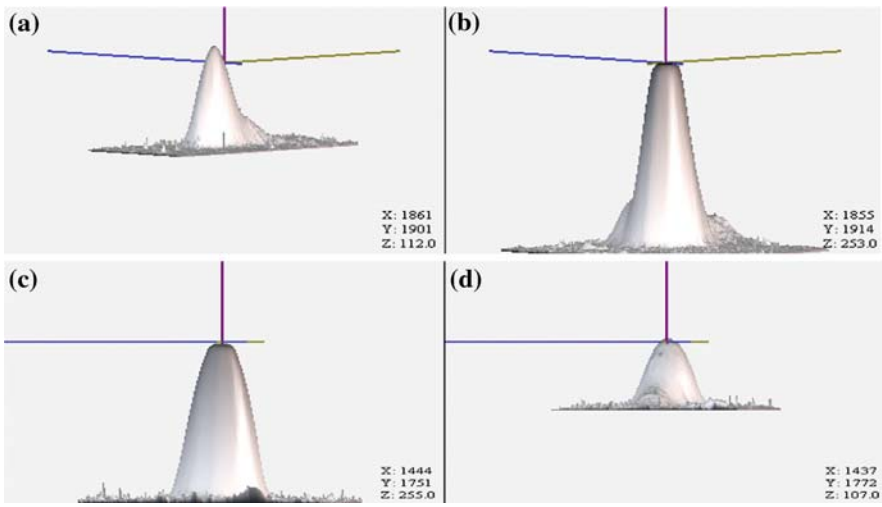

Fig. 2 3D simulation figures of protein spots acquired gel images by 2D-Elite5.0 software. (**a**) and (**b**) showed spot 1 identified as cystatin C was down-regulated in CSF of GBS (**a**) compared with the control (**b**). (**c**) and (**d**) showed spot 1 identified as haptoglobin was upregulated in CSF of GBS (**c**) compared with the control (**d**)

Table 2 Proteins changed in CSF from GBS patients compared with the control identified by 2-DE and MS

Protein number	Protein description	Swiss-prot summary	Theoretical (Mr/pI)	Measured (Mr/pI)	Score	Protein coverage (%)	% volume		*P*-value
							GBS	Control	
1	Cystatin-C	CYTC_HUMAN	15.80/8.76	15.8/9.00	112	47	0.28 ± 0.39	1.02 ± 0.56	0.0023
2	Transthyretin	TTHY_HUMAN	13.99/5.04	14.0/5.0	90	21	0.14 ± 0.61	1.21 ± 0.73	0.0015
3	Transthyretin	TTHY_HUMAN	13.99/5.24	14.0/5.2	81	20	1.13 ± 0.96	3.18 ± 1.81	0.0045
4	Transthyretin	TTHY_HUMAN	13.80/5.49	13.8/5.5	83	20	2.28 ± 1.77	6.85 ± 3.99	0.0036
5	Apolipoprotein-E	APOE_HUMAN	36.13/5.65	36.0/5.6	96	27	0.15 ± 0.09	0.28 ± 0.14	0.0163
6	Heat shock protein-70	HSP70_HUMAN	69.00/5.70	69.0/5.7	98	23	0.14 ± 0.11	0.49 ± 0.34	0.0060
7	Haptoglobin	HPT_HUMAN	17.12/5.67	18.0/5.6	81	13	0.18 ± 0.15	0.07 ± 0.037	0.0121
8	ApolipoproteinA-IV	APOA4_HUMAN	45.37/5.25	45.0/5.2	56	12	0.19 ± 0.10	0.11 ± 0.075	0.0277
9	Nurorofilament	NFL_HUMAN	61.38/4.56	61.0/4.5	75	9	0.25 ± 0.160	0.14 ± 0.081	0.0282
10	α-1-antitrypsin	A1AT_HUMAN	132.77/4.95	132.0/4.9	85	14	0.11 ± 0.089	0.05 ± 0.039	0.0263

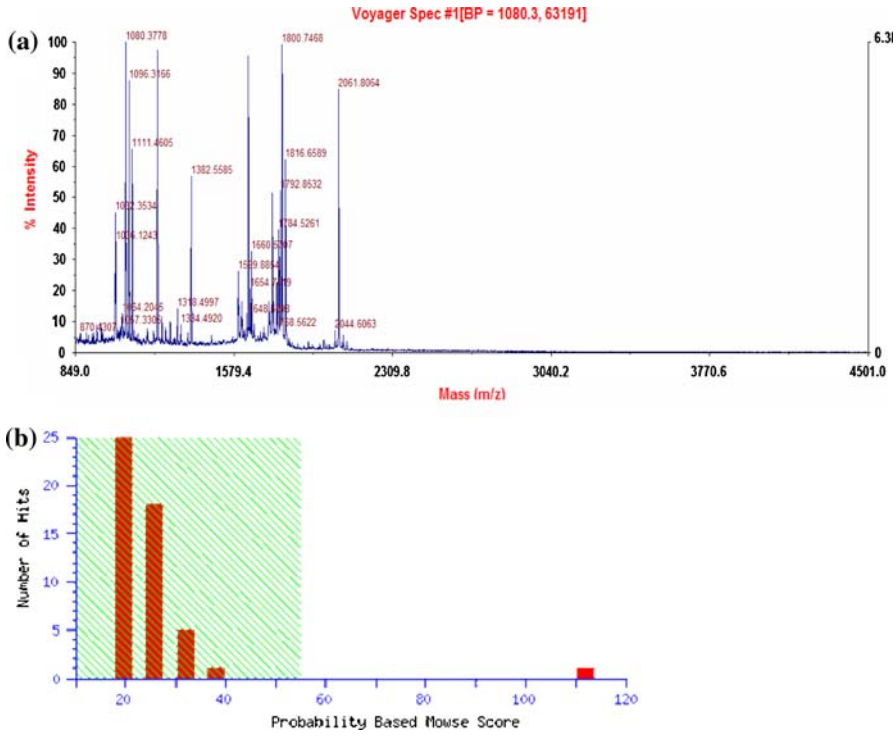

Fig. 3 (**a**) Typical MALDI-TOF peptide mass fingerprint spectrum of spot 1 identified as cystatin C later which is decreased in GBS CSF. The *x*-axis represents mass-to-charge ratio (m/z), whereas the *y*-axis represents relative abundance. (**b**) The result of the spot 1 searching Swissprot database. The *x*-axis represents probability-based mowse score whereas the *y*-axis represents numbers of hits

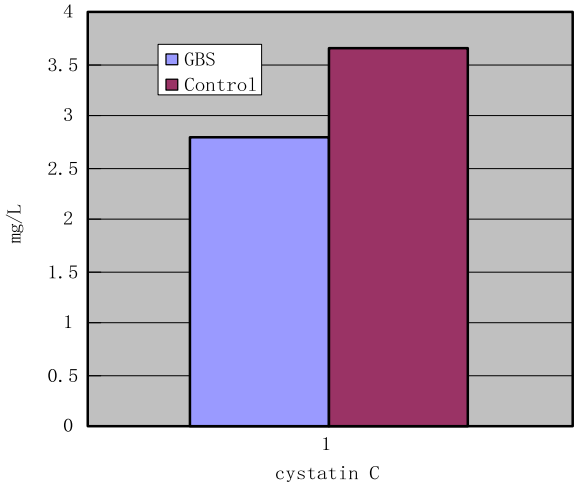

Fig. 4 ELISA results of CSF. The left post represented the concentrations of GBS CSF and the right represented the concentrations of control CSF. Data were expressed as Mean ± SD. Statistical differences are indicated: $P < 0.01$

is an obvious indicator for the population traits. Our data revealed 10 proteins spots showed significant differential expression between the two groups. After searching the database we found that in CSF of GBS patients, the expression of cystatin C, transthyretin, apolipoprotein E and heat shock protein 70 were significantly decreased. However, Haptoglobin, α-1-antitrypsin, apolipoprotein A-IV and neurofilaments were markedly increased compared to the control group. The subsequent ELISA measured the concentration of cystatin C and confirmed the results of proteomic analysis. It is assumed that the alteration of these proteins play an important role in the pathogenesis of GBS and may potentially serve as markers for participate in the nerve degeneration and regeneration.

In our study, Cystatin C was reduced distinctly in CSF of GBS group and was found to be the most significantly differential one between the two groups. Cystatin C is secrete mainly from plexus chorioideus in human CSF, the concentration of Cystatin C was [(2.04–3.58)mg/l] that was five times than [(0.50–0.96)mg/l] in serum, Therefore, Cystatin C in the CSF can be thought as cerebral protein, only less than1% is from serum (Reiber 2001). Cystatin C has been discovered to be a biomarker of pain (Mannes et al. 2003). The role of low level of Cystatin C in GBS has not been reported previously. Our finding indicated that its detection and down-regulation in the

CSF of GBS patients may be in relationship with numbness.

Transthyretin, a known negative acute phase protein, synthesized in liver cells and secreted into the CSF and plasma, was found significantly reduced in various acute liver disease (Yasmin et al. 1993; Volchkova et al. 2000), in the CSF of late stage Alzheimer's disease (Serot et al. 1997), and Amyotrophic lateral sclerosis patients (Ranganathan et al. 2005).

Haptoglobin is a plasma protein with haemoglobin (Hb)-binding capacity and is a positive acute-phase protein that functions as an inhibitor of prostaglandin synthesis and angiogenesis (Langlois and Delanghe 1996). Haptoglobin has been proposed to be involved in a highly interactive ensemble of lymphocytes, neutrophils and monocytes participating in inflammatory processes (Wagner et al. 1996; Langlois et al. 1997; Berkova et al. 1999). GBS is an acute inflammatory autoimmune disorder in the PNS. In the study, we found a high level of Hpt in the CSF of GBS patients.

However, the candidate proteins identified like transthyretin, haptoglobin and α-1-antitrypsin have been described by previous studies and implemented in a wide range of other neurological diseases including inflammatory diseases like viral meningitis, GBS or multiple sclerosis. Accordingly, these proteins seem to be unspecifically affected in different inflammatory neurological diseases and may be of limited value as disease-related biochemical markers in GBS (Lehmensiek et al. 2007).

ApoE has multiple biological properties, in addition to its physiological role in cholesterol transport, ApoE has immunomodulatory properties in vitro and in vivo (Avila et al. 1982; Laurat et al. 2001). It has been reported that ApoE acted as an inhibitor in experimental autoimmune neuritis (EAN), a model for GBS (Yu et al. 2004). Since ApoE is considered as a neurotrophic factor, consequently, any decrease in ApoE level would contribute to the progression of neurological diseases, such as GBS. Decreased CSF ApoE can also be found in multiple scleroses (MS), an analogous disease of the central nervous system (CNS) (Gaillard et al. 1998).

ApoA-IV is synthesized primarily in the intestine then secreted into the plasma, and an acute inflammation can disturb the plasma ApoA-IV concentration [Tu et al. 1987; Shen and Howlett 1993]. Up-regulation of ApoA-IV in the CSF of GBS patients is in agreement with previous reports that large concentrations of ApoA-IV aggregated in the regenerating nerve for myelin biosynthesis (Langner et al. 1991). So the level of ApoA-IV was significantly increased to function actively in the process of myelination.

Up-regulation of Nurorofilament (NFL) in CSF of GBS patients agrees with the previous report of NFL as a potential prognostic marker in GBS and axonal damage (Rosengren et al. 1996) in CSF. There is evidence that NFL mRNAs are transcribed and translated locally in a manner related to nerve regeneration, and they are up-regulated in response to nerve injury (Sotelo-Silveira et al. 2000). The level of NFL increase may be caused by the discharge of abnormal NFL, possibly by oxidative modifications, which were also found involved in the pathophysiologic process of GBS in this study and has been observed to be responsible for the NFL abnormalities in several oxidative-stress-related neurodegenerative diseases, notably AD (Hu et al. 2002), Parkinson's disease (Goldman et al. 1983), and amyotrophic lateral sclerosis (Manetto et al. 1988).

The function of heat shock protein-70 and in GBS is unclear and presently remains a matter of speculation. Further studies will be necessary to clarify a possible involvement of the protein in GBS pathology.

Althoug some papers in studying the proteome of CSF in GBS has been reported (Lehmensiek et al. 2007; Jin et al. 2007), the results of this study mainly based on 2-DE, in which cystatin C and neurofilament were first reported, was supplement to proteome of CSF in GBS. Because of complexity of 2-D method, the number of CSF samples cannot be too large in the action, which ELISA was to make up in this study. Further study with a larger group of GBS patients including different disease stages will evaluate the diagnostic sensitivity and specificity of the candidate biomarkers. More detailed ELISA validation will have to further confirm the present data.

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