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ORIGINAL PAPER

Protein Biomarkers in Serum of Patients with Schizophrenia

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Abstract This study was devised to identify potential biomarkers of schizophrenia (SP) using proteomics techniques. We obtained 44 serum specimens from patients with SP, 26 specimens from patients with depression, and 40 specimens from healthy controls. Immobilized metal affinity capture protein chips (IMAC30) and surface-enhanced laser desorption-ionization time-of-flight mass spectrometry were used to isolate and obtain mass spectrometric data of differentially expressed serum proteins. The sequences of the peaks discrepant among the study groups were obtained using matrix-assisted laser desorption/ionization mass spectrometry and proteins identified using Mascot database. In the SP group, there were 91 protein peaks that were different from other study groups at the p value of <0.05 and 54 peaks different at the p value of <0.01. Two protein peaks at the mass-to-charge ratio of 1,207.41 and 1,466.78 were markedly different among the study groups, with the lowest expression in specimens from patients with SP. The amino acid sequences were, respectively, Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg (EGDFLAEGG GVR) and Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg (DSGEGDFLAEGGGVR). These proteins were identified as the N-terminal fragments of fibrinogen. In conclusion, these biomarker proteins may be useful for molecular diagnosis of SP.

Keywords Schizophrenia · SELDI-TOF–MS · Proteomics · Diagnosis · Biomarker

List of abbreviations

SELDI-TOF–MS	Surface-enhanced laser desorption/ionization, time-of-flight mass spectrometry
SPA	Sinapinic acid
MALDI-TOF–MS	Matrix assisted laser desorption/ionization, mass spectrometry
CV	Coefficient of variance
m/z	Mass-to-charge ratio
IMAC30	Immobilized metal affinity surface
TFA	Trifluoroacetic acid
EAM	Energy absorption molecules

Introduction

Schizophrenia (SP) is a mental disorder with unknown etiology and unclear pathogenesis [1]. There is a clear need for molecular biomarkers for this disease. Proteomic techniques are becoming increasingly popular for biomarker identification, also in psychiatry [2, 3]. In the present study,

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we utilized surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technique to screen serum proteins of SP patients to find potential biomarkers. The proteins of interest were identified by enzymatic digestion and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS).

Materials and Methods

Patients and Healthy Controls

Patients with SP were enrolled in Psychiatry Department of the Affiliated Hospital of Luzhou Medical College between November 2007 and October 2008. The inclusion criteria were: diagnosis of SP made according to the International Diseases Classification and Diagnostic Criteria (10th edition, ICD-10) [4], first onset, no use of antipsychotic drugs after admission, and exclusion of mental disorders caused by cerebral organic or somatic diseases. Forty-four patients met these inclusion criteria. There were 20 male and 24 female patients whose age ranged from 19 to 48 years (mean ± SD age of 33.1 ± 8.4 years).

As control subjects, we enrolled 26 patients with depression. The inclusion criteria in this group were the following: diagnosis of depression according to the Classification and Diagnostic Criteria of Mental Disorders in China (CCMD3) [5], no use of antidepressant drugs after admission, and ruled out SP. There were 11 male and 15 female patients whose age ranged from 17 to 45 years (mean age of 32.5 ± 8.6 years).

We also utilized a healthy control group which comprised 40 healthy individuals (18 male and 22 female individuals, age range of 21–50 years, mean age of 34.0 ± 9.2 years).

Collection of Specimens

Venous blood (approximately 2–3 ml) was collected in the early morning and stored at 4 °C for a maximum of 30 min. Blood specimens were allowed to stand for 1 h to clot and were subsequently centrifuged at 3,000 rpm for 5 min at 4 °C. Serum samples were transferred into microtubes and re-centrifuged at 3,000 rpm for 5 min at 4 °C. Supernatants were separated into three tubes (100 µl/tube) and stored at −80 °C until further use.

Sample Preparation

The frozen serum samples were thawed on ice, centrifuged at 10,000 rpm for 2 min at 4 °C. Ten microliters of serum samples and 90 µl of phosphate buffer were combined in a microtube, and the mixture was mixed well.

Loading and Elution

Protein chip IMAC30 (Ciphergen Biosystems, Fremont, USA) was loaded on the chip holder. The chip was sequentially equilibrated in 50 µl of 0.1 M copper sulfate buffer (10 min), 200 µl of deionized water (1 min), 200 µl of 0.1 M sodium acetate buffer (pH 4.0; 5 min), 200 µl of deionized water again (1 min), and 200 µl of phosphate buffer (5 min). After phosphate buffer was removed, 50 µl of samples prepared as above were added and allowed to bind for 30 min at 4 °C. Then, the sample was removed, and the chip was washed twice with 200 µl of binding buffer (PBS, pH 7.2; 5 min). After the last wash with PBS, the chip was wiped dry and washed twice with 200 µl of HPLC-grade water. The chip was then removed and dried at room temperature.

Preparation of Energy Absorption Molecules (EAM)

Five microliters of trifluoroacetic acid solution (TFA) were added to 495 µl of HPLC-grade water and mixed well. Two-hundred microliters of diluted TFA were combined with 200 µl of acetonitrile solution in a sinapinic acid (SPA) tube (5 µg/tube) and homogenized for 3 min. The mixture was allowed to stand for 5 min and centrifuged for 3 min at 10,000 rpm.

Addition of EAM to the Sample

One microliter of SPA was pipetted onto the surface of the dried protein chip. The protein chip was then air-dried for 15 min and read in the instrument.

Mass Spectrometric Detection of Serum Proteins

Serum proteins attached to the surface of protein chip were detected using PBS II/C protein fingerprint spectrometer, with the optimization range of 1,000–20,000 mass-to-charge ratio (m/z), highest detectable molecular weight of 100,000 m/z, laser intensity of 210, and detection sensitivity of 9. The instrument was calibrated by all-in-one protein standard molecular chip (Ciphergen Biosystems) to confirm that the system's mass deviation was $\leq$0.1 %. The Ciphergen Protein chip software was used to automatically collect experimental data and store the original detection spectra. The pre-treatments of the obtained serum protein spectra included reduction of the baseline, homogenization, etc.

Statistical Analysis

The serum protein fingerprint patterns were analyzed by Biomarker Wizard 3.1 software (Ciphergen Biosystems), with the threshold value of occurrence frequency of

significant protein peak of 10 %, and signal–noise ratio (S/N) of 5 and 2 for filtering. The *t* test was performed to compare peak expressions between any two groups. The difference between protein peaks with the *p* value of <0.05 was considered statistically significant.

Results

Reproducibility of Experimental Results

A randomly selected sample was repeated twice within the same day on the same protein chip, and identical mass spectrometric peaks were obtained (Fig. 1). The same sample was further repeated four times at different days yielding the same mass spectrogram (Fig. 2). These experiments indicated good intra- and inter-assay reproducibility [6, 7].

Detection of Protein Peak Differentially Expressed Among Study Groups

The SELDI-TOF–MS technology was used to detect serum protein fingerprint patterns of 110 samples which included specimens from patients with SP or depression, or healthy individuals. The Ciphergen ProteinChip 3.0 software was utilized for data correction and analysis, and Ciphergen Biomarker Wizard 3.1 software was used for statistical analysis. Compared with the depression and healthy control groups, 91 protein peaks were significantly different in SP group at the *p* value of <0.05 and 54 protein peaks exhibited significant difference at the *p* value of <0.01. Fifteen discrepant peaks were identified via secondary mass spectrometry and could be distinguished with the fast classification algorithm (Table 1).

In the SP group, expression of protein peaks with the mass-to-charge ratio (*m*/*z*) of 1207.41, 1466.78, 3192.49, 3241.81, 3263.47, 5904.35, and 9287.25 was lower compared with healthy individuals. Further, in this group, protein peaks with *m*/*z* of 8932, 8987.59, 8601.29, 8136.86, 4467.69, 4201.54, 2082.83, and 1945.62 were more abundantly expressed (Table 2).

Among the three study groups, we found significant differences in two protein peaks with *m*/*z* of 1,207.41 and 1,466.78. Expression of these peaks was the lowest in the SP group, highest in the healthy control group, and intermediate in patients with depression (Table 3). These protein peaks were analyzed by direct sequencing method through MALDI-TOF/TOF tandem mass spectrometry (Figs. 3, 4). By searching in Mascot database, the 1,207.41 and 1,466.78 peaks were identified as the N-terminal fragment of fibrinogen with the following sequence: Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg (EGDFLAEGGGVR) and Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg (DSGEGDFLAEGGGVR).

Discussion

In recent years, mass spectrometry techniques have been increasingly used to identify biomarkers [8, 9]. These techniques have been applied to diseases such as leukemia,

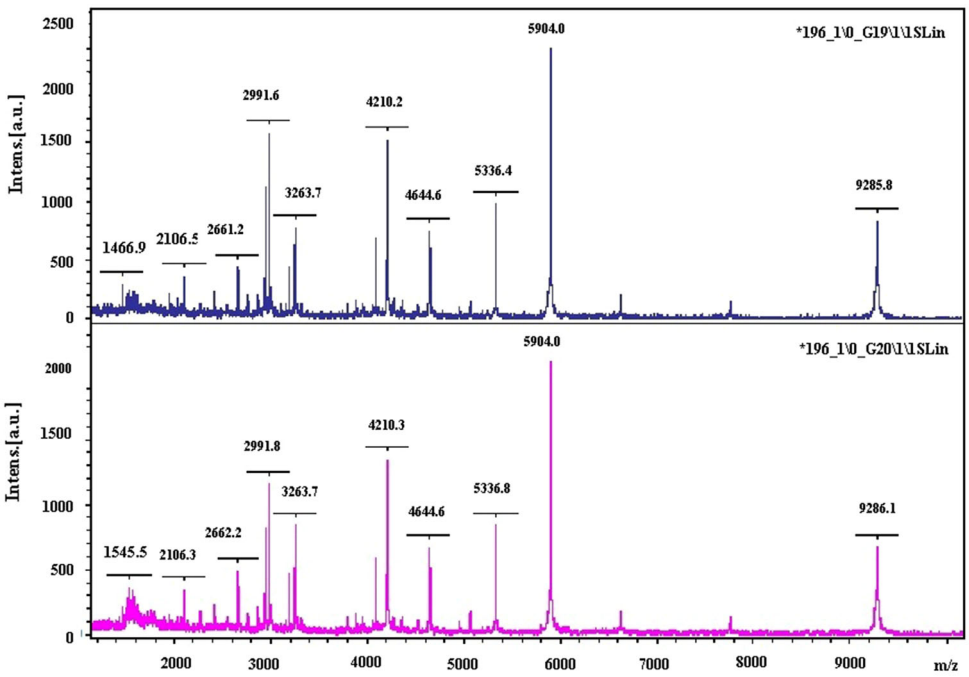

Fig. 1 Intra-assay variability: same sample was analyzed twice on the same day using the same protein chip

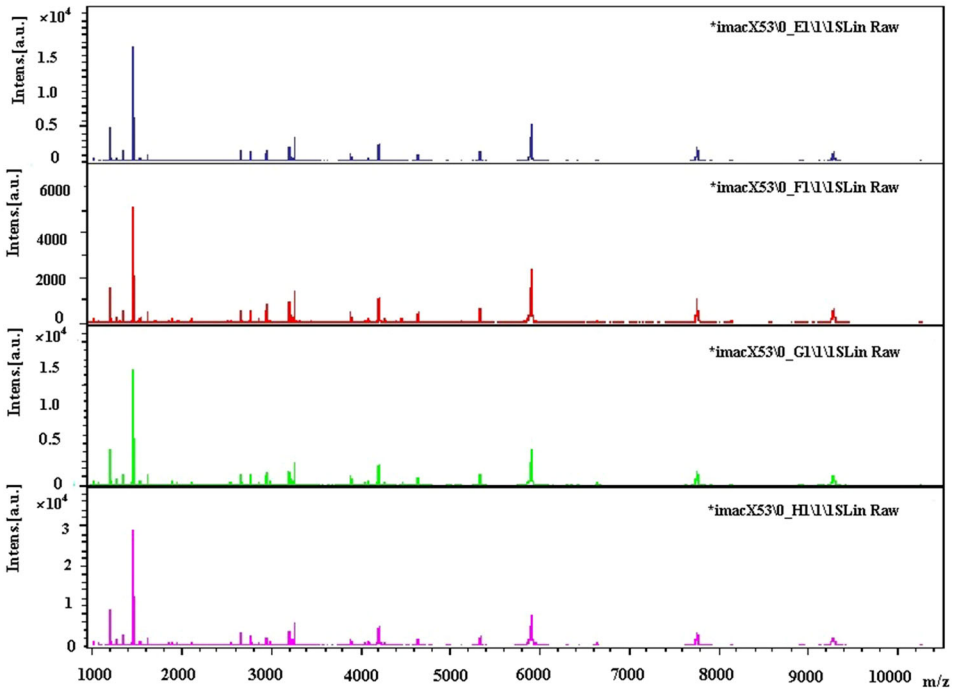

Fig. 2 Inter-assay variability: comparison of the mass spectra peaks of the same sample analyzed four times on different days using different protein chips

Table 1 Characteristics of 15 discrepancy peaks

Serial number	Mass-to-charge ratio	Mean	SD	Minimum	Maximum
4	1,207.41	83.53	17.19	2.77	159.49
10	1,466.78	388.71	79.21	8.25	759.75
31	1,945.62	10.82	14.04	1.06	43.93
35	2,082.83	13.61	17.92	0.98	53.46
71	3,192.49	9.64	8.21	1.04	114.96
74	3,241.81	11.26	9.43	1.69	201.82
75	3,263.47	15.28	14.82	0.97	45.83
99	4,210.54	21.46	5.92	9.72	36.49
106	4,467.69	6.42	4.93	0.72	216.24
129	5,904.35	24.65	19.63	1.41	85.77
152	8,136.86	2.83	1.36	1.21	207.76
154	8,601.29	2.27	2.50	0.36	210.82
156	8,932	6.76	5.63	0.49	820.24
157	8,987.59	1.48	1.08	0.32	104.77
161	9,287.25	11.60	3.47	4.51	17.02

breast cancer [10] and other cancers (e.g., colon, bladder, prostate, lung, and kidney cancers), as well as neuroblastoma [11]. Attempts have been undertaken to identify the biomarkers for SP. Thus, Mei et al. [12] used SELDI-TOF–MS technique to identify differentially expressed proteins in the dorsolateral prefrontal cortexes of patients with SP or control individuals. Further, Jiang et al. [13] utilized the same technique to screen serum protein biomarkers in patients with SP; these authors also developed an artificial neural network diagnostic model. The model was used to blindly test patients with SP, and exhibited diagnostic sensitivity and specificity of, respectively, 91.7 and 93.8 % [13]. Craddock et al. [14] found that α-defensins were increased in the serum of patients with SP. However, human α-defensins are closely related to the onset and development of many other diseases, such as oral cavity disease, cancer, inflammatory diseases, and HIV [15–17]. Therefore, the sensitivity and specificity of defensin biomarkers do not meet the requirements of differential diagnosis in SP.

Our study reveals higher expression of N-terminal fragment of fibrinogen in serum of patients with SP. Fibrinogen is a synthetic glycoprotein from liver and is abundantly present in blood [18]. However, expression of

Table 2 Characteristics of peaks discrepant among study groups at $p < 0.01$

Mass-to-charge ratio	p	Schizophrenia group		Depression group		Normal control group	
		Mean	SD	Mean	SD	Mean	SD
1,207.41	0.00044	28.46	4.99	79.12	15.3	153.5	21.02
1,466.78	8.80E−05	127.53	14.45	283	43.5	639.9	93.9
1,945.62	0.00049	11.91	3.38	8.16	3.89	4.25	0.84
2,082.83	0.00531	24.76	6.8	15.99	7.76	12.71	4.57
3,192.49	0.00054	28.3	8.3	58.72	51.81	105.8	38.15
3,241.81	0.00023	32.77	16	76.58	80.21	190.7	67.71
3,263.47	0.00387	7.56	2.52	11.02	3.07	14.25	3.92
4,210.54	0.00429	26.74	4.91	16.16	5.88	18.24	6.19
4,467.69	0.00058	202.57	78.14	131.4	77.75	40.39	25.65
5,904.35	0.00069	26.27	9.53	51.52	59.25	69.83	21.32
8,136.86	0.00495	185.06	74.36	133.8	81.67	72.54	9.62
8,601.29	0.00718	186.97	109.8	156.8	155.5	35.91	6.03
8,932	0.00058	773.51	289.1	489.8	326.1	144.1	119.4
8,987.59	0.00067	94.96	35.19	36.58	18.28	20.66	8.65
9,287.25	0.00096	5.49	2.14	8.94	3.21	13.21	3.53

Table 3 Protein peaks at the mass-to-charge ratio of 1,207.41 and 1,466.78

Mass-to-charge ratio	p	Schizophrenia group			Depression group			Normal control group		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1,207.41	0.00044	28.46	4.99	17.53	79.12	15.30	19.31	153.50	21.02	13.71
1,466.78	8.8E−05	127.53	14.45	11.30	283.00	43.50	15.41	639.90	93.90	14.71

CV coefficient of variation

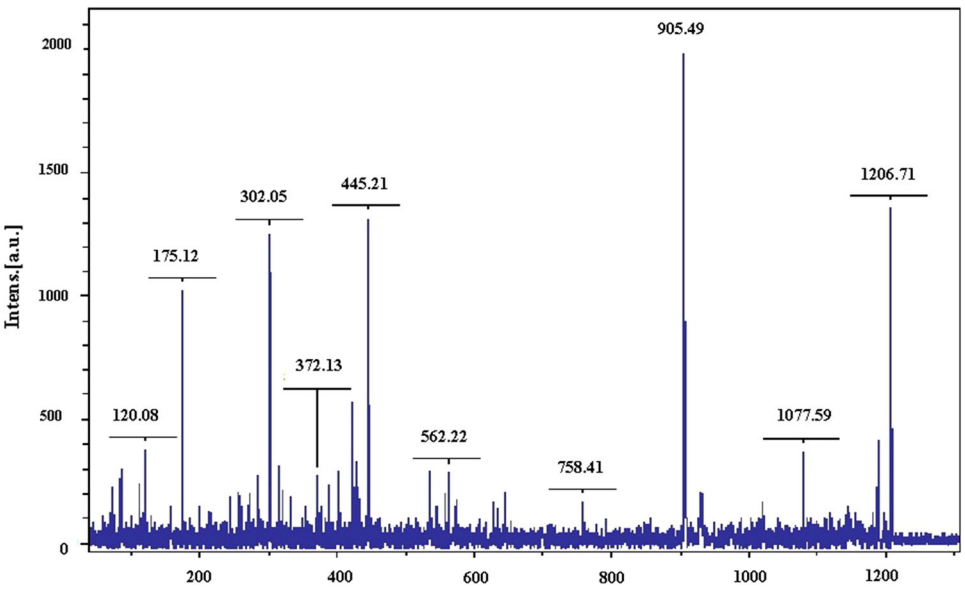

Fig. 3 The second order mass-spectrogram of protein peak at the mass-to-charge ratio (m/z) of 1,207

both 1,207.41 and 1,466.78 peaks was lower in serum of patients with SP in our study. The following may have caused the observed decrease: (1) lower content or reduced activity of fibrinogen in patients with SP, or (2) possible structural changes of fibrinogen in patients with SP that may have led to differential degradation in the serum.

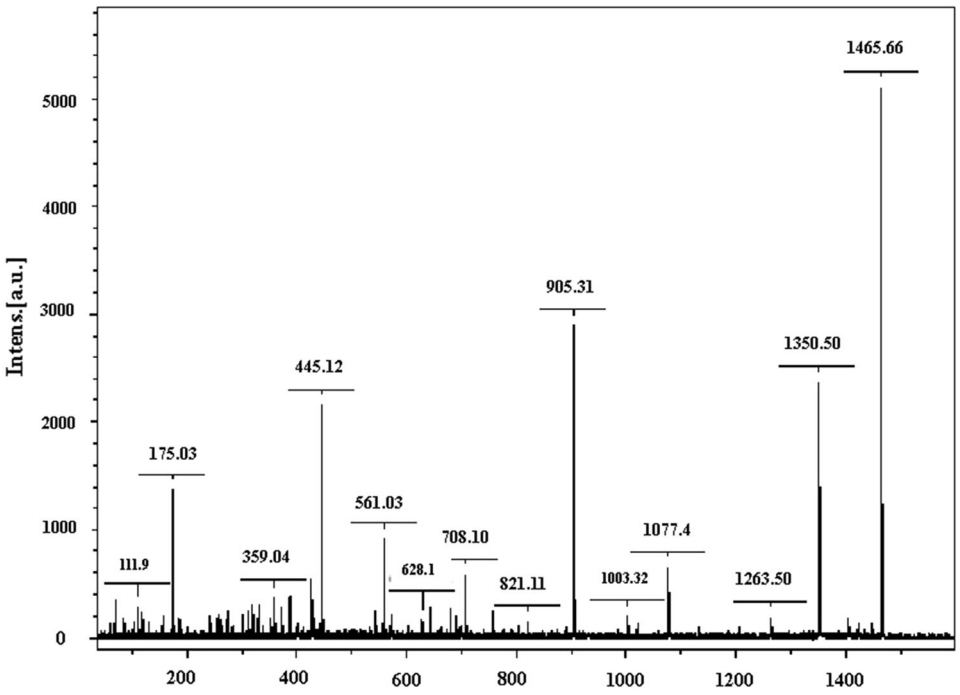

Fig. 4 The second order mass-spectrogram of protein peak at the mass-to-charge ratio (*m*/*z*) of 1,466

In conclusion, these biomarkers described in our study may be useful for molecular diagnosis of SP. The sequencing of biomarkers enables preparing anti-peptide antibodies to be tested for molecular diagnosis of SP. Studies involving higher numbers of patients should be conducted to ensure reproducibility and reliability of protein biomarkers in the diagnosis of SP.

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Conflict of interest The authors declare no competing interests.

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