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Microwave and magnetic (M²) proteomics of the experimental autoimmune encephalomyelitis animal model of multiple sclerosis

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Abstract

We hypothesized that quantitative MS/MS-based proteomics at multiple time points, incorporating rapid microwave and magnetic (M²) sample preparation, could enable relative protein expression to be correlated to disease progression in the experimental autoimmune encephalomyelitis (EAE)

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animal model of multiple sclerosis. To test our hypothesis, microwave-assisted reduction/alkylation/digestion of proteins from brain tissue lysates bound to C8 magnetic beads and microwave-assisted isobaric chemical labeling were performed of released peptides, in 90 s prior to unbiased proteomic analysis. Disease progression in EAE was assessed by scoring clinical EAE disease severity and confirmed by histopathologic evaluation for central nervous system inflammation. Decoding the expression of 283 top-ranked proteins ($p < 0.05$) at each time point relative to their expression at the peak of disease, from a total of 1191 proteins observed in four technical replicates, revealed a strong statistical correlation to EAE disease score, particularly for the following four proteins that closely mirror disease progression: 14-3-3 ϵ ($p = 3.4\text{E-}6$); GPI ($p = 2.1\text{E-}5$); PLP1 ($p = 8.0\text{E-}4$); PRX1 ($p = 1.7\text{E-}4$). These results were confirmed by Western blotting, signaling pathway analysis, and hierarchical clustering of EAE risk groups. While validation in a larger cohort is underway, we conclude that M² proteomics is a rapid method to quantify putative prognostic/predictive protein biomarkers and therapeutic targets of disease progression in the EAE animal model of multiple sclerosis.

Keywords

Isobaric chemical labeling; Microwave proteomics; Multiple sclerosis; Sample preparation

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis is the most common demyelinating disease of the central nervous system. Early lesions in the brain are characterized by the local accumulation of activated CD4⁺ and CD8⁺ T-cells around small venules [1]. This is followed by demyelination of oligodendrocyte axons associated with perivascular inflammation consisting of T-cells, B-cells, plasma cells, and activated microglia/macrophages [2]. Unfortunately, current quantitative measures, including magnetic resonance imaging and the expanded disability status scale disease score, poorly correlate to disease progression, particularly for patients with the relapsing-remitting form of the disease. Therefore, prognostic/predictive protein biomarkers and therapeutic targets, particularly those that reflect de/remyelination (e.g. proteolipid protein, PLP1) and/or inflammation (e.g. interleukin-17, IL17), are greatly needed to understand multiple sclerosis at the molecular level and to improve early detection, diagnosis, prognosis, and treatment.

1.2 Challenges for proteomics

Masking of low abundance proteins by high abundance proteins is a profound problem that limits the dynamic range of proteomics, the large-scale study of proteins. In humans, approximately 24 000 genes are translated into an estimated 2 million protein isoforms that may span up to 12 orders of magnitude in abundance in blood, where proteins originate from hundreds of different cell types. Paradoxically, there are less than 100 protein biomarkers that are routinely measured in blood today [3, 4]. Masking problems can be partially overcome by various sample preparation strategies, including protein enrichment/depletion and fractionation. While improving selectivity, these strategies suffer from poor sample throughput due to lengthy sample preparation times and poor sensitivity due to adsorptive losses at every step. Quantitative MS/MS-based proteomics, where peptides and proteins are assigned amino acid sequences by searching high-quality spectra from protease-specific peptides against spectra predicted from protein databases, place additional constraints on time and sensitivity. Consequently, previous studies have been statistically underpowered, focusing on qualitative or semiquantitative methods for identifying large numbers of proteins in relatively small numbers of brain tissue specimens.

1.3 M² proteomics

We hypothesized that quantitative MS/MS-based proteomics at multiple time points, incorporating rapid microwave and magnetic (M²) sample preparation, could enable relative protein expression to be correlated to disease progression in the experimental autoimmune encephalomyelitis (EAE) animal model of multiple sclerosis. M² proteomics was inspired by reports of high-throughput sample preparation by microwave-assisted digestion [5, 6] or by magnetic beads [7], high-sensitivity on-bead digestions [8], and isobaric labeling reagents for multiplexed protein quantification by MS/MS-based proteomics [9, 10]. To test our hypothesis, microwave-assisted reduction/alkylation/digestion of proteins from brain tissue lysates bound to C8 magnetic beads and microwave-assisted isobaric chemical labeling of released peptides were performed for all 32 samples spanning disease progression, and pooled reference material from the peak of disease, in a total of 90 s prior to unbiased proteomic analysis. Isobaric-encoding enabled 5-plex quantitative proteomic analysis of eight sample mixtures, corresponding to eight disease time points, where each mixture included four mice per time point and pooled reference material.

2 Material and methods

2.1 Murine EAE

C57BL/six female 5 week old mice were purchased from the Jackson Laboratory (Stock number 000664; Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions and all animal procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the University of Texas at San Antonio. Active induction of EAE was performed with a subcutaneous injection of each mouse with 300 µg of myelin oligodendrocyte glycoprotein 35–55 peptide (United Biochemical Research, Seattle, WA, USA) in 50 µL of complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37 RA (Difco Laboratories, Detroit, MI, USA) at a final concentration of 5 mg/mL. Two intraperitoneal injections of pertussis toxin (List Biological, Campbell, CA, USA) at 200 ng per mouse were given at the time of immunization and 48 h later [11]. Animals were monitored and graded daily for clinical signs of EAE using the following scoring system [12]: 0, no abnormality; 1, limp tail; 2, moderate and hind limb weakness; 3, complete hind limb paralysis; 4, quadriplegia or premonitory state; and 5, death. EAE scores are presented as the mean ± SD and were confirmed by histopathology (data not shown). Mice were sacrificed at eight disease time points, described by the number of days (d) postimmunization (−1 d (nonimmunized), 0 d (3-h postimmunization), 5 d, 7 d, 10 d, 15 d, 20 d, and 25 d) in biological quadruplicate ($n = 4$ per time point). These time points were selected to reflect a negative control, a positive control, the onset, of disease, the peak of disease, and disease remission as a practical compromise between the minimum number of mice and the minimum number of samples required to define the overall trajectory of disease progression. Half of all brain tissue was snap-frozen in liquid nitrogen and stored at −80°C for M² proteomics and Western blotting while the remainder was used for cytokine measurement and immunofluorescence analysis for inflammatory infiltrates (data not shown).

2.2 Cytokine measurement

Antigen-induced T-cell responses were assessed in dissociated brain and lymph node tissue by enzyme-linked immunosorbent spot (ELISPOT) assay for IFN γ and IL17A as previously described [13] after stimulation with myelin oligodendrocyte glycoprotein 35–55 peptide (United Biochemical Research). Briefly, ELISPOT plates (Multiscreen IP; Millipore) were coated with 1 µg/mL anti-IFN γ (clone: AN-18) or anti-IL-17A (clone 17F3) captures antibodies in PBS. The plates were blocked with 1% BSA in PBS for 1 h at room temperature and then washed four times with PBS. After 1 h of blocking with PBS/1% BSA,

cells were added with or without antigen and incubated for 24 h at 37°C. The plates were washed three times with PBS and four times with PBS/Tween 20, and biotinylated anti-IFN- γ (R4-6A2; 0.5 $\mu\text{g/mL}$) or -IL-17A (eBioTC11-8H4; 0.125 $\mu\text{g/mL}$) detection antibodies were added and incubated overnight, respectively. Plates were washed four times with PBS/Tween 20 and incubated with streptavidin-alkaline phosphatase (Invitrogen, Grand Island, NY, USA). Cytokine spots were visualized with a BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Image analysis of ELISPOT assays was performed on a Series 2 Immunospot analyzer and software (Cellular Technology, Shaker Heights, OH) as described previously [14, 15]. Results for antigen-specific T cells were normalized with a negative control containing peptide-free media. All measurements were performed in duplicate.

2.3 Brain tissue lysate

Whole cell protein was extracted from brain tissue using the RIPA Lysis Buffer Kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol. Briefly, an appropriate amount of RIPA complete lysis buffer was added to cell pellet. The mixture was incubated on ice for 5 min, followed by centrifugation at $14\,000 \times g$ for 15 min at 4°C. The supernatant was collected as brain tissue lysate and stored at -80°C until further use. Protein concentration was determined using Invitrogen EZQ Protein Quantitation Kit (Invitrogen). Protein from mice at the peak of disease (day 20) was pooled ($n = 4$) as reference material.

2.4 M² sample preparation

C8 magnetic beads (BeMg, Bioclone, San Diego, CA) were used in this study. Briefly, 50 mg of beads were suspended in 1 mL of 50% methanol. Immediately before use, 100 μL of the beads were washed three times with equilibration buffer (200 mM NaCl, 0.1% TFA). Brain tissue lysate (25–100 μg at 1 $\mu\text{g}/\mu\text{L}$) was mixed with preequilibrated beads and one-third sample-binding buffer (800 mM NaCl, 0.4% TFA) by volume. The mixture was incubated at room temperature for 5 min followed by removing the supernatant. The beads were washed twice with 150 μL of 40 mM triethylammonium bicarbonate (TEAB), and then 150 μL of 10 mM DTT was added followed by microwave heating for 10 s. DTT solution was then removed and 150 μL of 50 mM iodoacetamide was added followed by microwave heating for 10 s. Next, beads were washed twice with 150 μL of 40 mM TEAB and resuspended in 150 μL of 40 mM TEAB. In vitro proteolysis was performed with 4 μL of trypsin in a 1:25 trypsin-to-protein ratio (stock = 1 $\mu\text{g}/\mu\text{L}$ in 50 mM acetic acid) and microwave heated for 20 s in triplicate. The supernatant was transferred to a new tube for immediate use or stored at -80°C. Released tryptic peptides from digested brain tissue lysates, including the reference material described above, were modified at the *N*-terminus and at lysine residues with the tandem mass tagging (TMT)-6plex isobaric labeling reagents (Thermo scientific, San Jose, CA, USA). Each biological replicate ($n = 4$ –5) for each time point was encoded with one of the TMT-127–131 reagents, while reference material was encoded with the TMT-126 reagent. Then, 41 μL of anhydrous acetonitrile was added to 0.8 mg of TMT labeling reagent and 25 μg of brain tissue lysate was added and microwave heated for 10 s. To quench the reaction, 8 μL of 5% hydroxylamine was added to the sample at room temperature. To normalize across the time course of disease progression, TMT-encoded brain tissue lysates from individual mice, labeled with the TMT-127–131 reagents, respectively, were mixed with the reference material encoded with the TMT-126 reagent in 1₁₂₆:1₁₂₇:1₁₂₈:1₁₂₉:1₁₃₀:1₁₃₁ ratios. These sample mixtures, I–VIII, corresponding to eight disease time points, were stored at -80°C until further use.

2.5 Capillary LC-fourier-transform-MS/MS with protein database searching

Capillary LC-fourier-transform-MS/MS was performed with a splitless nanoLC-2D pump (Eksigent, Livermore, CA, USA), a 50- μ m id column packed with 7 cm of 3 μ m-od C18 particles, and a hybrid linear ion trap-fourier-transform tandem mass spectrometer (LTQ-ELITE; ThermoFisher, San Jose, CA, USA) operated with a lock mass for calibration. For unbiased analyses, the top six most abundant eluting ions were fragmented by data-dependent high-energy collision-induced dissociation. For targeted analyses, only ions corresponding to peptides observed for selected proteins were fragmented by high-energy collision-induced dissociation. The reverse-phase gradient was 2 to 62% of 0.1% formic acid in acetonitrile over 60 min at 350 nL/min. Probability-based and error-tolerant protein database searching of MS/MS spectra against the IPI_mouse protein database (release 2010_jan10; 56,729 sequences) were performed with a 10-node MASCOT cluster (ver. 2.3.02, Matrixscience, London, UK). Search criteria included: peak picking with Mascot Distiller; 10 ppm precursor ion mass tolerance, 0.8 Da product ion mass tolerance, three missed cleavages, trypsin, carbamidomethyl cysteines, and oxidized methionines as variable modifications, an ion score threshold of 20 and TMT-6-plex for quantification.

2.6 Statistical analysis

The M^2 proteomics results for each technical replicate estimate protein expression for individual mice, encoded in sample mixtures I-VIII, relative to pooled reference material at the peak of disease (day 20). Relative protein expression levels were transformed to log base 2 for quantile normalization. Outlier arrays were removed based upon the following quality control procedures: (i) overall intensity histograms of normalized expression were compared with kernel smoothed density plots, and (ii) hierarchical clustering of sample profiles was performed to assess the consistency of technical and biological variation. We tested the association between relative protein expression and EAE score using a linear mixed-effect while treating EAE score as a continuous predictor. First, we treated the EAE effect on relative protein expression singly, as a univariate predictor. Next, we considered the effects of EAE score by adjusting for time as a quadratic effect. We tested for changes in relative protein expression with postimmunization time using a linear mixed-effect model in which time was treated as a multilevel factor. We tested all the pairwise differences in relative protein expression between all disease time points and both nonimmunized mice (day -1) and 3-h postimmunization (day 0) using an unpaired, unequal variance *t*-test on the replicate averages. We examined the relationship between the overall expression profile with both time and EAE score using a hierarchical clustering display based upon Euclidean distance and complete linkage. For clustering analyses of relative protein expression profiles, we considered the subset of proteins that were most variable by selecting the proteins in the top quartile (top 25%) by their standard deviation ranking. All statistical analysis was performed with R v2.13 (R-Project, Vienna, Austria).

2.7 Western blotting

Brain tissue lysates (25 μ g at 1 μ g/mL) were treated with β ME (5% by volume) prior to boiling for 10 min and separating proteins in each sample with SDS-PAGE precast gels (Mini-PROTEAN gel catalog 456-9034 any kD 10-well by Bio-Rad, CA, USA) for 1DE. Then proteins were transferred to a 0.2- μ m nitro-cellulose membrane (catalog 162-0212 Bio-Rad) followed by blocking with 5% milk in TBS for 1 h. The membrane was then washed with TBS buffer for 5 min in triplicate and probed with (1:500) primary mouse monoclonal antibodies (anti-GPI (H-9) IgG1 or anti-14-3-3 e (8C3) IgG2a (sc-271459 or sc-23957, respectively; Santa Cruz Biotechnology)) in TBS with overnight incubation. Detection was performed by 1 h incubation with (1:2000) goat anti-mouse IgG HRP-conjugated secondary antibody (sc-2005) in TBS prior to chemiluminescence measurement using X-ray film and luminol reagents (sc-2048).

2.8 Pathway analysis

Biochemical pathway analysis was performed with Ingenuity Pathways Analysis (IPA, Ingenuity® Systems) according to the manufacturer's suggestions. Briefly, MASCOT results were imported to IPA as .csv files and IPA's core analysis was performed on each file. Proteins corresponding to genes in the IPA knowledgebase were mapped onto canonical signaling pathways per the manufacturer's recommendations. A vertical bar plot, showing the percentage of proteins quantified in each canonical signaling pathway, was visualized to investigate pathway enrichment during disease progression, where *p*-values for enrichment were assigned by IPA.

3 Results

Quantitative MS/MS-based proteomics at multiple time points, incorporating rapid M² sample preparation, was investigated as a means to correlate relative protein expression to disease progression in the EAE animal model of multiple sclerosis. Microwave-assisted reduction/alkylation/digestion of proteins from brain tissue lysates bound to C8 magnetic beads and microwave-assisted isobaric chemical labeling of released peptides were performed for all 32 samples spanning disease progression, and pooled reference material from the peak of disease, in a total of 90 s prior to unbiased proteomic analysis (Preliminary work to determine analytical figures of merit, such as linear dynamic range, is shown in Supporting Information Fig. 1). Disease progression in EAE was assessed by scoring clinical EAE disease severity and confirmed by histopathologic evaluation for central nervous system (CNS) inflammation (data not shown).

Decoding the expression of 283 top-ranked proteins at each time point relative to their expression at the peak of disease, from a total of 1191 proteins observed in four technical replicates, revealed a strong statistical correlation to EAE disease score, particularly for the following proteins that closely mirror disease progression: 14-3-3 ϵ ($p = 3.4\text{E-}6$); GPI ($p = 2.1\text{E-}5$); PLP1 ($p = 8.0\text{E-}4$); PRX1 ($p = 1.7\text{E-}4$). For example, Fig. 1 illustrates the correlation of relative 14-3-3 ϵ expression to disease progression in the EAE animal model of multiple sclerosis by M² proteomics. Figure 1A shows the M² proteomics experimental design superimposed on a plot of EAE disease score versus postimmunization time. Isobaric-encoding enabled 5-plex quantitative proteomic analysis of eight sample mixtures, corresponding to eight disease time points, where each mixture included four mice per time point and pooled reference material. TMT-encoded brain tissue lysates from individual mice, labeled with the TMT-127–131 reagents, respectively, were mixed with the reference material encoded with the TMT-126 reagent in 1₁₂₆:1₁₂₇:1₁₂₈:1₁₂₉:1₁₃₀:1₁₃₁ ratios to normalize across the time course of disease progression. Clinical onset of disease was observed on days 8–10, followed by disease peak at days 19–20 and remission. Figure 1B shows cytokine analysis of IFN γ (•, left axis) and IL17A (•, right axis) in the brain, showing that the relative expression of both low abundance cytokines, detected by ELISPOT but not M² proteomics, closely mirror disease progression, as expected. In contrast, cytokine analysis in the lymph node does not mirror disease progression (Supporting Information Fig. 2). Figure 1C shows the relative expression of 14-3-3 ϵ versus time, where levels are inversely proportional to disease progression, while Fig. 1D shows the inverse correlation of 14-3-3 ϵ relative expression to EAE score, where the overall p -value = $3.4\text{E-}6$. Likewise, Fig. 2A shows the relative expression of glucose-6-phosphate isomerase (GPI) versus time, where levels are directly proportional to disease progression, while Fig. 2B shows the direct correlation of GPI relative expression to EAE score, where the overall p -value = $2.1\text{E-}5$. Figure 1E and Figure 2C show annotated MS/MS spectra with expanded views of reporter ions for quantifying the relative abundance of representative peptides from individual mice, where (*) indicates isobarically labeled amino acid residues: (1E) 14-3-3 ϵ ;*LICCDILDVLDK*HLIPAANTGESK* and (2C) GPI;*IL-

LANFLAQTEALMK*. The relative expression of individual proteins from individual mice is inferred by decoding the relative abundance of multiple peptides for each protein.

Results were confirmed by Western blotting, signaling pathway analysis and hierarchical clustering of EAE risk groups. Supporting Information Fig. 3 shows signaling pathway analysis of relative protein expression, where *p*-values for representative 14-3-3 signaling and glycolysis/gluconeogenesis pathways were 1E-17 and 1E-15, respectively, also confirming the importance of 14-3-3 ϵ and GPI. The percentage of proteins observed in each top-ranked signaling pathway is shown as a vertical bar plot. Supporting Information Fig. 4 shows Western blotting analysis of relative protein expression for 14-3-3 ϵ , GPI, PLP1, and PRX1, confirming the M² proteomics results described above. Lastly, Fig. 3 shows hierarchical clustering of EAE risk groups by correlating relative protein expression to disease progression for a subset of proteins measured by M² proteomics. The heat map suggests proteomic classifiers that stratify mice into EAE risk groups (G1 $\rightarrow$ G4), where G1 includes mice with the lowest EAE scores and G4 includes mice with the highest EAE scores. For example, 14-3-3 ϵ and GPI, boxed in Fig. 3, closely mirror disease progression, confirming the M² proteomics results described above. Supporting Information Figs. 5 and 6 show Western blotting analysis of relative protein expression for PLP1 and PRX1, respectively.

4 Discussion

4.1 M² Proteomics

This proof-of-principle study shows, for the first time, that quantitative MS/MS-based proteomics at multiple time points, incorporating rapid M² sample preparation, enables relative protein expression to be correlated to disease progression in the EAE animal model of multiple sclerosis. Below, we provide an overview of the biological function of each of the proteins illustrated in the results section above. We also compare M² proteomics to previous work by laser capture microdissection (LCM) proteomics, other proteomics studies and transcriptomics studies. Lastly, we present some ideas on reactive oxygen species (ROS) for future proteomics studies.

4.2 14-3-3 ϵ

The 14-3-3 proteins are an evolutionarily conserved family consisting of seven isoforms that are abundantly expressed in the nervous system and involved in a variety of cellular functions, including: cell cycle; transcriptional control; regulation of ion channels; apoptosis; and neurodegeneration [16–19]. Patients with Miller–Dieker syndrome and severe lissencephaly were found to have a deletion in the 14-3-3 ϵ gene [20]. Furthermore, a deletion of this gene in a mouse model resulted in defects in brain development and neuronal migration, probably through a disruption in regulation of activity of dynein [21]. 14-3-3 ϵ plays an important role in nerve apoptosis by interaction with downstream molecules of the neurotrophin receptor p75NTR [22]. In normal cells, 14-3-3 ϵ interacts with the proapoptotic Bad protein and blocks its function. During apoptosis, caspase-3 cleaves 14-3-3 ϵ to release the Bad and Bcl-xL proteins that promote apoptosis [23]. The protein has been detected in the cerebrospinal fluid of patients with multiple sclerosis presenting with severe myelitis and in reactive astrocytes in demyelinating lesions [24,25]. Other studies have shown that 14-3-3 ϵ is differentially expressed in human brain capillary endothelium/endothelial cells when treated with sera from multiple sclerosis patients [26]. Lastly, 14-3-3 ϵ interacts with vimentin and glial fibrillary acidic protein in cultured human astrocytes, suggesting a role in the activation of astrocytes during CNS inflammation. Taken together, this suggests that 14-3-3 ϵ serves as a neurotrophic protein that may induce or prevent neuronal cell death.

4.3 GPI

GPI is a dimeric enzyme that catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. In the cytoplasm, GPI is involved in glycolysis and gluconeogenesis, while outside the cell it functions as a neurotrophic factor for spinal and sensory neurons. Major metabolic pathways converging from G6P include the pentose phosphate pathway to produce NADPH and ribose (nucleotides) and the glycolytic pathway/TCA cycle to produce ATP. In cancer cells, GPI is known as an autocrine motility factor that promotes cell survival by interfering with many apoptotic-signaling cascades, such as Rho-GTPase and VEGF receptor expression [27]. In healthy cells, GPI regulates calcium release from the ER during stress responses, and protects against ER stress and apoptosis [28]. The tumor suppressor protein p53 controls the basal level of GPI, where ROS or other signaling pathways cause p53 to induce transcription of GPI [29, 30]. GPI expression was elevated in mice infected with *Streptococcus pneumoniae*, suggesting that GPI is also involved in defense and inflammatory response to infection [31]. Lastly, 14-3-3 γ depletion causes an increase of GPI expression in the brain, suggesting a mechanism by which GPI compensates for the lack of 14-3-3 to enhance cell survival [32].

4.4 PLP1

PLP1 is the most abundant membrane protein of CNS myelin, playing an important role in maintaining axonal integrity. Consequently, PLP1 is a well-studied protein in demyelinating diseases such as multiple sclerosis and other neurological disorders [33]. The full-length PLP1 gene encodes an integral membrane protein of 276 amino acid residues with four hydrophobic membrane-spanning domains [34, 35]. Alternative splicing generates a functionally distinct isoform known as DM20 that lacks a 35 residue intracellular region. DM20 is selectively expressed in early development and is the most abundant isoform in immature oligodendrocytes. In contrast, PLP1 is restricted to myelinating Schwann cells [36]. Autoimmunity against PLP1 has been extensively studied in multiple sclerosis. B-cells secreting autoantibodies against PLP1 and PLP1-reactive T-cells have been identified in the peripheral blood and CSF of multiple sclerosis patients [37, 38].

4.5 PRX1

Peroxioredoxins are a family of enzymes comprised of six isoforms that protect cells against oxidation in various parts of the cell: PRX 1, 2, and 6 are cytosolic; PRX 3 and 5 are mitochondrial/peroxisomal; and PRX 4 is extracellular [39]. PRXs reduce and eliminate peroxides and other ROS that lead to cell apoptosis [40]. PRX1 also inhibits ASK1-mediated apoptosis, particularly in high peroxide environments, and long-term exposure of brain endothelial cells to ROS or activation of microglia by lipopolysaccharide stimulation leads to upregulation of PRX1 [41]. Therefore, PRX1 may be an indicator for activated microglia, where it functions as a free radical scavenger to protect cells from ROS-induced apoptosis, particularly in the inflammatory lesions of EAE [42, 43]. Interestingly, the cytokine macrophage migration inhibitory factor was found to interact with PRX1, where the complex decreased the D-dopachrome tautomerase activity to inhibit chemotaxis [44, 45].

4.6 M² Proteomics and LCM

In pioneering work, Han et al. showed that LCM of postmortem brain tissue specimens from multiple sclerosis patients followed by MS/MS-based proteomics could identify 2574 lesion-specific proteins, including GPI and PLP1 [46]. While gains in sensitivity and specificity are expected when identifying proteins extracted from spatially defined lesions instead of brain tissue lysates, low abundance Th1 and Th17 cytokines [47], including well-known cytokines such as IL17 (IL-17A and IL-17F) and IFN- γ [48] were not identified with

the exception of migration inhibitory factor, the most abundant proinflammatory cytokine that was identified with poor confidence (1.8% sequence coverage) [46]. This may be due to masking of low abundance proteins by high abundance proteins within the lesions themselves. Moreover, the qualitative and/or semiquantitative (i.e. spectral counting) nature of Han's study did not enable relative protein expression to be correlated to disease progression. In our hands, LCM of murine EAE requires impractically lengthy sample preparation times to pool the relatively large number of small-sized mouse brain lesions needed to partially overcome sensitivity limitations (data not shown). Unlike LCM proteomics, M² proteomics can be readily adapted to an automated, 384-well plate format for very high-throughput sample preparation strategies needed to validate biomarkers in a large number of specimens. For all these reasons, we believe that M² proteomics of brain tissue lysates is superior to LCM proteomics for EAE studies. However, combining M² proteomics with LCM may be a powerful tool for analysis of heterogeneous postmortem brain tissue specimens from multiple sclerosis patients.

4.7 M² proteomics and other proteomics studies

To overcome masking problems, enrichment/depletion strategies and/or fractionation approaches may need to be combined with M² proteomics. For example, microwave-assisted digestion of protein lysates bound to naked magnetite beads has been previously shown [49]. The microwave-absorbing, preconcentrating, and denaturing properties of magnetite are a promising alternative for unbiased analyses of positively charged proteins that may complement C8 enrichment of hydrophobic proteins as shown in this study. Likewise, enrichment of low abundance proteins or depletion of high abundance proteins with antibodies may be combined with M² proteomics. Measurement of low abundance species in the presence of high abundance species may also be enabled by fractionation of proteins or peptides before or after M² proteomics to improve dynamic range, respectively. Finally, validation of M² proteomics results by other gel- or gel-free proteomics approaches may become important. However, none of the proteins discussed in this work were observed in recent 2DE studies of EAE where 75 [50] and six differentially expressed proteins [19] were observed, respectively, nor in a recent gel-free isobaric labeling (iTRAQ) study of EAE where 41 differentially expressed proteins were observed [51]. While experimental differences partially explain disparate results from these proteomics studies of EAE, they are clearly complementary to M² proteomics rather than confirmatory. Therefore, validation of M² proteomics results by Western blotting, signaling pathway analysis, and hierarchical clustering are expected to remain important. Validation of M² proteomics results by targeted MS/MS analysis of selected peptides is also expected to remain important.

4.8 M² proteomics and transcriptomics studies

Protein biomarkers and therapeutic targets of multiple sclerosis, that reflect dynamic real-world socioeconomic and environmental factors (e.g. ethnicity, age, sex, nutrition, healthcare, and exposure to drugs, chemicals, infectious diseases or radiation), cannot be determined based solely on analysis of the relatively static genome [52]. Likewise, there is a poor correlation between the transcriptome and proteome due to single nucleotide polymorphisms, alternative splicing, posttranslational modifications (e.g. phosphorylation), limiting ribosomes available for translation, mRNA and protein stability, and various unknown actors (e.g. microRNA). More than 110 genes have been proposed as biomarkers of multiple sclerosis in 18 major transcriptomics studies [53]. Not surprisingly, none of these correspond to the proteins discussed in this work. Moreover, 288 differentially expressed transcripts, including PLP1, were observed in a transcriptomics study of EAE that was performed in parallel with the 2DE study of EAE discussed above, where only six differentially expressed proteins, and not PLP1, were observed [19]. Thus, careful

proteomics studies, and careful correlations to other “-omics” studies, are essential to the discovery of protein biomarkers and therapeutic targets of multiple sclerosis.

4.9 ROS

In the CNS of multiple sclerosis patients, infiltrating T cells release proinflammatory cytokines that activate monocytes and macrophages to release neurotoxic mediators, including nitric oxide and oxygen free radicals [54–56]. Furthermore, NADPH-oxidase is found in activated microglia in the inflamed CNS. This enzyme produces ROS that mediate oxidative stress [57]. In the EAE animal model of multiple sclerosis, IL-17A induces activation of NADPH-oxidase in monocytes, followed by ROS production, which results in the disruption of the blood-brain barrier [58]. In EAE mice treated with Edaravone, a free radical scavenger and a neuroprotective agent, a significant amelioration of clinical severity was observed [59]. These findings, combined with our own and others' observations of ROS-signaling proteins such as the peroxiredoxins, suggest that ROS-mediated stress in multiple sclerosis and EAE merits further investigation by proteomics.

4.10 Conclusions

While validation in a larger cohort is underway, we conclude that M² proteomics is a rapid method to quantify putative prognostic/predictive protein biomarkers and therapeutic targets of disease progression in the EAE animal model of multiple sclerosis. This was demonstrated by correlating the expression of 283 top-ranked proteins at each time point relative to their expression at the peak of disease to EAE disease score to reveal proteins that closely mirror disease progression, including: 14-3-3 ϵ , GPI, PLP1, and PRX1. Results were confirmed by Western blotting, signaling pathway analysis, and hierarchical clustering of EAE risk groups. Subtle changes observed in relative protein expression by M² proteomics may reflect significant differences in disease progression that could be difficult or impossible to ascertain by other methods. Future M² proteomics studies will focus on correlating longitudinal measurements of low abundance brain proteins in blood to quantitative measures of disease progression, including magnetic resonance imaging and/or the expanded disability status scale disease score, in patients with multiple sclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
ELISPOT	enzyme-linked immunosorbent spot
GPI	Glucose-6-phosphate isomerase
LCM	laser capture microdissection

M²	microwave and magnetic
PLP1	proteolipid protein
ROS	reactive oxygen species
TMT	tandem mass tagging

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Figure 1.

Illustration of the M² proteomics correlation of relative 14-3-3ε expression to EAE disease progression. (A) The M² proteomics experimental design is superimposed on a plot of EAE disease score vs. post-immunization time. Isobaric-encoding enabled 5-plex quantitative proteomic analysis of eight sample mixtures, corresponding to eight disease time points, where each mixture included four mice per time point and pooled reference material. TMT-encoded brain tissue lysates from individual mice, labeled with the TMT-127–131 reagents, respectively, were mixed with the reference material encoded with the TMT-126 reagent in 1₁₂₆:1₁₂₇:1₁₂₈:1₁₂₉:1₁₃₀:1₁₃₁ ratios to normalize across the time-course of disease progression. Clinical onset of disease was observed on days 8–10, followed by disease peak

at days 19–20 and remission. (B) Cytokine analysis of IFN γ (•, right axis) and IL17A (•, left axis) in the brain, showing that the relative expression of both low abundance cytokines, detected by ELISPOT but not M² proteomics, closely mirror disease progression, as expected. (C) Relative expression of 14-3-3 ϵ versus time, where levels are inversely proportional to disease progression. (D) Inverse correlation of 14-3-3 ϵ relative expression to EAE score, where the overall p -value = 3.4E-6. (E) Annotated MS/MS spectra with expanded views of reporter ions for quantifying the relative abundance of representative peptides from individual mice, where (*) indicates isobarically labeled amino acid residues: 14-3-3 ϵ ; *LICCDILDVLDK*HLIPAANTGESK*. The relative expression of individual proteins from individual mice is inferred by decoding the relative abundance of multiple peptides for each protein.

Figure 2.

(A) Relative expression of GPI versus time, where levels are directly proportional to disease progression. (B) Direct correlation of GPI relative expression to EAE score, where the overall P -value = $2.1E-5$. (C) Annotated MS/MS spectra with expanded views of reporter ions for quantifying the relative abundance of representative peptides from individual mice, where (*) indicates isobarically labeled amino acid residues: GPI; *IL-LANFLAQTEALMK*. The relative expression of individual proteins from individual mice is inferred by decoding the relative abundance of multiple peptides for each protein.

Figure 3. Hierarchical clustering of EAE risk groups by correlating relative protein expression to disease progression for a subset of proteins measured by M^2 proteomics. The heat map shows that these and other proteins can be used to construct proteomic classifiers that stratify mice into EAE risk groups (G1 $\rightarrow$ G4), where G1 includes mice with the lowest EAE scores and G4 includes mice with the highest EAE scores. 14-3-3 ϵ and GPI closely mirror disease progression, confirming the M^2 proteomics results shown in Figs. 1 and 2.