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Original Article

Application of SELDI-TOF-MS in screening serum biomarkers of newly diagnosed immune thrombocytopenic purpura

Chunyan Liu

Department of Pediatrics, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, China

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Abstract: Objective: To investigate the surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF-MS) method in screening the serum protein markers for the early diagnosis, disease monitoring, and prognosis determination of newly diagnosed immune thrombocytopenic purpura (NITP), and to establish a new molecular diagnostic method of NITP. Methods: SELDI-TOF-MS was used to detect the serum proteins binding on the protein chips so as to obtain the serum protein expression fingerprints of the NITP and healthy controls, respectively, and then Biomarker Wizard and Biomarker Paterns System 5.0 software were used to analyze the data and to establish the artificial neural network diagnostic model (ANNDM). Results: M/E was within 2000 to 20000, and the most significant proteins were those with M/E as 4109.23 and 5521.35, respectively, which can be seen as the serum biomarkers of NITP; the corresponding proteins were then searched in the protein library; the serum markers were then input into ANNDM to accurately group the children with NITP from the healthy children. Conclusions: The serum protein expression fingerprint has important clinical significance toward the early diagnosis of NITP, as well as in exploring the pathogenesis of NITP. The diagnostic model composed of the proteins with M/E as 4109.23 and 5521.35 can completely and accurately distinguish the children with NITP from healthy children.

Keywords: Acute immune thrombocytopenic purpura, surface enhanced laser desorption/ionization-time of flight-mass spectrometry, proteomics, biomarkers

Introduction

Immune thrombocytopenic purpura (ITP) is one common bleeding disorder in children, and a clinical syndrome of platelet destruction increasing caused by the immunologic mechanisms. Its incidence rate in pediatric populations is 4-5/100000 [1], and based on the clinical disease duration, ITP can be divided into newly diagnosed (≤3 months), persistent (3~12 months), and chronic (>12 months) [2]. Newly diagnosed ITP (NITP) is more common in children, especially more common in infancy and significantly reduced after 7 years old. NITP is mostly diagnosed by excluding other disease possibilities, which needs to be confirmed based on clinical manifestations and laboratory tests [3, 4], and sometimes, it cannot be distinguished from the early stage of some other bleeding disorders; therefore, there still lacks meaningful biomarkers for the prognosis of NITP after certain treatments, so it is an urgent problem to look for a simple and highly specific laboratory diagnostic method, as well as certain serum biomarkers, so as to help the early diagnosis, disease monitoring, and prognosis determination of NITP. Proteins are the end products of gene expressions, and the physiological changes of organs and tissues can cause the changes of serum proteomes, so studying the proteins and proteomics can directly obtain the characteristic signs of the disease. With the completion of human genome project, the research focus of life sciences has been transferred from structural genes to functional genes; as the gene products, proteins are the carriers of all life activities and the real performers of gene functions; therefore, the large-scale and systematic research about proteins has become hot, and thus the proteomics appears. In recent years, “Nature”, “Science”, and “Cell” publish proteomics-related articles continuous-

ly, indicating that proteomics has become the forefront and hotspot of life science research [5-8]; furthermore, many reference databases of various tissues, cells, and body fluid proteins identified based on biological information technologies have been constructed [9-11], so such information as structural domain, expression pattern, tissue specificity, subcellular localization, and functions of specific protein can be searched, based on which the statistics of proteins with rich-functions and their distributions can be performed so as to build the network of inter-protein interactions, as well as to screen potential biomarkers and drug targets, for further experimental verification.

This study used surface-enhanced laser desorption/ionization (SELDI) to analyze and compare the serum markers between the NITP children and healthy children, aiming to screen out specific biomarkers for the clinical early diagnosis of NITP. The author found that artificial neural network diagnostic model (ANNDM) composed of multi-protein markers established by surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF-MS) protein chip technology has important meaning toward the detection and screening of NITP. The study used SELDI-TOF-MS to compare the differential serum protein expressions between the NITP children and healthy children, and reported below.

Materials and methods

Sample source

All the patients were collected from the inpatients and outpatients of the department of pediatrics, the Affiliated Hospital of Southwest Medical University, from July 2013 to April 2014. All the patients met the basic diagnostic standards and classifications of NITP (2). (1) group NITP: 35 patients, including 18 boys and 17 girls, with the mean age as (4.3 ± 4.6) years; (2) healthy control group (group C): 35 healthy children, identified by medical examination in the department of pediatrics, the Affiliated Hospital of Luzhou Medical College, during the same period. After the informed consent was obtained, the blood was sampled, and medical inspection revealed normal peripheral blood conditions, including 18 boys and 17 girls, with the mean age as (4.6 ± 5.0) years. There was no significant difference in the age between the two groups (P>0.05). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Southwest Medical University. Written informed consent was obtained from all participants' guardians.

Serum samples

2-5 mL fasting venous blood was sampled in the morning, and then placed into BD vacuum tube (non-anticoagulated) and stored at 4°C within 0.5 h; the blood sample was then centrifuged at 4°C and 3000 $r \cdot min^{-1}$ for 5 min within 4 h of sampling. The supernatant was then added into one Eppendof tube, followed by re-centrifuged at 3000 $r \cdot min^{-1}$ for 5 min; 150 μL of the supernatant was then sub-packed into 0.25 mL Eppendof tubes, and stored at -80°C; each sample was prepared for at least three tubes.

Experimental design

The chip selected was one weak cation chip (CM10) (Ciphergen, USA), and all the samples were required to be mixed during the modeling (positive and control). All the samples were randomly arranged using the RAND function in Excel; seven samples of each chip were placed in, together with a mixed control serum (also randomly arranged on the chip), and one blank control was added into each model (deionized water was used instead of the sample), respectively.

Chip processing

The samples were firstly dissolved on ice (30 min~1 h), and then centrifuged at 10000 $r \cdot min^{-1}$ and 4°C for 2 min. After lined up, 96-well culture plates (FALCON, USA) were placed on ice boxes, and added 10 uL of phosphate buffer (Sigma) into each well using 10 μL micropipette; each well was then added 5 uL of serum, respectively, (directly added in with force and wall-adherently, but did not touch the bottom nor repeatedly blowing). The plates were then placed in one chromatography cabinet and oscillated at 3°C and 600 $r \cdot min^{-1}$ for 30 min; the chip was added and labeled when about 15 min was left, together with 200 μL of 50 $mmol \cdot L^{-1}$ sodium acetate (Sigma). After oscillated in the chromatography cabinet at 600 $r \cdot min^{-1}$ for 5 min, the plates were tapped dry (repeated once); the samples were then put on ice, and

Table 1. Mean protein peak intensity, SD, and intergroup P between the two groups

m/z	*p*	Mean protein peak intensity in group NITP	SD in group NITP	SMean protein peak intensity in group C	SD in group C
4109.23	0	2.21↓	1.69	18.57↑	10.20
5521.35	0	1.83↓	1.54	13.15↑	8.26
5697.53	1E-10	1.43↓	1.82	9.65↑	5.38
5879.15	2E-10	1.08↓	1.26	9.03↑	5.85
5954.03	2E-10	1.09↓	1.54	7.96↑	5.67
5978.34	3E-10	1.39↓	1.21	12.68↑	11.59
9432.56	1.23E-08	3.56↓	1.25	13.56↑	5.76
8622.07	1.68E-07	6.81↓	3.76	12.95↑	4.63
4077.03	2.05E-07	25.23↓	19.13	59.13↑	18.81
4159.86	5.64E-07	3.54↓	2.18	7.59↑	4.03
4535.22	6.65E-07	2.48↓	1.53	5.64↑	2.37
3465.12	1.24E-06	3.44↑	2.66	0.41↓	0.59
5775.21	2.04E-06	5.39↓	4.68	9.96↑	3.44
6720.43	2.08E-06	8.13↓	12.00	16.84↑	6.41
3836.37	7.54E-06	2.49↓	1.79	6.23↑	3.45
7856.70	2.46E-05	8.92↓	9.58	19.25↑	8.68
6945.56	2.61E-05	5.89↑	2.49	4.23↓	1.66
3786.03	3.16E-05	2.29↓	4.49	4.86↑	3.30
7539.01	0.000181	14.12↓	10.96	23.89↑	8.07
4667.79	0.000632	5.89↑	3.31	3.45↓	1.53
5613.73	0.000734	32.82↓	16.90	46.44↑	11.29
6223.27	0.001043	17.45↑	9.92	16.53↓	6.59
4321.07	0.001358	4.98↓	4.56	8.14↑	4.69
4356.56	0.001456	5.26↓	5.43	6.40↑	2.53
5123.36	0.001698	2.29↓	1.47	4.14↑	2.59
9914.34	0.003458	1.39↑	1.87	0.18↓	0.23
7986.32	0.003956	29.89↑	13.49	22.48↓	10.43
8653.23	0.004331	4.13↓	5.49	6.98↑	3.39
6569.71	0.006981	6.29↑	8.52	6.12↓	2.11

Note: ↑ indicates that the protein peak is highly expressed, and ↓ indicates that the protein peak islowly expressed.

quickly added 185 μL of sodium acetate (vertically added with force, but did not touch the bottom nor repeatedly blowing, followed by oscillation in the chromatography cabinet at 600 r·min^{-1} for 2 min); 100 μL of prepared samples were then added onto the chip rapidly, with the air bubbles inside hand-tapping away, or pipetted away using pipetting tips. The plates were the oscillated in the chromatography cabinet at 4°C and 600 r·min^{-1} for 1 h, followed by rinsing with 200 μL of sodium acetate (600 r·min^{-1}, 5 min× 3 times, at room temperature). After removing the sodium acetate, the plates were tapped dry with force; 200 μL of deionized pure water (Fluka, Germany) was then added in rapidly, twice, followed by being splashed away and tapped dry; the dry plates were then added 1 μL of half saturated meson acid (Sigma) and another 1 uL 5 min later, followed by the detection.

Data collection

The serum proteins on the chip CM10 surface were detected by PBS II/C type protein fingerprint chromatogram instrument (Ciphergen Inc., USA). Under the action of the accelerated electric field, the flight time of proteins with different mass to charge ratios were different in a certain length of the vacuum pipe. The protein of LL (M/Z) is proportional to the square of the ion flight time. Instantaneous current produced by electron multiplier transducted into the relative content of proteins once the charged protein ions beam was detected (instantaneous current I=Q/T, Q was the number of charges detected). The optimizing scope of mass to charge ratios was 2000~20000 (m/z), and the highest detection of molecular weight was 100000 ngz, laser intensity was 230, detection sensitivity was 9. All-in-one protein molecular weight standards (Ciphergen company) was applied for the instrument correction, and the parameters of the apparatus was setted (quality deviation ≤0.1%). Ciphergen ProteinChip software was used to collect data, lower baseline and normalize the data. The protein spectrum was draw, and the vertical coordinate was the relative content of proteins and the horizontal coordinate was mass to charge ratios.

Statistical analysis

Biomarker Wizard software was used for the data analysis, which is a relatively simple statistical software, and uses the analysis of variance to preliminarily analyze the data obtained by the Proteinchip software; the means, stan-

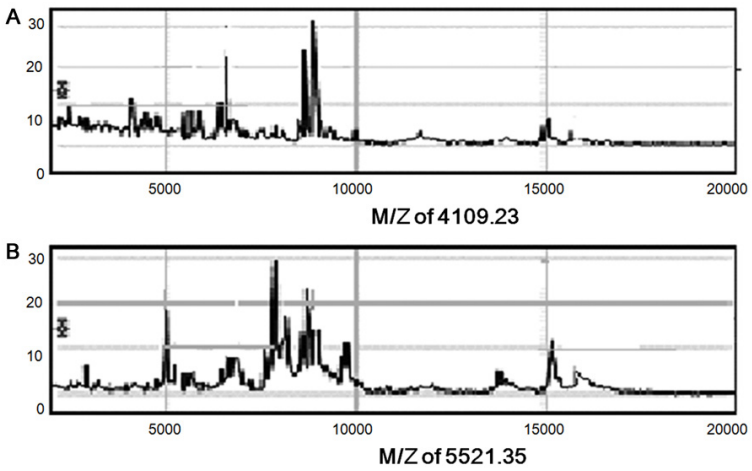

Figure 1. Fingerprints of the proteins with m/z as 4109.23 and 5521.35, respectively. The x-axis represents the molecular mass calculation (m/z values), and the y-axis represents relative intensity.

Table 2. Diagnostic efficiencies in group NITP and group C

Group	n	Prediction in group NITP (1)	Prediction in group C I (0)	Prediction rate (%)
AITP	20	19	1	95%
C	10	1	9	90%

dard deviations, and *P* values of the proteins were then calculated, and provided to the BP artificial neural network so as to establish ANNDM. The M/Z and intensity of each peak were expressed as $\bar{x} \pm s$, respectively, and the comparison between peak intensities used the T-test; the Biomarker Wizard software was used for the statistical analysis, with P<0.01 considered as significant difference.

Results

Detection of protein peaks

The serum protein fingerprints of the 35 NITP patients and 35 healthy controls were obtained by the SELDI-TOF-MS detection. Then, after the original serum protein fingerprints detected were standardized, the Biomarker patter software was used for the analysis, the protein peaks differentially detected within m/z range 0~20,000 were then performed the comparative T test, and the results showed that: (1) there existed a total of 29 protein peaks significantly different between group NITP and group C (P<0.01) (**Table 1**), among which seven proteins were highly expressed in group NITP, and 22 proteins were lowly expressed. The analysis reveal the most significant proteins, which had the most significant difference and the minimum *P* value (zero), were the proteins with m/z as 4109.23 and 5521.35, respectively (**Figure 1**).

Protein spectral diagnostic model

The BP neural network used the back-propagation algorithm (conjugate gradient learning function is used to improve trainscg). The specific settings were: a total of 4 layers (the input layer, the output layer, and the hidden layer). Each layer used the randomly initialized tangent s-shaped transferring function (Tansig). The expected output values in group NITP and group C were set as 1 and 0, respectively, together with 0.5 determined as the CUT-OFF PIONT, so the subject with this value less than 0.5 can be determined as normal, otherwise as NITP. The proteins with m/z as 4109.23 and 5521.35 were used to establish the diagnostic model of NITP. The 60 samples were blindly grouped into the modeling group and the validation group, with 30 cases in each group. The above two protein peaks in the protein spectra of the modeling group were input into the BP neural network so as to establish the diagnostic model of NITP. The cases in the validation group were blindly tested, which revealed the sensitivity as 95% and the specificity as 90%. The predictive results obtained by ANNDM toward the 30 NITP children and the 30 healthy children were shown in **Table 2**.

Protein identification

The m/z values of the two proteins significantly different between group NITP and group C were input into protein databases, and the proteins corresponding to the m/z values were then obtained. Procedures: Entered the name of the following protein database: http://us.expasy.org/tools/tagident. html→entered the website→entered m/z in the box of "Mw", clicked the "Start TagIdent"→the corresponding protein then appeared (**Table 3**).

Table 3. m/z-corresponding proteins significantly different between group NITP and group C

m/z	Mean protein peak intensity in group NITP	Mean protein peak intensity in group C	Corresponding protein name
4109.23	2.21	18.57	Large subunit of ribosome
5521.35	1.83	13.15	Unknown, may be new protein

Discussion

The diagnosis of NITP mainly relies on clinical manifestations and laboratory tests, among which the number and maturity of bone marrow megakaryocytes, as well as the platelets in peripheral blood, are the main diagnostic basis, but it still lacks specific criteria. The etiology and pathogenesis of NITP are still not completely clear yet; currently, it's believed that this disease is caused by autoimmune dysfunctions, which result in the generation of antiplatelet autoantibodies in the NITP children, so the platelets were largely damaged, and thrombocytopenia is thus resulted [12]. The protein chip technology has great potentials, and the revolutions induced by it in science and technology are much more profound than any previous technologies, so it has become a powerful tool in the fields of new century medicine and biological research [13]. Proteins are the end products of gene expressions, and the physiological changes of organs and tissues can cause the changes of serum proteomes; therefore, investigating proteins and proteomics can directly obtain the characteristic signs of diseases [14]. In recent years, the developments of mass spectrometry are creating a new era of proteomic research [15, 16]. The storage, analysis, and interpretation of proteomic data have become new challenges in bioinformatics [17]. More and more journals require researchers to upload high-throughput data into public databases, so ordinary researchers can mine and integrate these data via simple bioinformatical methods for the possible re-analysis of these data using their own unique perspectives. Recently, two research groups simultaneously published the draft of human proteomics [18, 19], which, together with more follow-up studies, will become a valuable gold mine, thus promoting our understanding of biological markers. The appearance of SELDI protein fingerprinting technology firstly provided an important tool to detect early tumor biomarkers; nowadays, it has been used for the early detection and early diagnosis of a variety of tumors, and has obtained breakthrough in screening biomarkers of various autoimmune diseases, infectious diseases [20, 21], and blood system diseases.

Cochran et al. [22] reported that the existence of high mutation of IVIG or overexpression of CD38 in vivo can be used to divide chronic lymphocytic leukemia into different subtypes; Hegedus et al. [23] also used proteomics to analyze the differences in the protein expression profiles in the childhood with acute nonlymphocytic leukemia, and the results suggested the molecular mechanisms of the clinical and biological characteristic differences between acute non-lymphocytic leukemia and acute lymphocytic leukemia. Although widely used in blood system diseases, proteomic techniques are mostly used in the early screening of tumor markers, and it's still blank worldwidely using proteomics against ITP. In this study, we used SELID to purposely investigate and analyze the serum spectra between the NITP children and the healthy children, and the results revealed that a total of 29 protein peaks exhibited significant differences between group NITP and group C ($P<0.01$), among which seven proteins were highly expressed in group NITP, and 22 proteins were lowly expressed; the two proteins with the smallest *p* values exhibited the m/z values as 4109.23 and 5521.35, which may be the most meaningful proteins or the biomarkers of NITP. After inputting these two proteins into ANNDM, the sensitivity was 95%, and the specificity was 90%. The protein database search tells us that the protein with m/z 4109.23 is the large subunit of ribosomal, but the other one with m/z 5521.35 is a new protein. Further studying these proteins can further analyze the cause of NITP, and thus discover possible medication-related treatment targets. In short, we used SELDI-TOF-MS and detected the characteristic serum proteins of NITP; the further established BP neural network diagnostic model can thus provide scientific basis for the studies of NITP etiology, clinical diagnosis, or genetics, so it can exhibit great economic values and social significance.

Disclosure of conflict of interest

None.

Address correspondence to: Chunyan Liu, Department of Pediatrics, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, China. Tel: +86 13982729788; Fax: +86 830 2392753; E-mail: chunyanliudoc@13.com

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