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Differential Expression of Complement Proteins in Cerebrospinal Fluid From Active Multiple Sclerosis Patients

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ABSTRACT

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system with complex immunopathogenesis. Using the 2-D DIGE technology, we separate CSF proteins from patients with active MS and control subjects. Three of the seven differential proteins identified were related with complement system, and the network analysis of the differential proteins revealed complement activation involvement in active MS. Complement C4b (gamma chain) was confirmed elevated by performing western blotting analysis ($P < 0.01$). The present results are an independent quantitative proteomic measure in CSF from active MS patients. The differential expression of the complement C4b and related proteins in CSF provides potential biomarkers as well as evidence for the involvement of complement activation in the pathogenesis of MS disease. J. Cell. Biochem. 112: 1930–1937, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: MULTIPLE SCLEROSIS; 2-D DIGE; CEREBROSPINAL FLUID; COMPLEMENT

Multiple sclerosis (MS) is a common demyelinating disease of the central nervous system (CNS) with complex immunopathogenesis, in which the myelin sheaths and axons are destroyed with unclear etiology and pathogenesis [Tumani et al., 2009]. The disorder results from an interplay between as yet unidentified environmental factors and susceptibility genes. Up to now, MS is recognized throughout the world, with around 2.5 million affected individuals. It is a potentially disabling disease for young adults [Compston and Coles, 2002]. Relapsing-remitting MS (RRMS) is the most common clinical subtype, which is characterized by a series of exacerbations that result in varying degrees of disability [Ottervald et al., 2010]. Previous studies revealed that poor recovery from the first two attacks of MS is the most important significant single risk factor for disease progression [Mowry et al., 2009; Scott and Schramke, 2010], and it is hoped that treating patients during the early phase of MS may provide an opportunity to prevent relapses [Vollmer, 2007]. Therefore, earlier diagnosis and new therapeutic strategies are urgently needed for MS, especially in the crucial early stages of the illness.

Complement plays a central role in the innate immune system, providing an important defense against infection and immune complex disease including MS [Gasque et al., 2000]. Within the CNS the majority of complement proteins are produced by glial cells and neurones and expression is increased in response to inflammation [Morgan and Gasque, 1996; Gasque et al., 2000]. In patients with MS, numerous studies have indicated that there is a change in complement activity in CSF or serum. However, the actual pathogenic role of complement in MS remains poorly understood. Results obtained from different studies measuring the same complement component (C3 and C4) have often been conflicting, which were reviewed in detail by Ingram [Ingram et al., 2009]. In two latest studies, a fragment of C4 was found increased in serums of MS [Sawai et al., 2010], and plasma and CSF levels of C4a were elevated in acute RRMS [Ingram et al., 2010]. This indicates the potential role of C4 related proteins as biomarker of MS.

To find new biomarkers for MS and the possible pathogenesis, we studied CSF proteins of active MS patients and controls by two-dimensional fluorescence differential in gel electrophoresis (2-D

Abbreviations: 2-D DIGE, two-dimensional fluorescence differential in gel electrophoresis; IEF, isoelectric focusing; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; MS, multiple sclerosis; ONDs, other neurological disorders; PMF, peptide mass fingerprinting; RRMS, relapsing-remitting MS; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

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DIGE). This approach is based on the separation of proteins pre-labeled with fluorescent dyes according to their charge in a first dimension by isoelectric focusing (IEF) and according to their size in a second dimension by SDS polyacrylamide gel electrophoresis (SDS-PAGE) [Marouga et al., 2005; Shaw et al., 2009]. In this unbiased approach, abnormal expression of seven proteins was found in CSF from patients with active MS and controls with other neurological disorders (ONDs). The protein–protein interactive network analysis of the seven differential proteins revealed complement activation involvement. Our results provide independent evidence for involvement of complement activation in active MS, and these findings may have a relevant impact on the identification of disease-specific biomarkers.

MATERIALS AND METHODS

CSF SPECIMENS

CSF samples were obtained by lumbar puncture in the Department of Neurology of Shandong Provincial Hospital (Jinan, ShanDong province, China). We collected CSF samples from 30 patients suffering from RRMS, and 30 controls with ONDs, of which CSF samples from 12 RRMS patients and 12 controls were selected at random to perform 2-D DIGE. One sample was used for 2-DE western blotting. All the 60 samples were used for SDS-PAGE western blotting. In all our patients, lumbar puncture was performed prior to any immunomodulatory therapy. The samples of RRMS were obtained during an acute phase at the first or secondary attack of disease (active MS). Patients with RRMS enrolled in this study received their diagnosis on the basis of the criteria of McDonald [McDonald et al., 2001]. The ONDs patients had no obvious clinical and laboratory inflammation signs. Immediately after sampling, CSF was then centrifuged at 16,000 g (4°C) for 10 min to eliminate cells and other insoluble materials. The concentration of total protein of each sample was measured by Bradford assay using BSA as standard (Protein Assay Kit, Bio-Rad, Hercules, CA) after centrifugation. Then samples were stored at −80°C before the succeeding manipulation.

This study was approved by the local ethical committee at Shandong University in China. All the patients were from the Department of Neurology of Shandong Provincial Hospital and gave informed consent to participate in the study. The clinical and laboratory features of the patients are summarized in Table I.

SAMPLE POOLING STRATEGY AND REMOVAL OF HIGH-ABUNDANCE PROTEINS FOR 2-D DIGE

The 12 CSF samples (500 μl each) from RRMS or control group were mixed into a pool. The CSF sample pool was concentrated with centrifugal filter devices (Ultracel YM-10 membrane, Millipore, Bedford, MA) into less than 100 μl, and then albumin and IgG were depleted with albumin and IgG removal Kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's recommendations. Subsequently, sample was precipitated with 100% ice-cold acetone in a 4:1 ratio of acetone and the CSF and stored overnight at −20°C to remove salt. Then, the mixture was centrifuged at 16,000 g at 4°C for 30 min. The pellet was washed with 90% acetone and then air-dried. The dried pellet was solubilized in a lysis buffer containing 8 M urea, 4% CHAPS and 30 mM Tris. Finally, the protein concentrations were determined using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

PROTEOMICS ANALYSIS

The follow details on fluorescence labeling, IEF and electrophoretic procedure, gel-scanning, image analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) and data searching has been published before by our group [Liu et al., 2009]. The experiment was repeated three times to reduce system errors.

Briefly, for each repeated gels, we labeled 50 μg proteins of each sample pool with 400 pmol of the appropriate CyDye DIGE fluors (GE Healthcare). Commercial immobilized pH gradient (IPG) dry strips (18 cm, pH 4–7, GE Healthcare) and the corresponding IPG buffer (pH 4–7, GE Healthcare) was used in supplement buffer and rehydration buffer. After IEF and SDS-PAGE, the gels were scanned by Typhoon TRIO (GE Healthcare) to gain digital Cy2, Cy3, and Cy5 images. Image analysis and statistical quantification of relative protein abundances was performed using DeCyder software (Version 6.0, GE Healthcare). Finally, one of the gels was stained with Coomassie brilliant blue R-350 (Amresco, Solon, OH, USA), and the protein spots that were found altering significantly by the statistical analysis were manually excised from the gel, destained and analyzed on a MALDI-TOF/MS 4700 (ABI Corp, Carlsbad, CA). The peptide mass data were searched against Swiss-Prot databank.

WESTERN BLOTTING

The isoforms of C4 were isolated and further identified by 2-DE western blotting, and CSF levels of C4b were measured by the method of traditional SDS-PAGE western blotting.

SAMPLE PREPARATION FOR WESTERN BLOTTING

CSF sample (500 μl) stored at −80°C was precipitated with 2 ml 100% ice-cold acetone and stored overnight at −20°C. After centrifugation and washing as mentioned above, the dried pellet was solubilized in a lysis buffer containing 8 M urea, 4% CHAPS, and 65 mM DTT. Protein concentrations were determined using 2-D Quant Kit (GE Healthcare).

TABLE I. The Clinical and Laboratory Features of the RRMS and Control Patients Included in 2-D DIGE and Western Blotting

	N (male/female)	Age (years) Mean ± S.D.	No. of OCBs[a]	CSF concentration (g/L)	Disease duration (days)
Active RRMS	30 (12/18)	38.6 ± 12.5	1–5	0.47 ± 0.15	17.0 ± 8.1
Controls	30 (15/15)	37.8 ± 14	N/A	0.39 ± 0.14	N/A

[a]OCBs, oligoclonal bands.

2-DE WESTERN BLOTTING

Proteins (70 μg) were separated on 2-D gels (11 × 8 cm). One as the master gel was stained with silver according to the previous article published by our group [Yang et al., 2008] to check for the quality of sample preparation and electrophoresis. The resolved proteins in the other gel were electrotransferred to a PVDF membrane (Millipore, Billerica, MA, USA) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3). The membrane was blocked with 5% non-fat dry milk in TBS-T buffer (20 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween-20) for 1.5 h at room temperature and incubated overnight at 4°C with the following primary antibody: mouse against human complement C4 mAb (1:500, Santa Cruz). After washing three times in TBS-T buffer, blots were incubated with a secondary antibody of goat against mouse IgG-peroxidase conjugate (diluted 1:2,000, Santa Cruz) for 1 h at room temperature. After the membrane was washed three times in TBS-T buffer, the immunoreactive complexes were visualized by the Lumin enhanced chemiluminescent Kit (DBI Bioscience, Shanghai, China). Finally, the light was detected by photographic film. In order to match the immunoreactive spots to the master map, the scanned western blotting image was overlapped to the color inverted master map using Adobe Photoshop software.

SDS-PAGE WESTERN BLOTTING

Proteins (20 μg) were mixed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue) and resolved by SDS-PAGE on 12% polyacrylamide gels. Proteins were transferred to an appropriate PVDF membrane and tested by mouse against human C4 mAb as primary antibody as described in the 2-DE western blotting. Protein bands were scanned and quantification of band densities was performed using Quantity One software (V.4.6.2, Bio-Rad, Hercules, CA).

STATISTICAL ANALYSIS

SPSS 12.0 software (Abbott Laboratories, North Chicago, IL) was used for statistical analysis. Assay differences between the active MS and control groups were analyzed by student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

NETWORKS AND FUNCTION ANALYSIS USING METACORE

To utilize all the data obtained through proteomic analysis, proteins that exhibited statistically significant changes in Table II were imported to MetaCore (GeneGo, St. Joseph, MI) for protein–protein interaction network analysis. "Canonical" pathway and "auto expand" algorithm were used to build our network. Based on alter of identified nodes (from the 2-D DIGE data), up- and down-regulation of the inferred nodes can be predicted upon the interpreted biochemical phenomenon [Chance et al., 2010].

RESULTS

IDENTIFICATION OF CANDIDATE BIOMARKER USING 2-D DIGE AND MALDI-TOF/MS

A total of 1,408 protein spots were detected in the sample pools from active MS and control patients. Analysis of these spots with DeCyder software revealed 17 spots with more than 1.5-folds difference in expression levels between the two pools. The difference was assessed by the independent samples *t*-test ($P \leq 0.05$). The identification of selected spots was accomplished by MALDI-TOF/MS based peptide mass fingerprinting (PMF) analysis, and database searched in Swiss-Prot showed 7 distinct proteins corresponded to the 17 spots in gels. They are complement C4b (C4b), complement C3 (C3), complement factor H (CFH), tetranectin (TNA), haptoglobin (HP), apolipoprotein E (APOE), and gelsolin (GSN). An example of 2-D DIGE image is shown in Figure 1. The spots that showed obvious difference are labeled and each protein isoform is numbered.

In identified seven proteins, three were related to complement system. There were C3, C4b, and factor H. The information of these seven proteins and their isoforms were described in Table II.

WESTERN BLOTTING FOR C4b

In the following study, complement C4b was verified by 2-DE western blotting. The master gel showed satisfying matching of

TABLE II. Proteins and Their Isoforms Identified in CSF by 2-D DIGE and MOLDI-TOF/MS

Protein name	Swiss-Prot entry name	Swiss-Prot accession No.	Isoforms	Theoretical Mw(kDa)/pI	Experimental Mw(kDa)/pI	Ratio[a] (MS/controls)
Complement C4b	CO4B_HUMAN	P0C0L5	1	33.07/6.37	30.87/6.50	1.86
Complement C3	CO3_HUMAN	P01024	1	187.03/6.02	83.18/6.63	1.52
			2	187.03/6.02	81.13/6.73	1.61
Complement factor H	CFAH_HUMAN	P08603	1	139/6.2	48.17/6.31	−1.57
Tetranectin	TETN_HUMAN	P05452	1	19.96/5.8	21.76/5.64	1.80
Haptoglobin	HPT_HUMAN	P00738	1	45.86/6.13	42.55/5.03	4.29
			2	45.86/6.13	41.76/5.18	4.99
			3	45.86/6.13	41.29/5.33	8.42
			4	45.86/6.13	18.45/5.38	8.20
			5	45.86/6.13	18.72/5.71	8.28
			6	45.86/6.13	18.41/6.06	10.94
Apolipoprotein E	APOE_HUMAN	P02649	1	36.25/5.65	31.53/5.04	−6.18
			2	36.25/5.65	32.22/5.20	−9.15
			3	36.25/5.65	32.22/5.24	−4.48
Gelsolin	GELS_HUMAN	P06396	1	85.63/5.9	109.16/5.78	−2.44
			2	85.63/5.9	108.32/5.88	−3.01
			3	85.63/5.9	109.16/5.95	−2.62

[a]The average ratio is calculated by BVA model of DeCyder software. A positive average ratio value indicates an increase from controls to MS group. Conversely, a decrease in abundance is denoted by a negative average ratio.

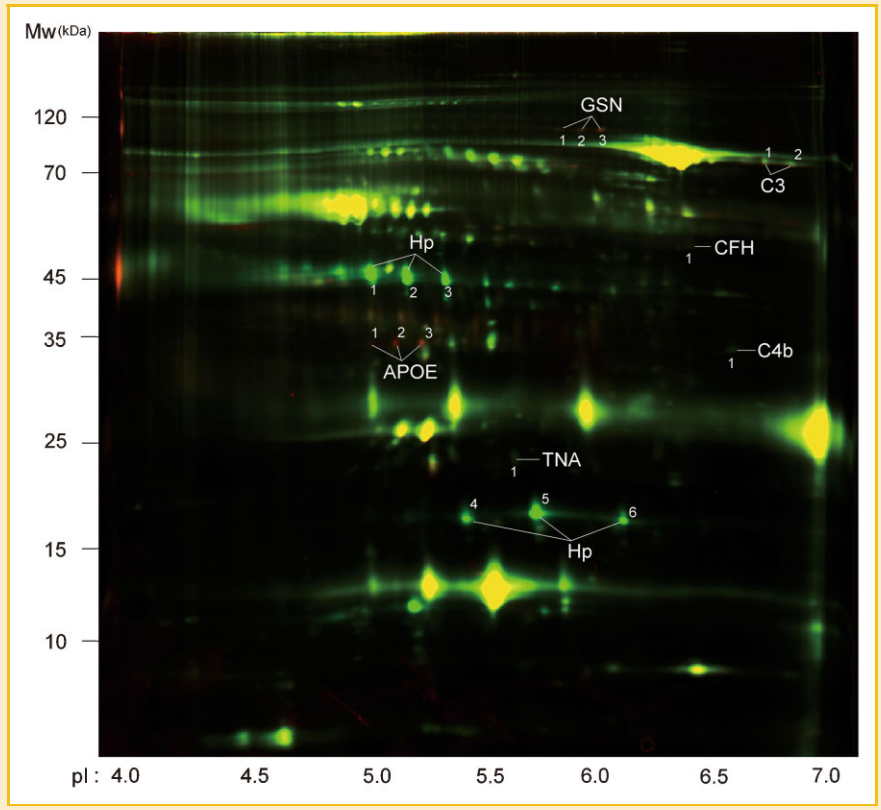

Fig. 1. Overlapped gel scanned by Typhoon TRIO from 2-D DIGE experiment. A sample pool including 12 CSF samples was used. This gel contained 50 μg protein from active MS (Cy5-labeled, green), 50 μg protein from controls (Cy3-labeled, red), and 50 μg internal standard protein (Cy2-labeled, excluded in this image). Seven identified differential proteins and their isoforms were labeled with white line and numbers, which are included in Table II. Green spots reveal up-regulated protein spots and red spots reveal down-regulated protein spots in active MS compared with controls.

proteins, and the other map of proteins immunoblotted by anti-C4 mAb was overlapped to the color inverted master gel using Adobe Photoshop software as exemplarily shown in Figure 2. At pH 4–7, there were three isoforms of complement C4 at 33 kDa and one isoform at 58 kDa.

Two bands (58 and 33 kDa) presented in SDS-PAGE western blotting of C4, and the 33 kDa band was much more clearly discernable. The locations of the C4 immunobinding spots (33 kDa) as well as the sequence coverage by mass spectroscopy correspond to the gamma chain of C4b. Therefore, the 33 kDa band was selected for further statistical analysis.

To evaluate and compare the CSF level of C4b expression, Volume Tool was used to calculate the relative volume percentage (%vol.) of each band based on band-densities. The volume report revealed that the C4b (33 kDa) of active MS group expressed at an average level of 2.37-folds higher than the control group ($P < 0.01$). The average %vol. in the active MS group was 13.74 ± 8.57, while in the control group it was 5.80 ± 4.16. The typical western blot image is shown in Figure 3.

PROTEIN–PROTEIN INTERACTION NETWORKS

Protein–protein interaction network analysis of all differentially expressed proteins was performed with MetaCore software (Fig. 4). The analysis result revealed that the differentially expressed proteins were mostly involved in the process of complement activation (50%, $P = 1.097\text{E-}22$).

DISCUSSION

2-D DIGE was used to detect differential CSF proteins between active MS and control patients. As a gel based platform of quantitative proteomic methods, 2-D DIGE can minimize gel-to-gel variance and quantify protein levels between samples comparing with traditional 2-D gel electrophoresis [Karp and Lilley, 2005], and it has a powerful resolution of separating thousands of proteins in a single gel, including different isoforms or fragments of one protein, a capacity that no other method can currently overpass in protein separation [Tumani et al., 2010]. A problem in CSF proteome analysis is that high-abundance proteins, such as albumin and immunoglobulins, may overlay brain-specific low-abundance CSF proteins in the electrophoresis. At present, many commercial albumin-removal kits have been specifically designed for use with serum and plasma. Because the protein concentration is much lower in CSF than that in serum, those kits and their recommended procedures are not compatible with CSF directly [Yuan and Desiderio, 2005]. Our study proves that affinity removal of high-abundance proteins after concentration of the CSF with commercial kits suggested by Yuan is

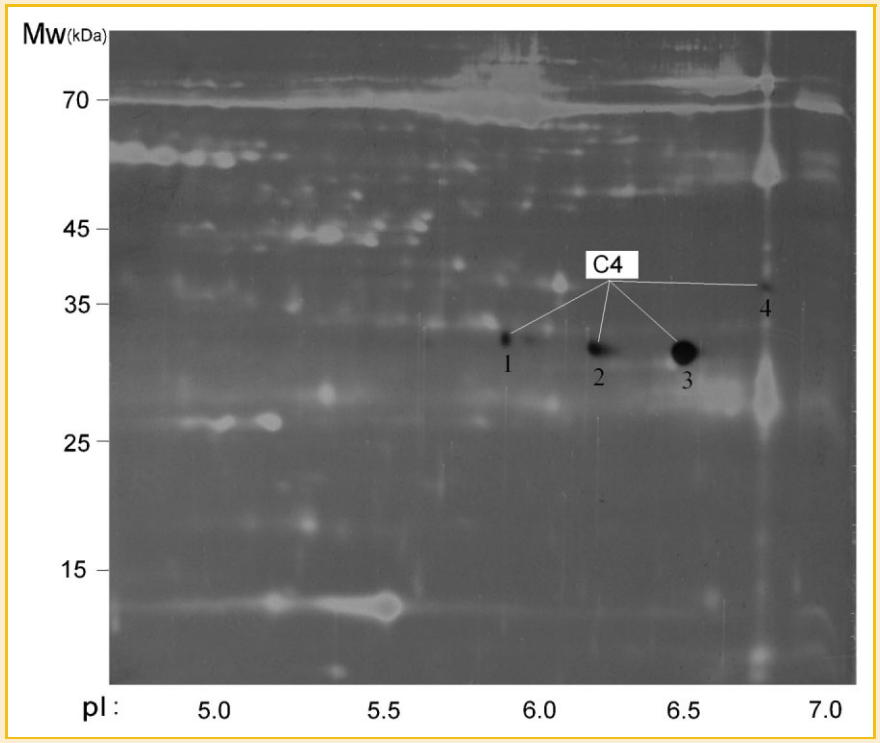

Fig. 2. The overlapped image of the color inverted master gel and exemplary 2-DE Western blots. Proteins were separated by 2-DE and stained with silver to serve as a master gel. Separated proteins in the other gel were electrotransferred onto a PVDF membrane and immunoblotted by anti-C4 mAb. Four isoforms of C4 with two different molecular weights were detected as shown with black spots. The isoform 3 of C4 corresponds to the spot that was identified as C4b in 2-D DIGE.

feasible. Though no novel proteins were found, it is still an effective method to allow enrichment of the remaining protein content of CSF, which could otherwise be below the detection limit. For example, in our previous study, HP used to be found at only two locations in 2-DE analysis [Bai et al., 2009], but six isoforms of HP presented in this study. Accordingly, much more CSF was needed to provide enough remaining proteins for electrophoresis and identification of picked differential protein spots [Lilley and Friedman, 2004], so sample pooling strategy was necessary.

In this study, three of the seven identified proteins were related to complement system, including C3, C4b, and factor H, which indicates the potential role of complement system in pathogenesis of MS. The protein interaction network analysis also suggested complement activation in active MS. The complement cascade is initiated by one of three pathways (the classical, alternative, or lectin pathways). All three pathways converge at the level of C3 cleavage and proceed to the assembly of the terminal, membrane attack pathway [Rus et al., 2006]. In the present study, C4b and C3 showed increased expression, while factor H decreased in active MS. C4b is one of the activated peptide generated by cleavage of C4. It is an essential subunit of the C3 convertase (C4b2a) and the C5 convertase (C3bC4b2a) enzymes of the classical complement pathway. C3 participates in activation of the three pathways and therefore represents total complement activation [Loeffler et al., 2004]. Factor H is one of the important soluble regulators of complement activation, which inhibit complement activation by causing dissociation of the subunits of the C3 and C5 convertase [Sahu and Lambris, 2000]. Taken together, elevated C4b and C3 and reduced factor H levels consistently indicate the involvement of complement activation and regulation in active MS. The present results are an independent quantitative proteomic measure in CSF of MS. The expression of C4 precursor was found decreased in RRMS patients in another proteomic study [Liu et al., 2009], in which CSF were collected from RRMS patients with more than 5 years disease duration. There is also evidence of reduced serum and CSF C3 in clinically stable RRMS [Jongen et al., 2000], and C4 may be elevated

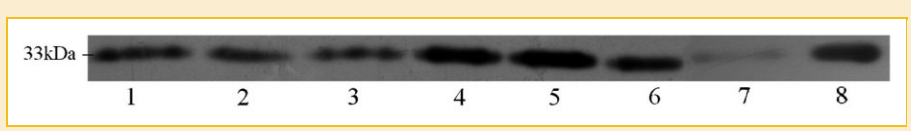

Fig. 3. Western blot analysis of C4 CSF levels in partial study subjects. 4–6 were from active MS patients and 1–3 were from controls. 7 and 8 were the sample pools used for DIGE analysis after removing albumin and IgG. 8 was from active RRMS patients and 7 was from controls.

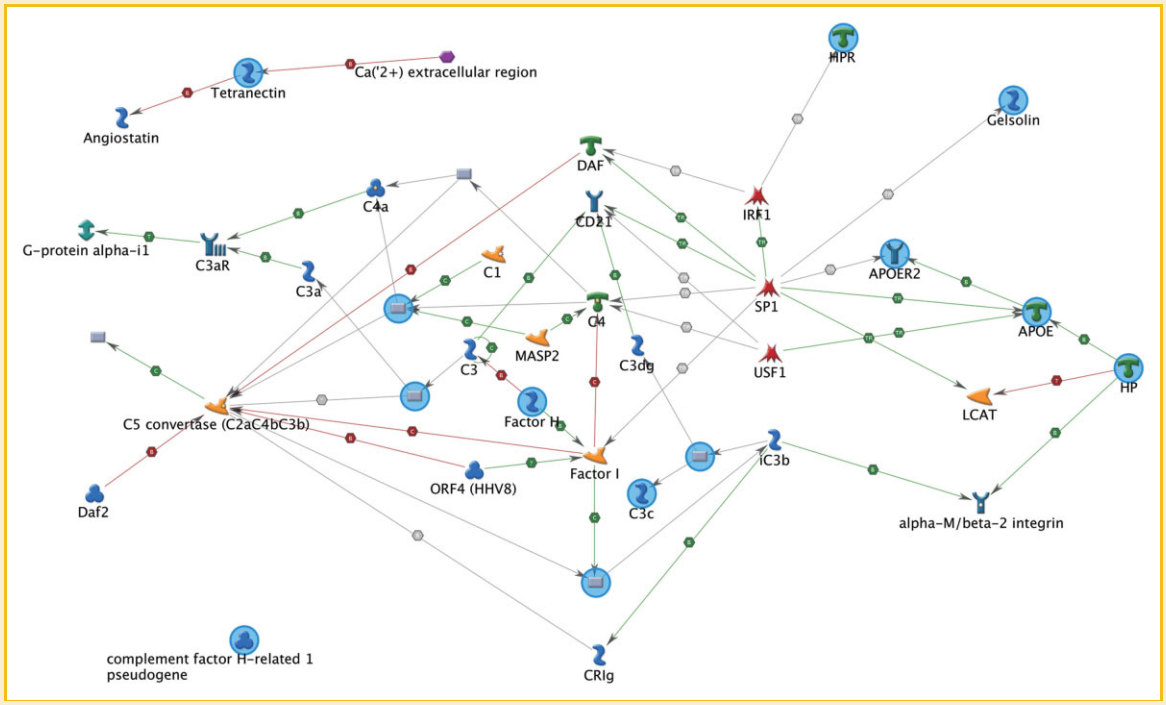

Fig. 4. Protein–protein interaction network derived from MetaCore analysis. Seven proteins that exhibited statistically significant changes in Table II were imported to MetaCore. Protein targets detected in the 2-D DIGE experiment are labeled by large blue circles. The red line indicates down-regulation interactions, while green indicating up-regulation and gray indicating no effect. The "TR" and "B" on the line denote relationship such as transcription regulation and binding interaction.

in chronic progressive MS serum and reduced in CSF compared to controls [Jans et al., 1984]. In a lastest study, the plasma and CSF levels of C4a were elevated in acute RRMS, and C4a levels decreased after the acute inflammatory event [Ingram et al., 2010]. In view of the different expression of complement components in different disease stages, it is likely that C3 and C4 elevate in acute RRMS and reduce in clinical stable RRMS. However, more studies on different disease stages of the same patients are expected.

Barnett and colleagues reported that the unusual microglial nodules containing short, linear deposits of activated complement may constitute a specific biomarker with pathogenetic significance in MS [Barnett et al., 2009]. Previous studies showed that in the presence of complement, activated microglia showed significant phagocytosis of myelin basic protein in vitro [Zajicek et al., 1992], and strong expression of CD59 (a complement regulatory protein molecule) occurs was observed in areas of myelin production [Zajicek et al., 1995]. Complement activator cobra venom factor (CVF) treatment had been used to reduce serum complement in MBP-induced EAE and caused suppression of disease [Pabst et al., 1971; Linington et al., 1989; Hinman et al., 1999]. Although complement activation is not specific to MS, the complement activation in active MS may indicate initial myelin attack, assessed in combination with other inflammatory and immune markers, may be of value as biomarkers in the early stage of MS.

The result of 2-DE western blotting for C4 further confirmed the identification of C4b. It was reported that patients with Alzheimer's disease (AD), Parkinson's disease (PD), and MS all showed more than one complement isoform with a significant change in CSF expression level compared to normal subjects, in which the isoforms were separated with 2-DE and spots were identified by MOLDI TOF/TOF [Finehout et al., 2005]. As shown in our study, C4 contains at least 4 immunobinding spots with different pI, but in 2-D DIGE analysis, only one spot (C4b) was identified having obvious differences between the two pools. The result of western blotting also confirmed the obviously elevated expression of C4b in active MS patients, which suggests that C4b CSF levels may be used as potential biomarkers in active MS.

Among these differential proteins, TNA has little correlation with the others in protein-protein analysis. TNA is a glycoprotein and C-type lectin thought to play a prominent role in tissue remodeling. It is a plasma protein secreted by many different tissues, including brain [Strausberg et al., 2002]. TNA have been proved showing limited variation between individual CSF samples [Stoop et al., 2010]. A recent proteomic study reported TNA as being one of four proteins in the CSF of patients with MS that is absent in controls [Hammack et al., 2004], and soon a research using ELISA method revealed that in definite MS and patients with first attack of MS, TNA secretion did not change in the brain [Stoevring et al., 2006]. In a recent similar research, CSF TNA levels were proved increased in epileptic patients while serum-TN levels decreased [Wang et al., 2010]. All these results indicate the potential role of TNA as a biomarker of disease of CNS. TNA can stimulate plasminogen activation and is expressed during bone development [Iba et al., 2001]. However, whether TNA has any biological relation to pathogenesis of MS, such as demyelination and remyelination, is remain a challenging question. In our gel based proteomic research,

TNA CSF expression was increased by 1.8 folds in active MS pool. Further follow-up studies on TNA involving large number of MS patients even animal models are warranted.

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