Identify Biomarkers of Neuropsychiatric Systemic Lupus Erythematosus by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Combined with Weak Cation Magnetic Beads

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ABSTRACT. ***Objective.*** To identify proteomic biomarkers in cerebrospinal fluid (CSF) and develop a diagnostic proteomic model for neuropsychiatric systemic lupus erythematosus (NPSLE).

Methods. CSF proteomic spectra were generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) combined with weak cation exchange (WCX) magnetic beads. The spectra were taken from 27 patients with NPSLE before and after treatment, and 27 controls including 17 patients with scoliosis and 10 patients with SLE but without neuropsychiatric manifestation. Discriminating peaks were processed by Biomarker Patterns Software to build a decision tree model for NPSLE classification. In addition, CSF samples of 12 patients with NPSLE, 12 patients with lumbar disc herniation, and 9 patients with other neurological conditions were used as a blind test group to verify the accuracy of the model.

Results. Twelve discriminating mass-to-charge (m/z) peaks were identified between NPSLE and controls: m/z peaks 7740, 11962, 8065, 7661, 6637, 5978, 11384, 11744, 8595, 10848, 7170, and 5806. The diagnostic decision tree model, built with a panel of m/z peaks 8595, 7170, 7661, 7740, and 5806, recognized NPSLE with both sensitivity and specificity of 92.6%, based on training group samples, and sensitivity and specificity of 91.7% and 85.7%, respectively, based on the blind test group. In addition, the root node m/z peak 8595 protein, which was downregulated in the CSF of patients with NPSLE after treatment, was identified and confirmed as ubiquitin by immunoprecipitation and ELISA.

Conclusion. Potential CSF biomarkers for NPSLE are identified by MALDI-TOF-MS combined with WCX magnetic beads. The novel diagnostic proteomic model with m/z peaks 8595, 7170, 7661, 7740, and 5806 is highly sensitive and relatively specific for NPSLE diagnosis. The level of ubiquitin in CSF is a promising biomarker for active NPSLE. (First Release Jan 15 2011; J Rheumatol 2011;38:454–61; doi:10.3899/jrheum.100550)

Key Indexing Terms:
NEUROPSYCHIATRIC LUPUS
MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY
WEAK CATION EXCHANGE MAGNETIC BEADS   UBIQUITIN

Neuropsychiatric lupus (NPSLE)[1,2] is a common yet severe manifestation of systemic lupus erythematosus (SLE), with prevalence of 15%–75% and mortality of 7%–13%[3]. Early diagnosis and prompt treatment could significantly improve

its prognosis[4]. However, the diversity of its clinical presentations, the multiple potential etiologies, and the absence of sensitive and specific tests often make NPSLE diagnosis difficult[5]. In our study, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) combined with weak cation exchange (WCX) magnetic beads to screen and identify biomarkers from cerebrospinal fluid (CSF) in patients with NPSLE[6]. A decision tree model was built, and we found this novel diagnostic model was highly sensitive and specific for NPSLE.

MATERIALS AND METHODS

Adult patients aged > 18 years who were admitted to the Peking Union Medical College (PUMC) Hospital between November 2008 and May 2009 were enrolled. All patients with SLE fulfilled at least 4 of the 1997 American College of Rheumatology (ACR) revised criteria for SLE[7]. Each patient was defined as having NPSLE if he/she had significant clinical manifestation that fulfilled at least 1 of the 19 neuropsychiatric syndromes summarized by the ACR in 1992, identified by history, physical examination, and laboratory or radiographic tests, and further proved by clinical course and response to treatment. NPSLE was excluded if ≥ 1 of these criteria were present: hypertension (diastolic blood pressure > 120 mm Hg), hypoxia (PaO_2 < 50 mm Hg), uremia (blood urea nitrogen > 35.5 mmol/l), serious electrolyte imbalance, or culture-proved central nervous system infection.

In all, 39 patients with NPSLE were enrolled, including 11 with seizure disorder, 8 with cognitive dysfunction, 5 with acute confusional state, 7 with psychosis, 3 with myelopathy, 4 with lupus headache, and 1 with cerebrovascular infarction. All enrolled patients with NPSLE were treated with methylprednisolone 1 g/day for 3 consecutive days, followed by prednisone 1 mg/kg/day plus cyclophosphamide or mycophenolate mofetil. They were also treated with a weekly intrathecal injection of 10 mg dexamethasone, plus 10 mg methotrexate, because this treatment regimen had been shown effective in treating NPSLE[4,8]. The NPSLE symptoms were improved by these treatments in 36 patients. Symptoms did not improve in 2 patients with acute confusional state and 1 patient with cerebrovascular infarction.

A total of 114 CSF samples were obtained. CSF proteomic spectra were generated by MALDI-TOF-MS combined with WCX magnetic beads. A decision tree model for NPSLE classification was constructed with data from a training group, and validated with independent data from a blind test group. The training group consisted of (1) paired samples of 27 patients with NPSLE before and 2 weeks after treatment; (2) 27 samples from a control group, including 10 patients with SLE without neuropsychiatric manifestations (non-NPSLE); and (3) 17 patients with scoliosis, a noninflammatory disease. The blind test group consisted of 12 patients with NPSLE before treatment, 12 patients with lumbar disc herniation, and 9 patients with other autoimmune diseases that had neuropsychiatric involvements, including 2 patients each with primary Sjögren's syndrome, multiple sclerosis, Wegener's granulomatosis, and Behçet's disease, and 1 patient with relapsing polychondritis (Table 1).

In addition, the root node mass-to-charge (m/z) peak of this decision tree model was searched against an online proteomic database, and the potential candidate protein was confirmed with immunoprecipitation experiment plus MALDI-TOF-MS combined with WCX magnetic bead analysis as well as Western blot, and further validated by protein-specific ELISA.

Our study was approved by the ethics committee of PUMC Hospital, and informed consent was obtained from each patient or family.

Reagents and equipment. The MALDI-TOF-MS (Protein Biological System IIc) and Au-chip were both provided by Vermillion, Fremont, CA, USA. The WCX magnetic beads were purchased from Beijing SED Science and Technology, Beijing, China. Antiubiquitin antibody, protein A/G Plus-Agarose, horseradish peroxidase (HRP)-conjugated goat polyclonal to rabbit IgG and normal rabbit IgG were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Antiubiquitin ELISA kit was purchased from Abcam, Cambridge, MA, USA. Other reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Sample collection. CSF samples were collected in sterilized tubes by lumbar puncture, and were immediately centrifuged at 3000 g for 10 min at 4°C. The supernatant was divided into 1-ml aliquots and stored at –80°C for subsequent proteomics analysis. The sample collection time was within 1 week after the onset of neuropsychiatric symptoms in SLE and other autoimmune disease controls.

Magnetic bead-based sample preparation for MALDI-TOF-MS. WCX magnetic bead was activated according to the manufacturer's operation manual[9]. A CSF sample of 40 µl was incubated with 40 µl of U9 solution (9 M urea, 32.5 mM CHAPS, 64.8 mM dithiothreitol, 50 mM Tris-HCl buffer, pH 9) for 30 min at 4°C, then diluted with 320 µl of reaction buffer (150 mM sodium acetate, pH 4). The diluted sample was incubated with the activated magnetic beads for 1 h at room temperature, and washed twice with 100 µl binding buffer (50 mM sodium acetate, pH 4). Then the proteins bound to the beads were eluted with 10 µl 1% (v/v) trifluoroacetic acid (TFA). Finally, 3 µl of elute supernatant and 1.5 µl saturated SPA (50% ACN, 0.5% TFA) was spotted onto Au-chip for subsequent MALDI-TOF-MS analysis.

MALDI-TOF-MS analysis. Prepared Au-chips were placed on the Protein Biological System IIc mass spectrometer reader (Vermillion), and time-of-flight spectra were generated by averaging 81 laser shots collected on each spot at laser intensity 140–145, detector sensitivity 8. The optimization range was from 2000 to 30,000 m/z ratio, with the highest m/z 50,000[6]. Data reproducibility was tested and validated[10]. All the samples were test-

Table 1. Baseline characteristics of the study population.

Groups	Subgroup	Disease	Number	Sex (F/M)	Age, yrs, SD (range)	SLEDAI
Training	NPSLE	NPSLE	27	23/4	29 ± 11 (16–60)	26 ± 11 (10–61)
	Control	Non-NPSLE	10	10/0	28 ± 9 (20–41)	11 ± 4 (8–18)
		Scoliosis	17	13/4	19 ± 10 (16–51)	N/A
Blind test	NPSLE	NPSLE	12	12/0	37 ± 11 (21–54)	16 ± 6 (8–28)
	Control	Other autoimmune disease	9	7/2	40 ± 8 (28–51)	N/A
		Lumbar disc herniation	12	5/7	57 ± 6 (47–65)	N/A

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; NPSLE: neuropsychiatric systemic lupus erythematosus; N/A: not applicable.
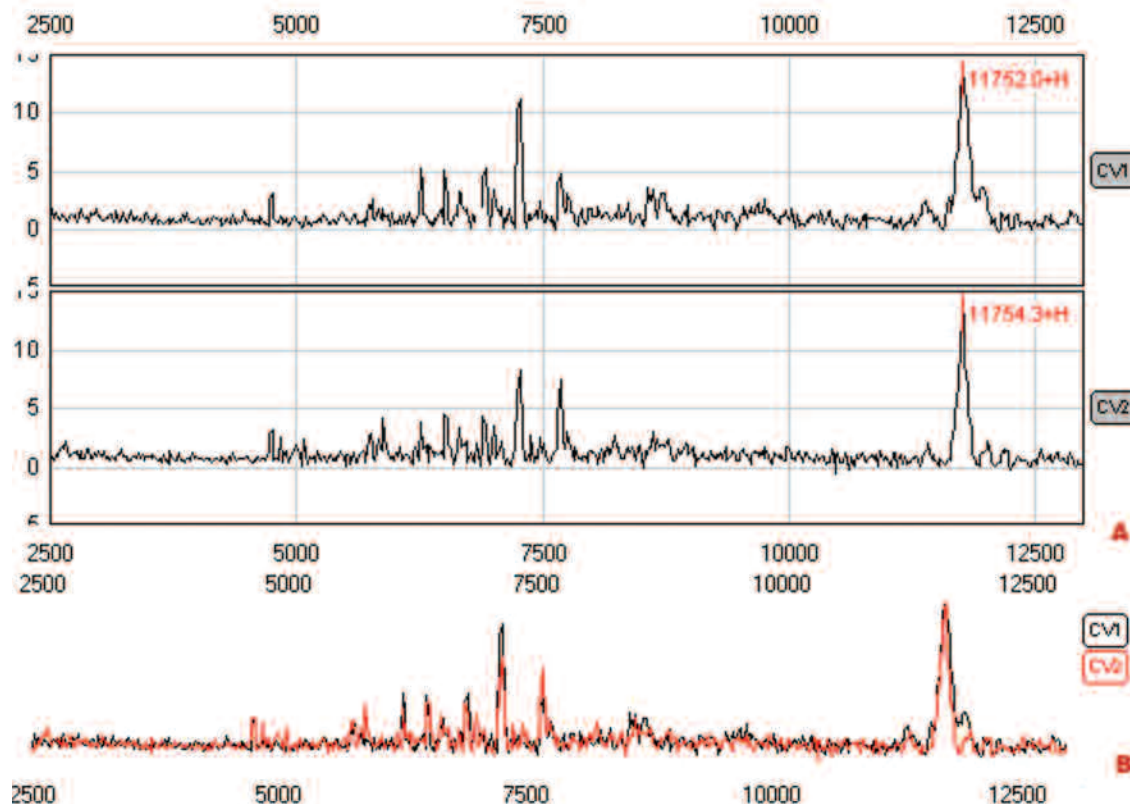
Figure 1. Cerebrospinal fluid spectrum of 1 sample tested at 2 different timepoints (A) and overlap analysis (B). The mass difference in peak 11752 was 2.3% (< 0.3%). The coefficient of variability (CV) of intensity in peak 11752 was 8.2%.
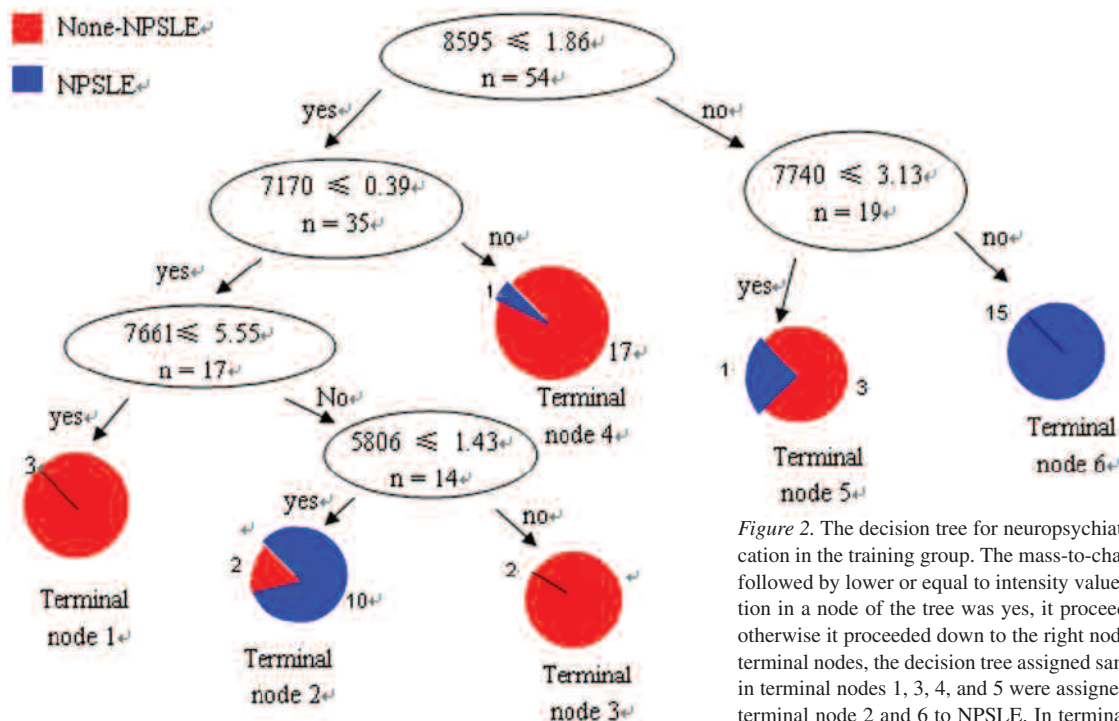
Figure 2. The decision tree for neuropsychiatric SLE (NPSLE) classification in the training group. The mass-to-charge value in the nodes was followed by lower or equal to intensity value. If the answer to the question in a node of the tree was yes, it proceeded down to the left node, otherwise it proceeded down to the right node. When proceeding to the terminal nodes, the decision tree assigned samples to 2 groups. Samples in terminal nodes 1, 3, 4, and 5 were assigned to the control group, and terminal node 2 and 6 to NPSLE. In terminal node 2, 2 out of 12 control subjects were assigned incorrectly to the NPSLE group, and in terminal node 4 and 5, 2 out of 22 NPSLE samples were assigned incorrectly to control group.

ed by MALDI-TOF-MS in 1 batch to optimize the stability and accuracy. In addition, mass accuracy was calibrated externally by standard procedures using the all-in-one peptide molecular mass standards (Vermillion), and the spectra were calibrated by ProteinChip software (Vermillion). A representative CSF spectrum of 1 patient tested at 2 different times is shown in Figure 1. The mass difference in peak 11752 was 2.3% (< 0.3%). The coefficient of variability (CV) of intensity in peak 11752 was 8.2%.

Data analysis. The data were analyzed with ProteinChip software[6,9]. Step 1: Peak detection including (1) baseline subtraction, (2) mass accuracy calibration, and (3) automatic peaks detection. Using Biomarker Wizard Version 3.1.0 (Vermillion), biomarkers were generated that represent consistent protein peak sets across multiple spectra[9]. Baseline subtraction was performed on all spectra. The settings for autodetect peaks to cluster were first 5, mini peak 0%, cluster mass 0.3%, second pass 2. Step 2: Selection of differently expressed peaks that may represent potential biomarkers of NPSLE with SPSS software, p values < 0.05, were considered statistically significant (after normality analysis, data in normal distribution were analyzed with independent-samples T test or paired-samples T test; data in nonnormal distribution were analyzed with nonparametric Kruskal-Wallis test or Wilcoxon signed-rank test). Step 3: Construction of a decision tree model for NPSLE classification with Biomarker Patterns Software (BPS) 5.0 (Vermillion) with data from the training group, and validated with independent data from the blind test group. BPS is a pattern analytical tool developed by Breiman, et al[11]. BPS builds a binary decision tree algorithm with the peak information of the training set. The algorithm assigns each sample into 1 of the 2 nodes by rules based on the intensity of certain peaks[12,13].

Protein identification. For immunoprecipitation, a volume of 180 μl CSF was incubated with 2 μg antiubiquitin polyclonal antibodies for 2 h. As negative controls, aliquots of the same CSF sample were incubated with normal rabbit polyclonal antibodies. Then 20 μl protein A/G agarose was added and incubated overnight. The samples were then spun down and the supernatants were analyzed by MALDI-TOF-MS combined with WCX beads.

For Western blotting, samples included ubiquitin standard protein, NPSLE CSF, NPSLE CSF supernatant after immunoprecipitation with antiubiquitin antibody, non-NPSLE CSF, and scoliosis CSF. They were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted to immobilon P membrane. The antiubiquitin antibody was incubated with the membrane at 1:200 dilution for 2 h. Then goat polyclonal IgG to rabbit (HRP-conjugated) was added for 1 h and detected with an enhanced luminol-based chemiluminescent kit.

For ubiquitin ELISA, CSF samples from 16 cases of NPSLE and 7 cases of non-NPSLE were tested by antiubiquitin ELISA kit according to the manufacturer's instructions. OD 450 nm was read with a 2-wavelength microplate photometer.

RESULTS

Within the m/z peak range of 2000–20,000, there were 12 discriminating m/z peaks between NPSLE and the control group (p < 0.05), including 10 peaks that were upregulated in NPSLE and 2 peaks downregulated (Table 2).

Construction of decision tree model for NPSLE classification. The discriminating m/z peaks were analyzed by Biomarker Patterns Software 5.0 (BPS) to establish the optimal classification tree (Figure 2), with the following measurements[9]: method, 0; advanced, 10; testing, 10; costs, 1:1. The m/z peaks 8595, 7170, 7661, 7740, and 5806 were selected in the classification tree, and m/z peak 8595 was the root node. The peaks of m/z 7740, 7661, and 8595 were upregulated in patients with NPSLE compared with the con-

Table 2. The discriminating mass-to-charge (m/z) peaks between the patients with neuropsychiatric systemic lupus erythematosus and the control group.

	m/z	p	m/z	p
Upregulation	7740	0.001	5978	0.004
	11962	0.010	11384	0.012
	8065	0.016	11744	0.017
	7661	0.022	8595	0.022
	6637	0.044	10848	0.046
Downregulation	7170	0.002	5806	0.024

trol group, while the peaks of m/z 5806 and 7170 were downregulated (Figure 3). All 54 training group CSF samples were allocated to 6 terminal nodes. Samples allocated to terminal nodes 2 and 6 were classified as NPSLE; those allocated to terminal node 1, 3, 4, and 5 were classified as control. For example, if an unknown sample had peaks of m/z 8595 (intensity > 1.86) and m/z 7740 (intensity > 3.13), then the sample was placed in terminal node 6 and classified as NPSLE. If the sample was placed in terminal node 1, it was ruled out from NPSLE diagnosis. The model showed both sensitivity and specificity of 92.6% in classifying the training group CSF samples, and a sensitivity of 91.7% and a specificity of 85.7% in the blind test group (Table 3). The corresponding receiver-operating characteristic (ROC) curve of the optimal decision tree was supplied by the BPS. The ROC curve integral was 0.963 (Figure 4).

When CSF proteomic profiles of patients with NPSLE before and after treatment were compared, 4 discriminating peaks were identified. Peaks of m/z 4963 and 8595 were downregulated, and peaks of m/z 6637 and 6896 were upregulated after treatment (Figure 5).

Identification of m/z peak 8595 protein. Because the m/z peak 8595 was the root node of this decision tree model, and it was downregulated in the CSF of patients with NPSLE after treatment, we set out to search this discriminating peak against an online proteomic database (Geneva: Swiss Institute of Bioinformatics; http://expasy.org), and ubiquitin was identified as the potential protein. We then conducted an immunoprecipitation experiment to validate this candidate protein. Antiubiquitin antibody was used to deplete the CSF sample of ubiquitin. When the depleted sample was analyzed by MALDI-TOF-MS combined with WCX magnetic beads, we found that the m/z peak 8595 was dramatically reduced in the MALDI-TOF-MS spectra as compared with the sample treated with control antibody, while other protein peaks remained unchanged (Figure 6A-6C). This was also confirmed by Western blot after antiubiquitin antibody immunoprecipitation (Figure 6D). In addition, with a ubiquitin ELISA, we also found that the optic density of the NPSLE group (0.200 ± 0.080) was significantly higher than that of the non-NPSLE group (0.090 ± 0.021; p < 0.001; Figure 7).
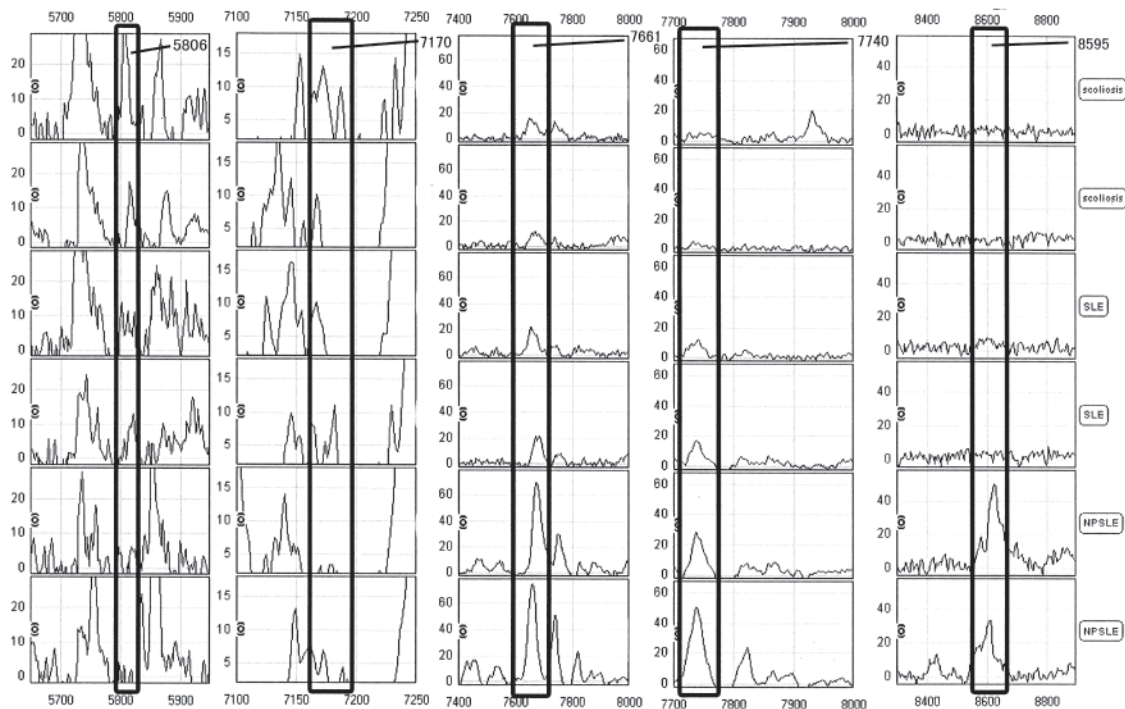
Figure 3. Detection of 5 mass-to-charge (m/z) peaks in the decision model. Protein spectra of cerebrospinal fluid samples from 2 patients with scoliosis, 2 patients without neuropsychiatric SLE (NPSLE), and 2 patients with NPSLE were shown. X axis represents the m/z and Y axis represents the relative intensity. The spectrographic profiles reveal downregulation of the m/z peaks 5806 and 7170 and upregulation of m/z peaks 7661, 7740, and 8595 in NPSLE samples.

Table 3. Sensitivity and specificity in classifying the cerebrospinal fluid samples of the training group and of the blind test group.

| Actual class | Total cases | Prediction Success for Training Group Samples | | |
		Percent correct	0 (n = 27)	1 (n = 27)
0	27	92.593	25	2
1	27	92.593	2	25

| Actual class | Total cases | Prediction Success for Blind Test | | |
		Percent correct	0 (n = 14)	1 (n = 19)
0	12	91.667	11	1
1	21	85.714	3	18

DISCUSSION

MALDI-TOF-MS is a reliable, high-throughput technique for identifying proteins and/or peptides. WCX magnetic beads separate the proteins and/or peptides of different isoelectric points from complex biological fluids with specific anionic ligands. The techniques of MALDI-TOF-MS combined with WCX magnetic beads incorporate both of their advantages[9], allowing the identification of comprehensive "fingerprints" of protein profiles within biological fluids, and were used to identify biomarkers of various diseases[14]. In the field of SLE, Mosley, *et al*[15], Suzuki, *et al*[16], and Rovin, *et al*[17] used this technique to study the urine pro-

teomics of lupus nephritis, and profiled the urine proteomic signature to distinguish lupus nephritis from lupus without nephritis, and/or active lupus nephritis from inactive lupus nephritis, independently. Huang and colleagues profiled the serum proteome of patients with SLE, established a decision tree for SLE classification, and provided a novel approach for the diagnosis of SLE[18].

We compared the CSF proteomic profiles of patients with NPSLE and patients without NPSLE, and established a decision tree model for NPSLE classification, with both a sensitivity and a specificity of 92.6% in learning data classification, and a sensitivity of 91.7% and specificity of 85.7% in
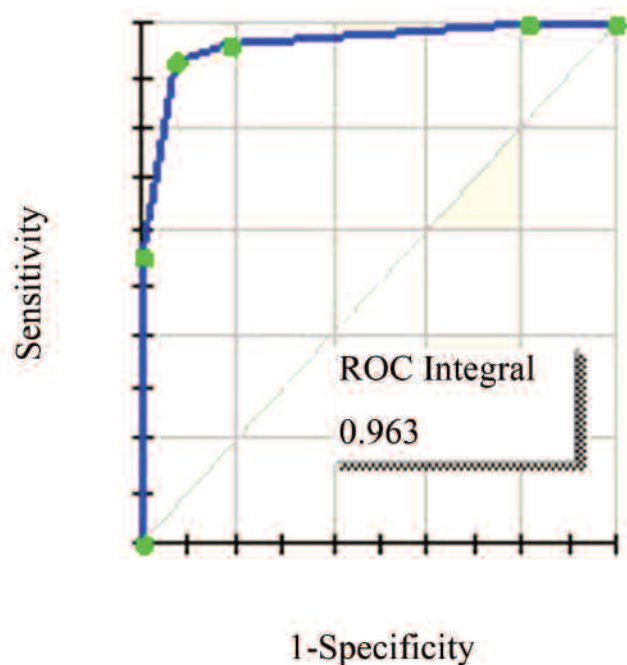
Figure 4. Prediction success and ROC curve of the decision tree model for neuropsychiatric SLE classification. The data of 54 patients were used to construct the model, including 27 with NPSLE, 10 without NPSLE, and 17 with scoliosis. The data used to test the model included 12 patients with NPSLE before treatments, 12 patients with lumbar disc herniation, and 9 patients with other autoimmune diseases who had neuropsychiatric involvement.

a blind test group, indicating that this model had high sensitivity and specificity for the diagnosis of NPSLE.

The discriminating m/z peaks included in the nodes of the decision tree model were those with the most significant difference, especially the root node peak at m/z peak 8595. We found the peak intensity at m/z peak 8595 was significantly higher in the CSF of patients with NPSLE than in the control group (Figure 3), and was downregulated after effective treatment (Figure 5), indicating that protein of m/z peak 8595 strongly correlates with NPSLE activity. In our study, the protein at m/z peak 8595 was identified and validated as ubiquitin by immunoprecipation experiment plus MALDI-TOF-MS combined with WCX magnetic bead analysis as well as Western blot. In addition, ELISA also found that the level of ubiquitin in the CSF of patients with
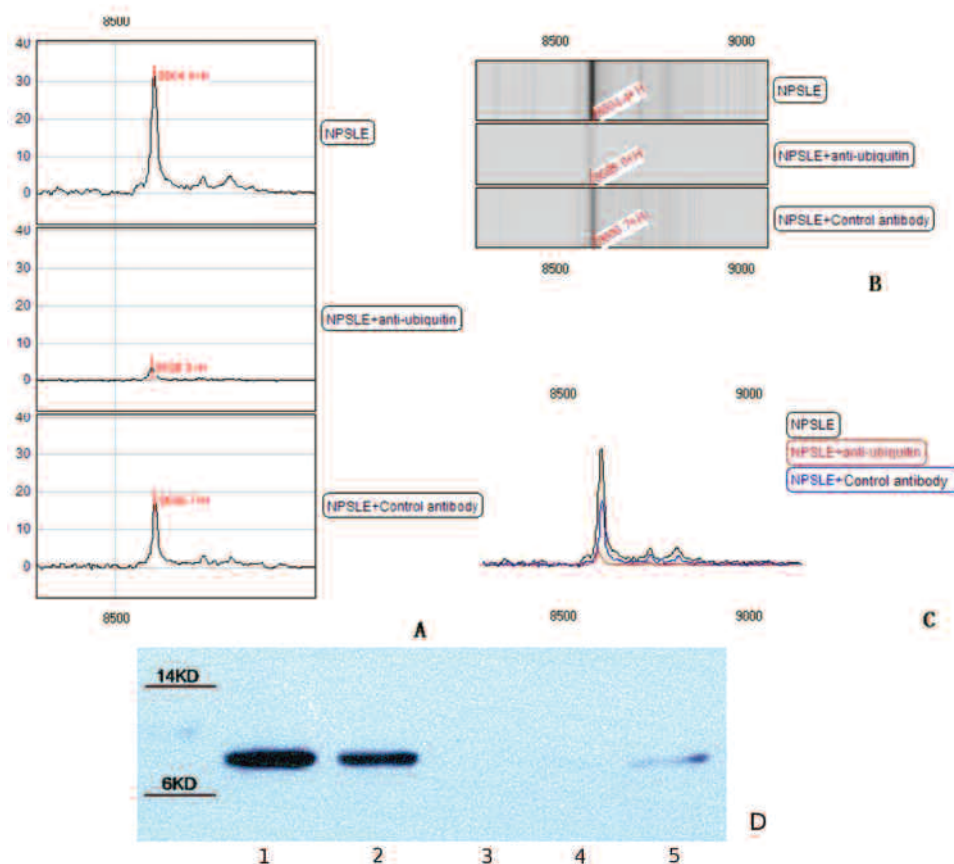
Figure 6. Ubiquitin identification. A-C: Mass spectra of cerebrospinal fluid (CSF) and its supernatant after immunoprecipitation with antiubiquitin antibody and control rabbit polyclonal antibodies in a patient with neuropsychiatric SLE (NPSLE). The intensities of mass-to-charge peak 8595 in CSF samples without immunoprecipitation, immunoprecipitated with antiubiquitin, and immunoprecipitated with control antibodies were 31.4, 4.9, and 19.8, respectively. D: Ubiquitin Western blot after immunoprecipitation. From left: (1) standard ubiquitin protein; (2) NPSLE CSF without immunoprecipitation; (3) NPSLE CSF supernatant after immunoprecipitation with antiubiquitin antibody; (4) negative control; and (5) non-NPSLE CSF without immunoprecipitation.
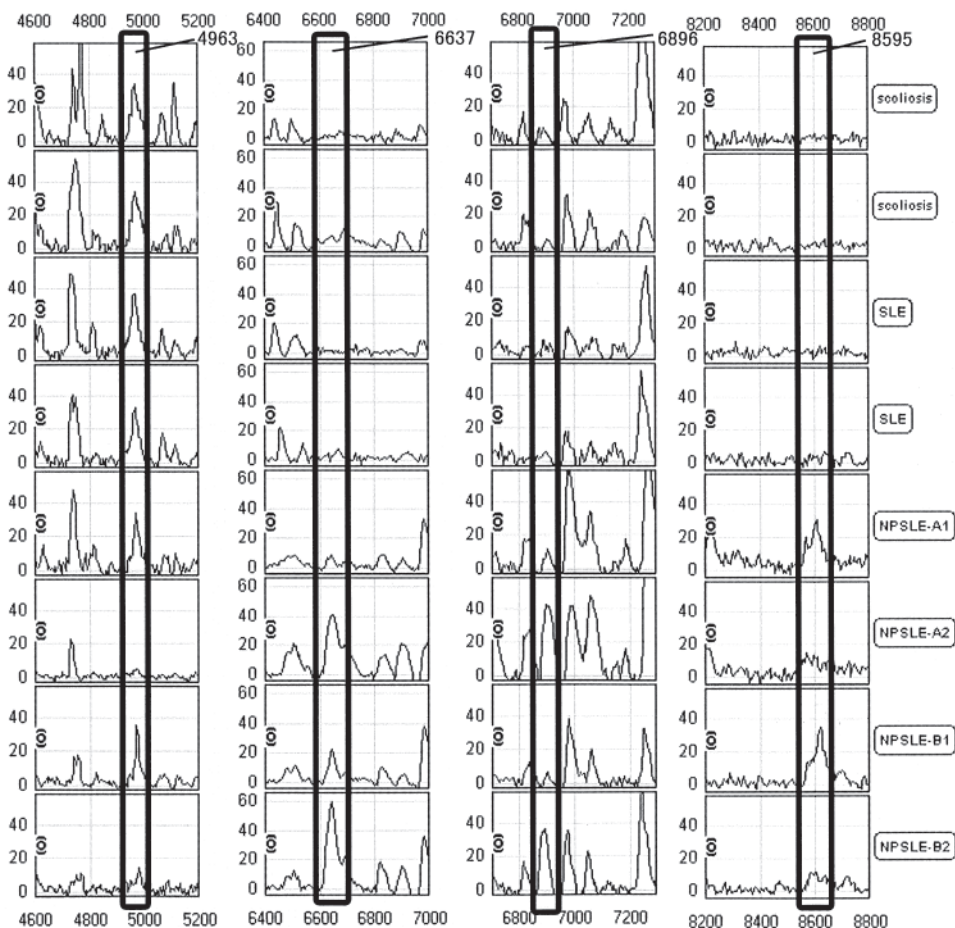
Figure 5. Cerebrospinal fluid (CSF) proteomic profiles of patients with neuropsychiatric SLE (NPSLE) before and after treatment. NPSLE-A1 and NPSLE-B1 represent 2 patients with NPSLE before treatment, and NPSLE-A2 and NPSLE-B2 represent the same 2 patients after treatment. The spectrographic profiles reveal downregulation of mass-to-charge (m/z) peaks 4963 and 8595 and upregulation of m/z peaks 6637 and 6896 in the CSF of patients with NPSLE after treatment.

NPSLE was significantly higher than that of patients without NPSLE, suggesting ubiquitin could be a promising biomarker indicating active NPSLE.

Ubiquitin is a small protein that is ubiquitously expressed in eukaryotes[19]. It consists of 76 amino acids and has a molecular weight of about 8.5 kDa. The most important function of ubiquitin is labeling various target proteins for proteasome degradation. The ubiquitination system functions in various cellular processes, including apoptosis, immune response, and inflammation, as well as neural and muscular degeneration. There were also reports showing that ubiquitin was elevated in the CSF of patients with some other neurological conditions, including Alzheimer's disease[20,21] and Creutzfeldt-Jakob disease[22], suggesting ubiquitin might participate in neural apoptosis and degeneration. We showed that in patients with SLE, an elevated level of ubiquitin in CSF indicates neuropsychiatric involvement and it correlates with NPSLE disease activity.

We identified several discriminating protein peaks for NPSLE by MALDI-TOF-MS combined with WCX magnetic beads. The decision tree model with m/z peaks 8595, 7170, 7661, 7740, and 5806 was highly sensitive and relatively specific for NPSLE classification. In addition, the level of ubiquitin in CSF was identified as a promising biomarker for active NPSLE. This study provides useful biomarker data in NPSLE, and more studies are needed before these biomarkers can be introduced in clinical practice.

ACKNOWLEDGMENT

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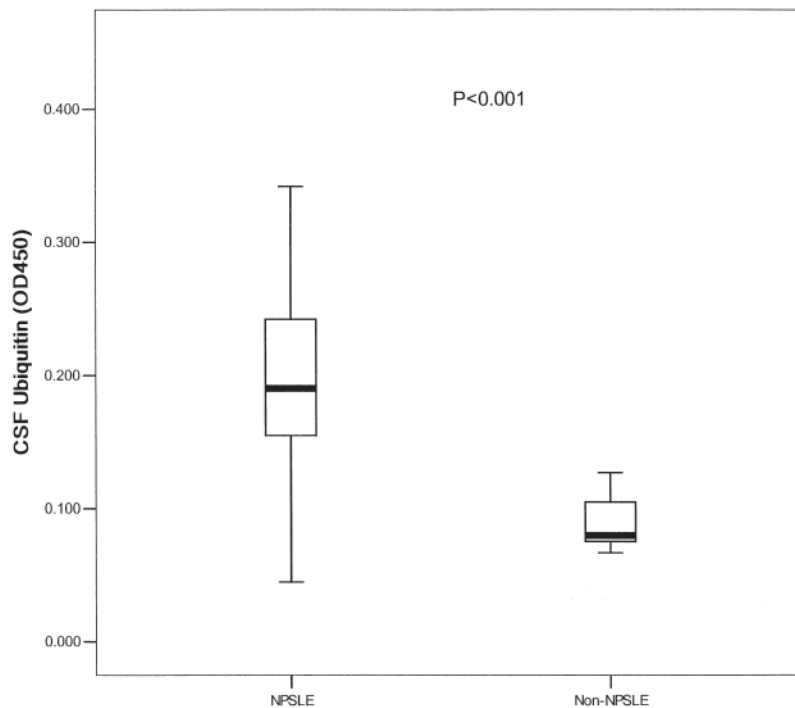
Figure 7. Ubiquitin ELISA results from neuropsychiatric SLE (NPSLE) and non-NPSLE cerebrospinal fluid (CSF). The OD450 of the NPSLE group was 0.20 ± 0.08 and of the non-NPSLE group 0.090 ± 0.021 (mean ± SD) (p < 0.001).

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