Early urinary candidate biomarkers in a rat model of experimental autoimmune encephalomyelitis

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Abstract

Biomarker is the change associated with the disease. Without homeostatic control, urine can accumulate early changes in the body. We expect that urinary proteome can also reflect early changes in the nervous system and urine is a better biomarker source for nervous system diseases. Multiple sclerosis is a chronic autoimmune demyelinating disease of the central nervous system and is difficult to diagnose in early stages. In this study, a tandem mass labeling approach coupled with high-resolution mass spectrometry was used to analyze seven-day urinary proteome changes in a rat model of experimental autoimmune encephalomyelitis when the clinical scores in the EAE group were "0" and no obvious histological changes were observed. Thirty-one urinary proteins were altered, based on Ingenuity Pathway Analysis, seventeen of these proteins were associated with neurological functions. The top canonical pathways represented by these dysregulated proteins included the acute phase response and metabolic processes. The acute phase response was characterized by an increase in inflammatory factors that are known to cause multiple sclerosis. Additionally, lipid or glucose metabolic alterations may provide clues for future mechanistic studies on multiple sclerosis. Fourteen proteins were identified to have catalytic activities that may contribute to neuronal damage. Furthermore, among the seven proteins that were most affected, six were reported to be expressed in the serum/cerebrospinal fluid/brain tissue of multiple sclerosis patients, thereby indicating that urine can be a good source of biomarkers for the early detection of multiple sclerosis.

Introduction

Multiple sclerosis (MScl) is a chronic autoimmune demyelinating disease of the central nervous system (CNS) that are characterized by both inflammatory components and neurodegeneration[1]. It contributes to a significant proportion of neurologic morbidity in young adults [2] , but the causes of MScl are not fully understood. Magnetic resonance imaging (MRI) is the most common diagnostic tool for MScl. Additionally, some clinical features may help to diagnose the disease. However, few clinical manifestations are specific to MScl, and MRI lacks specificity for the early stages of the disease.

Without homeostatic control, urine can reflect most changes in the body and thus can be a better source of biomarkers [3]. Numerous biomarkers of various diseases can be detected in urine, and some of these biomarkers perform even better than plasma biomarkers [4]. The urinary proteome may provide clues for brain diseases [5-8]. Because MScl is difficult to diagnose at early stages in clinical practice, in this study, the urinary proteome of an experimental autoimmune encephalomyelitis (EAE) model was used for the discovery of early biomarkers of MScl. For animal models, it is convenient to limit confounding factors to understand the onset of diseases [9].

Advancements in mass spectrometry (MS) have made it possible to uncover new distinct molecular components. During the past few years, proteomics approaches have been used to investigate changes in urinary proteins/metabolites of MScl patients. The results of those studies indicated that metabolites or proteins in the urine of MScl patients may reflect pathogenic processes. For example, proteins such as trefoil factor 3 and lysosome-associated membrane protein 2, both of which are related to immune responses, were differentially expressed in two

phases (the third trimester of pregnancy and the postpartum period) of MScl patients, and these proteins may be associated with MScl [10]. MScl shares many overlapping clinical features with neuromyelitis optica spectrum disorders (NMO-SD), but the treatment strategies differ substantially for these two diseases, and misdiagnosis can often result in wrong treatment [11]. Several urinary proteomic/metabolic studies have been conducted to differentiate these two diseases [12, 13].

EAE is the most commonly employed model for MScl [14], and it has been a powerful tool for studying relevant mechanisms in MScl as well as for translating the findings into clinically meaningful therapeutic approaches [15]. EAE lesions in Lewis rats are characterized by the infiltration of T cells and macrophages into the CNS followed by activation of the microglia, apoptosis of immune cells, impaired axonal conduction in the CNS, and progressive hindlimb paralysis [16]. The clinicopathologic characteristics of the EAE model, including inflammation and demyelination of the CNS, are similar to those of MScl [17]. Thus, studies using the EAE model have provided new insights into the pathogenesis and pathophysiology of MScl.

In the present study, we used two-dimensional high-resolution MS to investigate changes in the urinary proteome during the early stages of MScl in a rat model of EAE. Urine samples were collected at three time points, namely, at the onset of normal condition (day 0), at the onset of disease symptoms (day 7) and at the peak of symptoms (day 14). These samples were analyzed to detect early indicators and correlate them with the hallmarks and pathogenesis of MScl.

Materials and methods

Experimental rats

Thirty male Lewis rats (8 weeks old) were purchased from the Institute of Laboratory Animal

Science, Chinese Academy of Medical Science & Peking Union Medical College. The experiment was approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Animal Welfare Assurance Number: ACUC-A02-2014-007). The study was performed according to guidelines developed by the Institutional Animal Care and Use Committee of Peking Union Medical College.

Rat models of experimental autoimmune encephalomyelitis

Thirty rats were randomly divided into two groups, namely, the EAE group and the control group. EAE was induced in Lewis rats with myelin basic protein (MBP) as previously described [18]. Rats in the EAE group were immunized with subcutaneous injections of 100 μg MBP (Sigma) emulsified in 5 mg/mL complete Freund's adjuvant (CFA) containing Mycobacterium butyricum (Sigma). Rats in the control group were administered with CFA and infused with an equal amount of saline. On days 0 (before MBP immunization, baseline), 7, 14 and 21, all rats were placed in metabolic cages to collect urine. Three pairs of rats in the two groups were sacrificed at each time point, and tissues were collected for histological analyses. Body weight and neurological impairment scores were evaluated daily. The progression of EAE was measured daily based on neurological impairment and scored from 0 to 5 as follows [19]: grade 0, no symptoms; 0.5, mild floppy tail; 1, floppy tail; 2, hindlimb weakness; 3, severe paraparesis; 4, tetraparesis; and 5, moribund.

Histological analysis

On days 0, 7, 14 and 21, spinal cord samples were harvested from both EAE and control groups and dissected after blood withdrawal. After being fixed in 4% paraformaldehyde, the samples were embedded in paraffin. Sections of paraffin-embedded heart samples were stained with

hematoxylin and eosin (H&E) for the evaluation of inflammatory foci.

Urine sample preparation

After collection, urine was immediately centrifuged at 2,000 g to remove pellets. Urinary proteins were extracted by adding three volumes of acetone. After centrifugation, proteins were dissolved in lysis buffer (8 M urea, 2 M thiourea, 25 mM dithiothreitol and 50 mM Tris). The urinary proteins were then denatured with dithiothreitol, alkylated with iodoacetamide and digested with trypsin (Promega) (1:50) at 37°C overnight using filter-aided sample preparation methods as previously described [12]. The digested peptides were desalted using Oasis HLB cartridges (Waters, USA).

Six urine samples (from three rats with EAE and three control rats) collected on day 7 were used for MS analysis. Peptides in each sample were labeled with 126, 127, 128, 129, 130 and 131 tandem mass tag (TMT) reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. The labeled peptides were mixed and then analyzed with two-dimensional liquid chromatography-MS/MS (LC-MS/MS).

Reverse-phase liquid chromatography separation

TMT-labeled peptides were fractionated using offline high-pH reverse-phase liquid chromatography (RPLC) columns (XBridge, C18, 3.5 μm, 4.6 mm × 250 mm, Waters). Peptides were diluted in buffer A1 (10 mM NH4FA in H2O, pH = 10) and then loaded onto the RPLC column. The elution buffer consisted of 10 mM NH4FA in 90% acetonitrile (pH = 10; flow rate = 1 mL/min; 60 min). The eluted peptides were collected at a rate of one fraction per minute. After lyophilization, 60 dried fractions were resuspended in 0.1% formic acid and combined into 15 fractions; for example, fractions 1, 16, and 31 were combined with fraction 46.

LC-MS/MS analysis

Each fraction was analyzed in duplicate using a reverse-phase C18 (3 μm, Dr. Maisch, Germany) self-packed capillary LC column (75 μm × 120 mm). The elution gradient was 5–30% buffer B (0.1% formic acid in acetonitrile; flow rate 0.3 μL/min) for 60 min. A TripleTOF 5600 MS system was used to analyze the eluted peptides. The MS data were acquired using data-dependent acquisition mode with the following parameters: 30 data-dependent MS/MS scans per full scan; acquisition of full scans at a resolution of 40,000, and acquisition of MS/MS scans at a resolution of 20,000; rolling collision energy; charge state screening (including precursors with +2 to +4 charge states); dynamic exclusion (exclusion duration 15 s); an MS/MS scan range of 250-1800 m/z; and a scan time of 50 ms.

Data analysis

All MS/MS spectra were analyzed using the Mascot search engine (version 2.4.1, Matrix Science), and proteins were searched against the SwissProt_2014_07 database (taxonomy: Rattus, containing 7,906 sequences). Carbamidomethylation of cysteines and TMT labeling were set as fixed modifications, the precursor mass tolerance and the fragment mass tolerance were set to 0.05 Da, and two missed trypsin cleavage sites were allowed. To obtain convincing results, proteins were filtered using the decoy database method in Scaffold (version 4.3.2, Proteome Software Inc., Portland). The false discovery rate (FDR) of proteins was set below 1.0%. Each protein contained at least two unique peptides. Scaffold Q+ software was employed for the quantification of TMT labeling. The statistical test used in Scaffold Q+ was permutation; the changed proteins were defined based on a fold change > 1.2 and a p value < 0.05.

Western blotting

Primary antibodies against neutrophil collagenase (MMP-8) and angiotensinogen (AGT) (Abcam, Cambridge) were used to validate the MS results. A total of 30 μg of urinary proteins from the EAE group on days 0, 7 and 14 (four samples per time point) was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Whatman, UK). The membranes were blocked in 5% milk and then incubated overnight with primary antibodies. After the membranes were washed, they were incubated with peroxidase-conjugated anti-rabbit IgG (ZSGB-bio). Finally, the proteins were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific). ImageJ analysis software (National Institutes of Health) was used to analyze the intensity of each band.

Functional analysis

For functional analysis, deregulated urinary proteins identified by MS were further annotated by Ingenuity Pathway Analysis (IPA). After importing the information on the deregulated proteins and their fold changes into the Ingenuity website, we analyzed the affected canonical pathway, networks and related diseases. Biological functions assigned to each canonical pathway were ranked according to the significance of that biological function in the pathway.

Results

Workflow for quantitative proteomics analysis of EAE

To determine the urinary candidate biomarkers of EAE, we immunized rats with MBP to establish an EAE model. Body weight indexes, neurological impairment scores and histopathological characterization were used to evaluate disease progression. On day 7 after immunization, the rats with EAE began to display some clinical symptoms, and this phenomenon was identified as the time point to study early candidate biomarkers. Urine samples were collected from three rats that

showed EAE and three control rats seven days after MBP immunization. For comprehensive and comparative analyses, the peptides were labeled with TMT. The TMT-labeled samples were separated into 15 fractions by offline RPLC, and each fraction was then analyzed in duplicate by LC-MS/MS. The workflow for the quantitative proteomics analysis is shown in Figure 1.

Raw data were searched against the SwissProt database for the taxonomy Rattus and then imported into Scaffold Q+ software for protein identification and quantification. If the identified peptides were shared between two proteins and could not be separated based on the MS data, the two proteins were grouped and annotated as one protein group. In total, 613 proteins that consisted of at least two peptides were identified at a 1% FDR at the protein level. Among these proteins, 566 high-confidence proteins were quantified by TMT labeling analysis in duplicate.

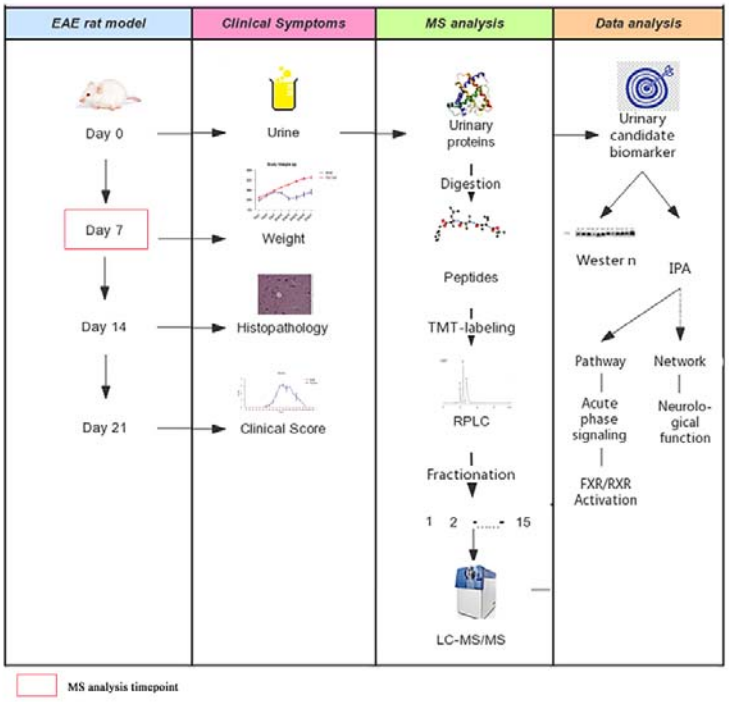

Figure 1. Workflow of the study. Urine was collected on days 0, 7, 14 and 21. After urine collection at each time point, histological analyses of the spinal cord were conducted. Urinary proteins were identified by TMT labeling followed by LC-MS/MS.

Body weight and neurological impairment characterization

Parameters of the EAE and control groups were evaluated from day 0 to day 21. Both MBP-immunized Lewis rats and control rats exhibited a consistent 10 to 15% increase in body weight from the first day to the seventh day. In the first week, no significant difference in body weight was observed between the two groups. From the eighth day onward, the weight of rats in the control group remained on the rise and were higher than that of rats with EAE. However, the weight began to decline in the EAE group. From day 8 to day 10, the weight of rats with EAE gradually decreased (242.1 ± 10.8 g vs. 237.6 ± 17.1 g), but the change was not significant. From day 11 onward, there was a marked decrease in the weight of the EAE group (225.1 ± 16.9 g), whereas the weight of the normal group was 268.1 ± 10.1 g. From day 13 to day 14, the weight of the EAE group stopped decreasing and remained unchanged. From day 15 to day 16, the body weight in the EAE group began to quickly increase. By day 21, the last time point examined in the study, the weight of the EAE group returned to the value on day 7 (242.0 ± 16.9 g), whereas the weight of the control group considerably exceeded the value on day 7 (314.1 ± 11.1 g). As shown in Figure 2A, the difference in body weight between the EAE and control groups was large, indicating possible impairment due to MBP immunization. Additionally, Lewis rats with EAE displayed a monophasic clinical course and spontaneous recovery, both of which are also consistent with previous results [20].

In the present study, no rats died or reach “grade 5” in disease severity. From day 0 to day 7, consistent with the lack of negative changes in body weight, neither the MBP-immunized Lewis rats nor the control rats displayed any abnormal clinical symptoms. All rats immunized with MBP demonstrated typical clinical manifestations of neurological impairment lasting from 8 to 21 days

post-immunization. On the eighth day, which is also the time when the weight began to decrease in rats with EAE, some of the MBP-immunized rats developed mildly flaccid tails, and this symptom became obvious in nearly all rats with EAE by the ninth day. From day 12 to day 14, the rats in the EAE group exhibited progressive bilateral hindlimb paralysis (grades 2 to 3), which was also the most severe symptom observed in this study. Then, the rats recovered from paralysis. By the end of the experiment, these effects disappeared (Figure 2B).

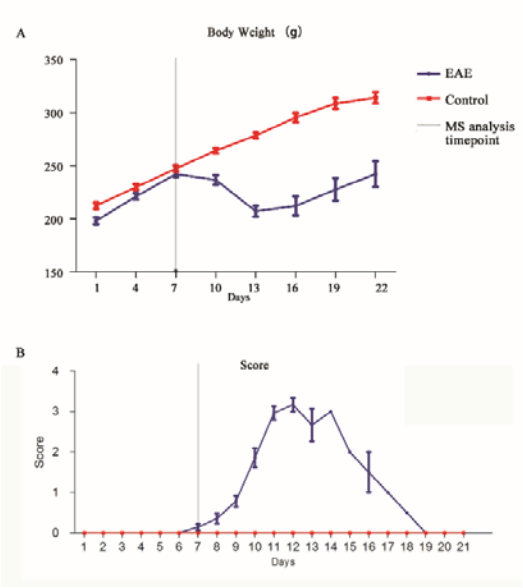

Figure 2. Changes in body weight and neurological damage score during the experiment. The red line indicates the control group, and the purple line indicates the EAE group.

Histopathological findings during the progression of EAE

Histopathological examinations were performed by H&E staining of spinal cord sections from rats with EAE to evaluate the severity of disease (Figure 3). During the early stages after initial immunization, very few infiltrated inflammatory cells were observed. On day 14, H&E staining revealed the presence of numerous inflammatory infiltrates in the parenchyma of the spinal cord and perivascular area. On day 21, also known as the recovery stage, some inflammatory lesions

had disappeared; nevertheless, unlike the absence of inflammatory cell infiltration in the control samples, a number of inflammatory cells were still detected in the EAE samples. These pathological changes demonstrated the successful induction of EAE in Lewis rats.

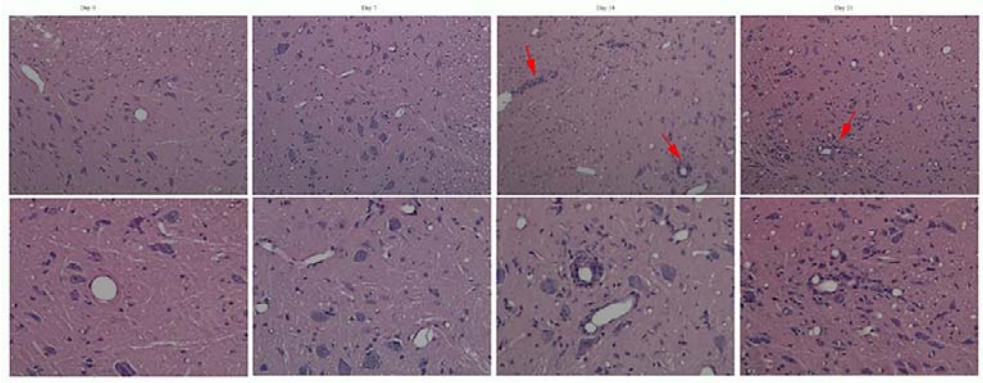

Figure 3. Histological characterization of the spinal cord in rats with EAE. A) Histological changes in the four stages in rats with EAE (H&E staining, 10×). B) Histological changes in the four stages in rats with EAE (H&E staining, 100×).

Early changes in urinary proteins in EAE models

As indicated above, on day 7, the clinical scores in the EAE group were "0" and were similar to the scores in the control group; no obvious histological changes were observed. Therefore, urine samples collected on day 7 after immunization were used for early biomarker detection. All identified proteins were quantitated by Scaffold Q+ software. The quantitative data are listed in Supplemental Table 1. The altered proteins were defined based on the following parameters: $p < 0.05$ (t-test) and fold change ratio > 1.2. Statistical analyses indicated that 31 proteins were significantly affected by MBP immunization (15 up- and 16 downregulated proteins). The proteins are listed in Table 1.

Table 1. All of the altered urinary proteins identified by MS.

Accession	Gene	Human orthologs	Fold change	P value	Location	Type(s)	Reported before	Ref.
P55054	FABP9	Yes	2.5	2.71E-02	Cytoplasm	transporter	Serum	[21]
P01048	Map1	Yes	2.3	1.93E-03	Extracellular Space	other	CSF	[22]

Q62714	RatNP-3b	Yes	2.2	7.50E-03	Extracellular Space	other		
P12020	Crisp1/Crisp3	No	1.8	6.42E-07	Extracellular Space	other		
P01015	AGT	Yes	1.6	1.33E-03	Extracellular Space	growth factor	Serum/CSF	[23]
Q62930	C9	Yes	1.6	1.13E-03	Extracellular Space	other	CSF	[22]
O88766	MMP8	Yes	1.5	3.10E-03	Extracellular Space	peptidase	CNS	[24]
P70490	MFGE8	Yes	1.5	8.95E-03	Extracellular Space	other		
P20059	HPX	Yes	1.4	1.14E-03	Extracellular Space	transporter		
Q8VI04	ASRGL1	Yes	1.4	2.00E-02	Cytoplasm	enzyme		
Q9EQV9	CPB2	Yes	1.4	7.88E-04	Extracellular Space	peptidase		
P13635	CP	Yes	1.3	3.75E-03	Extracellular Space	enzyme		
P02764	ORM1	Yes	1.3	4.33E-02	Extracellular Space	other		
P00787	CTSB	Yes	1.2	1.05E-02	Cytoplasm	peptidase		
P08649	C4A/C4B	Yes	1.2	5.41E-03	Extracellular Space	peptidase		
P02625	PVALB	Yes	0.8	4.58E-04	Cytoplasm	other		
P10959	Ces1c	No	0.8	8.85E-03	Extracellular Space	enzyme		
Q811X6	CRYL1	Yes	0.8	1.55E-02	Cytoplasm	enzyme		
O88989	MDH1	Yes	0.8	3.56E-03	Cytoplasm	enzyme		
P13596	NCAM1	Yes	0.8	1.38E-03	Plasma Membrane	other		
P48500	Tpi1	Yes	0.8	1.38E-03	Cytoplasm	enzyme		
P09656	Spink1l	Yes	0.8	1.68E-02	Extracellular Space	other		
P10111	PPIA	Yes	0.8	8.84E-03	Cytoplasm	enzyme		
P47853	BGN	Yes	0.8	1.31E-02	Extracellular Space	other		
O08839	BIN1	Yes	0.8	2.15E-02	Nucleus	other		
P11232	TXN	Yes	0.7	1.04E-03	Cytoplasm	enzyme		
Q9QZ76	MB	Yes	0.7	8.82E-03	Cytoplasm	transporter		
P50116	S100A9	Yes	0.7	8.02E-03	Cytoplasm	other		
P10760	AHCY	Yes	0.7	9.26E-03	Cytoplasm	enzyme		
P07092	SERPINE2	Yes	0.7	4.15E-05	Extracellular Space	other		
P02631	OCM	Yes	0.7	1.32E-03	Cytoplasm	other	Tissue	[25]

To better understand how the 31 proteins were altered by MBP immunization, we performed principal component analysis (PCA). Figure 4 shows the effect of MBP immunization on the 31 proteins and highlights the differences in the urinary proteome between the control and the EAE groups. In addition, 95% confidence interval values were calculated for each protein.

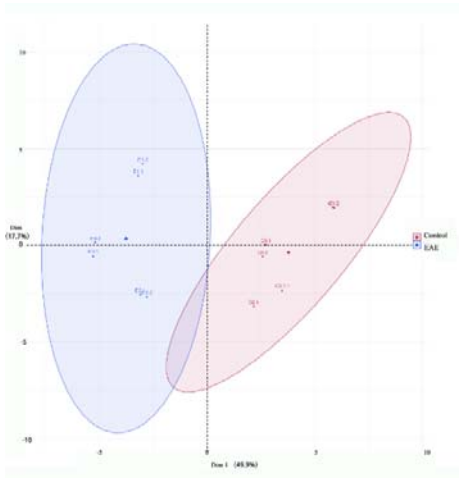

Figure 4. PCA of deregulated urinary proteins. Each axis is labeled with the variation in the data.

Proteomic interactomes of the changed proteins

To investigate the analytical underpinning of this urinary proteomics study, the proteomic interactome of altered proteins after MBP immunization was determined. The IPA software highlights interactions among specific molecules and demonstrates how they might work together at the molecular level. Within these networks, highly interconnected hub molecules are more likely to have important biological functions. Thus, these networks might represent an efficient and unified interactions among the deregulated proteins and highlight potential causal molecules that may be used as targets for preventing MScl progression.

As shown in Figure 5, we uploaded 31 deregulated proteins into IPA and determined their interactions with network proteins associated with “neurological diseases”. A total of 17 deregulated molecules were identified in this functional interaction. In particular, specific peptidases (C4, CPB2, CTSB, MMP-7 and MMP-8) and enzymes (CP, PPIA and TXN) were identified as being altered in MBP-induced EAE.

The hub proteins identified in the neurological disease networks, namely, IL-1, LDL and P36

MAPK, are located in the extracellular space, plasma membrane and cytoplasm, respectively. The upregulation of P38 MAPK is considered to be closely related to 4-1BB signaling in T cells and is consistent with the induction of EAE because EAE is initiated by immunization with autoantigens presented to MHC class II-restricted CD4+ T helper (Th) cells [14]. Additionally, the inhibition of active mouse p38 MAPK in CD4+ T cells was shown to decrease the severity of EAE in mice [26]. IL-1 is one of the commonly used inflammatory factors. Although it is rarely detected in the normal brain, IL-1 is significantly upregulated and plays a central role in neuroinflammation, especially under neurodegenerative conditions [27].

Therefore, the proteins deregulated in response to MBP immunization include interactors that preferentially function in CD4+ T cells, neuroinflammation and lipid metabolism and hence may affect neurological functions.

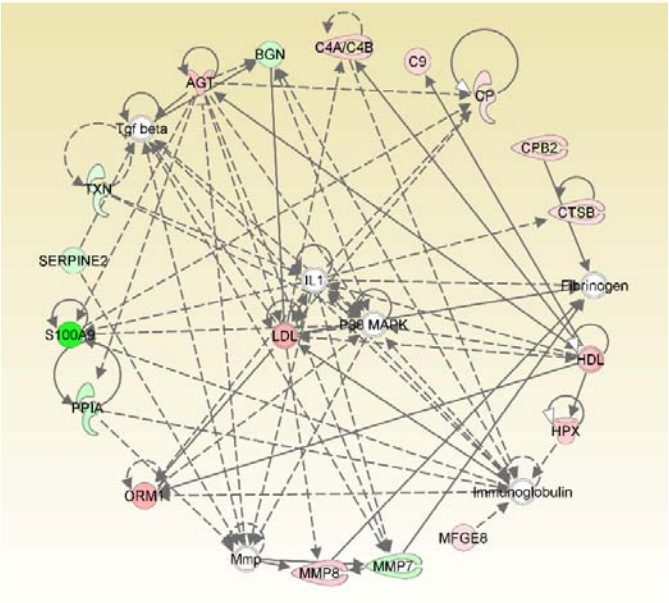

Figure 5. Network related to neurological functions annotated by IPA. Red indicates upregulated genes, and green indicates downregulated genes. Blank indicates that the gene was not deregulated in the study.

Functional annotation of the altered urinary proteins

Altered proteins that are homologous to human proteins are potentially useful in clinical practice and may be candidate biomarkers of MScl. When we imported the 31 altered proteins into the InParanoid database [28] to search for human homologs, 30 of the 31 proteins were found to have human counterparts. Because these 30 proteins may be useful for clinical practice, we will discuss these proteins that have human homologs in the following part of the manuscript.

Because MS analysis of TMT-labeled samples was conducted seven days after MBP immunization when clinical manifestation and histological characterization were not obvious, a majority of urinary proteins were relatively unchanged. When the fold change was set at 1.2, only 31 proteins were found to be dysregulated. When the filtration criteria were made more stringent, for example, the fold change was set at 1.5, eight proteins were identified. Among these proteins, seven have human homologs. Among the seven urinary proteins that were noticeably affected, five are expressed in the serum, cerebrospinal fluid (CSF) or brain tissues of patients with MScl or brain injury (Table 1). Other proteins have been used as markers for neurological disorders. These findings indicate the accuracy of the MS analysis and demonstrate urine as a good source of biomarkers for MScl. The following examples illustrate the importance of these proteins identified in urine and serum/CSF/brain tissues:

Both kininogen (a precursor for kinin) and complement component C9 are mediators of inflammation and play important roles in response to inflammatory injury. Elevated levels of kininogen and C9 in the CSF have been reported in rats that have EAE [22] . Additionally, expression of the kinin B1 receptor mRNA on peripheral blood mononuclear cells can serve as an index of disease activity in MScl [29]. Therefore, in EAE or MScl, the expression of kininogen is upregulated in the plasma, CSF and urine.

Protease family members, including metalloproteases, serine proteases, and cysteine proteases, can be markers of disease activity in MScl [24]. The neutrophil collagenase MMP-8, a metalloprotease, has been shown to increase in the CNS in response to EAE and is correlated with symptom severity [24]. Compared with MMP-8 in the CNS, MMP-8 in urine is upregulated by as much as 1.5-fold during the early stages of EAE and can further increase with disease progression.

ANG is involved in maintaining blood pressure and in the pathogenesis of essential hypertension and preeclampsia. Interestingly, the upregulation of serum angiotensin-converting enzymes is related to disease activity in longitudinal analysis [23], while reduced levels of intrathecal angiotensin II in the CSF are indicators of neural damage and repair processes in MScl [30]. Consistent with plasma ANG, urinary ANG was also increased after immunization in rats that have EAE.

Serum fatty acid binding protein (FABP) is thought to distinguish subtypes of MScl, because it is expressed at the highest level in secondary progressive MScl and increased during early stages of pediatric-onset MScl [21]. In the current study, urinary FABP 9 level was also increased in the early stages of disease.

Oncomodulin, a factor produced by macrophages, promotes axon growth in neurons and is an indicator of CNS injury [25], including MScl. In the early stages of EAE, the levels of oncomodulin are reduced, which may partly be due to axonal injury.

Most of the significantly altered proteins in this study are related to MScl; this finding may indicate the accuracy of MS analysis. Thus, a greater number of altered proteins can be used as candidate biomarkers of spinal cord injury in the future.

Western blot validation

Because the MS analysis was based on urine samples collected at baseline and on day 7, to further study the overall trend in the expression of urinary proteins during disease progression, two proteins were selected for western blot analysis at three timepoints (baseline, day 7 and day 14) in twelve additional samples (Figure 6). The two proteins (ANG and MMP-8) were chosen based on the following criteria: the presence of human orthologs, a relatively large fold change value, the availability of commercially antibodies, and no previous indication of an increase or decrease in the urine from MScl patients.

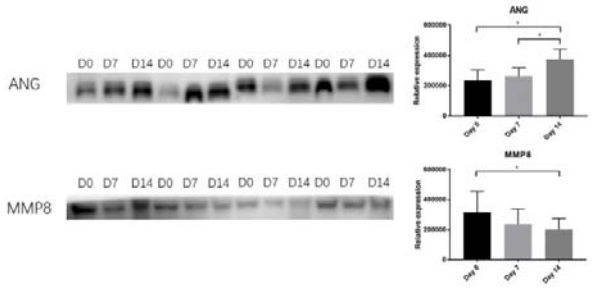

Figure 6. Western blot analysis of AGT and MMP-8. The proteins examined were AGT and MMP-8. * indicates $p < 0.05$; D0, day 0.

Urinary ANG levels in the EAE group on day 7 were similar to the levels at baseline. Then, the levels began to markedly increase, and on day 14, the levels became more that 1.5-fold higher than those in the control group ($p < 0.05$). Likewise, urinary MMP-8 was deregulated in rats that have EAE on day 7 and day 14. On day 7, the level of MMP-8 slowly decreased in rats with EAE and remained low in the following days. On day 14, the urine of rats with EAE exhibited a significantly lower level of MMP-8 ($p < 0.05$) than did the urine of control rats.

ANG and MMP-8 levels significantly differed between EAE and control rats on day 14; at an earlier time point (day 7), this difference was not significant. The western blotting results for ANG

were consistent with shotgun proteomics data. In this study, urinary MMP-8 identified by MS analysis was upregulated in rats with EAE. However, the western blotting results were not consistent with the MS results. The differences could partially be because proteins identified by MS are often grouped based on common peptide fragments, whereas western blotting identifies proteins based on molecular weight. Therefore, when interpreting results, attention should be paid to the methods used.

Discussion

The purpose of this study was to investigate whether urinary proteins can reflect changes in MScl. Urine is highly likely to reflect the functional status of the CNS; however, few studies have directly confirmed this hypothesis. In this study, the levels of 31 urinary proteins were significantly altered at an early stage of disease between the EAE and control groups.

Among the 31 deregulated urinary proteins, six (AGT, C9, C4A, CP, HPX and ORM1) were involved in the acute phase response, which is a rapid inflammatory response and the most enriched canonical pathway represented by the altered proteins. The acute phase response is characterized by an increase in inflammatory factors and changes in several plasma proteins. MScl is an inflammatory and neurodegenerative disorder of the brain and spinal cord [1]. Shortly after MBP immunization, inflammatory factors were upregulated, leading to an immune response within the brain of rats. Inflammatory factors that are the main components of the acute phase response may also be elevated in the CSF and plasma and can eventually be detected in urine. In addition to the acute phase response-related proteins, farnesoid X receptor, which plays a crucial role in the interactions between bile acid regulation and lipoprotein, lipid and glucose metabolism

[31] , was also activated in the early stages of EAE. Although the relation between metabolic disorders and MScl is not yet clear, these findings may provide new clues and directions for future studies.

When the 31 deregulated proteins were analyzed for gene ontology (GO), 14 proteins (AHCY, ASRGL1, Ces1c, CP, CRYL1, MDH1, PPIA, Tpi1, TXN, C4A/C4B, CPB2, CTSB, MMP-7 and MMP-8) were found to exhibit catalytic activities. Among these proteins, nine were enzymes and five were peptidases. MScl is characterized by the demyelination and necrosis of white and gray matter of the spinal cord. Thus, it is likely that enzymes and peptidases with catalytic activities play important roles in destroying normal spinal tissues and causing neuronal damage. Therefore, it is important to determine how to reduce the abundance of these proteins to protect normal spinal cord tissues. In addition, these proteins may be used as therapeutic targets in MScl.

Conclusion

In this study, early candidate urinary biomarkers of MScl were identified in a rat model of EAE before histological changes and clinical symptom. All of the proteins that were noticeably altered had been previously reported to be expressed in the CNS or plasma of MScl patients. Additionally, more than half of the differentially regulated proteins were identified as participants in neurological functions. Most of the altered proteins have catalytic activities and mainly participate in the acute phase response and metabolic processes. These findings may provide clues for the pathogenesis of MScl. In conclusion, this study showed that urine can be a good source of MScl early biomarkers.

Author Contributions

M.Z. and Y.G. prepared the first draft. M.Z., X.L. and Y.G. conceived and designed the experiments. M.Z., X.L. and J.W. performed the experiments. M.Z. and J.W. analyzed the data. All authors approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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