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Platelet proteomics in diagnostic differentiation of primary immune thrombocytopenia using SELDI-TOF-MS

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ABSTRACT

Background: Primary immune thrombocytopenic purpura (pITP) is defined as isolated autoimmune thrombocytopenia with idiopathic low platelet count, normal bone marrow, and unexplained causes of thrombocytopenia. Currently there is no definite criterion for ITP diagnosis.
Methods: We conducted proteomic screen of patients with pITP, secondary immune thrombocytopenia (sITP), and healthy controls using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). The proteomic profiles were obtained from platelet lysate samples of 82 healthy adult controls, 64 pITP, and 70 sITP patients, from which we screened marker proteins with significant differences, and constructed a diagnosis model using the artificial neural network (ANN) technique.
Results: We identified 6 marker proteins in the platelet lysates of pITP patients. This diagnosis method differentiated pITP patients from sITP effectively with a sensitivity of 96.9% (31/32), a specificity of 71.0% (54/76), and the area under the ROC curve of 0.864 in the training set, and a sensitivity of 87.5% (28/32), a specificity of 69.7% (53/76), and a positive predictive value of 75.0% (81/108) in the test set.
Conclusion: The artificial neural network model based on platelet protein profiling established a potential pITP diagnosis platform.

1. Introduction

Immune thrombocytopenia (ITP), also known as idiopathic thrombocytopenic purpura, is an acquired autoimmune disorder which results from low levels of platelet and affects both adults and children. The annual incidence of ITP is about 1.9 to 6.4 per 10^5 in children and 3.3 per 10^5 in adults [1–4]. An international working group has proposed and recommended "immune thrombocytopenia" to replace the original naming of "idiopathic thrombocytopenic purpura" for ITP, highlighting the pathogenesis of immune-mediated mechanism [5]. The term "purpura" is not used because most ITP patients do not have or only have mild bleeding symptoms. Primary immune thrombocytopenia (pITP) is defined as isolated thrombocytopenia without other causes, and secondary immune thrombocytopenia (sITP) represents the type of immune thrombocytopenia which results from other diseases or drug exposure [5].

Currently pITP is a diagnosis of exclusion and clinical decisions are largely dependent on the patient's history, physical examination, blood count and bone marrow biopsy, etc. [3,4,6,7]. In the past 40 years, researchers have evaluated a number of different methods for reliable diagnosis of pITP, mainly focusing on detection of platelet-associated antibodies and platelet-specific autoantibodies. Detection of platelet-specific autoantibodies such as the approach of Monoclonal Antibody Specific Immobilization of Platelet Antigens (MAIPAs) usually requires a large sample size and lacks sufficient sensitivity, therefore it is not suitable for clinical use [7–9]. The methods for platelet-associated immunoglobulins (PAIgs) detection such as ELISA and flow cytometry (FCM) have also been investigated intensively. Although with high sensitivity, their specificity is usually too low to distinguish pathological from non-pathological PAIgs for clinical applications [7,10–12].

More recently, there were 3 reports focusing on screening candidate biomarkers to support differential diagnosis and treatment decisions in ITP. Chousa et al. found significantly higher levels of ceruloplasmin in ITP patients by one-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometric analysis [13]. Zheng et al. pinpointed significantly higher haptoglobin levels in ITP patients responsive to splenectomy than in the non-responders with two-dimensional gel electrophoresis [14]. Bal G et al. identified 161 differentially expressed proteins including oncoproteins, tumor-suppressor proteins, and 6 anti-nuclear autoantibodies through microarray-based serum protein profiling [15]. However, the markers in these three

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studies are clinically less conclusive and limited in clinical application. Until today, there are no known specific biomarkers for pITP to support differential diagnosis and treatment decisions, which urges us to explore novel markers.

Proteomics, with the concept first proposed by Wilkins et al., is a large-scale study of proteins on the level of structures and functions, including the expression levels, post-translational modifications, and protein–protein interactions [16]. A comprehensive understanding of disease, cell metabolism, and other processes can be achieved by studying the overall level of protein composition in the biological system [17]. Due to the lack of cell nuclei, platelets have limited levels of protein synthesis through the small amount of residual mRNA (3000–6000 transcripts in platelets) left in the mitochondria when they derived from megakaryocytes. This characteristic limits the application of genomics and transcriptomics for platelet study, while rendering proteomics a more suitable approach for evaluating platelets in both physiological and pathological conditions [17–20]. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a powerful technology in proteomic studies and has been explored for screening of various cancer biomarkers and disease diagnosis [21–24]. With continuous development of MS-based proteomics and protein isolation methodology, the field of platelet proteomics has attracted more and more attention in recent years. The similarities and differences of protein expression and modification under various physiological and pathological conditions can provide novel insights into protein identification and classification, as well as protein interaction and function analysis [17]. The application of platelet proteomics has been explored in areas such as platelet-related diseases [25], type 2 diabetes [26,27], and acute coronary syndrome [28], providing pathogenic information of the diseases and highlighting biomarkers for new drug targets.

In this study, we use SELDI-TOF-MS to analyze the platelet proteome of patients with pITP, sITP, and normal platelets. Protein profiles of pITP patients were obtained and screened for platelet-specific protein markers. Combined with artificial neural network (ANN) model, we examined the potential of using SELDI-TOF-MS for differential diagnosis of pITP.

2. Materials and methods

2.1. Study population

The platelet samples of 134 patients collected between October 2011 and December 2013 were provided by the Department of Hematology in the Affiliated Hospital of Luzhou Medical College. The diagnosis of all patients was according to The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia [3]. A total of 64 cases of pITP and 70 cases of sITP patients were included in this study. Among the sITP group, 30 cases of systemic lupus erythematosus (SLE), 16 cases of antiphospholipid antibody syndrome (APS), 18 cases of immune thyroid disease, and 6 cases of Evans syndrome were included. The control group was comprised of 82 healthy adults who visited the General Health Check-up Division at the Affiliated Hospital of Luzhou Medical College. Patients did not use adrenal corticosteroids, immunomodulatory agents, or drugs that affect platelets within three months prior to blood sampling. There was no statistically significant difference with respect to gender or age between the groups. Written informed consent was obtained from all participating subjects. This study was performed according to the guidelines of Luzhou Medical College, which abides by the revised World Declaration of Helsinki on ethical principles for medical research involving human subjects (Table 1).

Table 1
Demographics of the subjects.

	pITP	sITP	HC	*P* value
Number of subjects	64	70	82	N/A
Sex (male/female)	15/49	22/48	23/59	NS
Age (y, mean ± SD)	39.4 ± 13.07	44.32 ± 10.1	39.3 ± 12.03	NS
PLT ($\times 10^9$/l)	41 ± 9.7	43 ± 12.1	173 ± 25.4	<0.0001

HC: healthy controls; PLT: blood platelet count.

2.2. Platelet lysate preparation

BD vacutainer blood collection tubes with sodium citrate (Fisher Scientific) were used to collect whole blood samples from fasting subjects in early morning. Four microliters of prostacyclin (Sigma-Aldrich) was then quickly added to 4 ml of each blood sample and mixed gently. After centrifugation at 150 $\times g$ for 15 min, the upper layer of platelet-rich plasma was collected and gently mixed with another 4 μL of prostacyclin followed by centrifugation at 1000 $\times g$ for 20 min. The supernatant was removed and platelets were resuspended in acid-citrate-dextrose buffer (ACD Buffer, Sigma-Aldrich) and centrifuged at 1000 $\times g$ for 20 min again. Deionized water was then added, and the platelet samples were treated with five freeze–thaw cycles of −80/30 °C followed by centrifugation at 7000 $\times g$ for 20 min. The supernatant of platelet lysate was cryopreserved at −80 °C for further analysis.

2.3. SELDI-TOF-MS

Samples were divided into training and test groups for SELDI-TOF-MS analysis. The platelet proteins captured on the surface of the gold (Au) chips were examined on a PBS-IIc mass spectrometer reader (Ciphergen Biosystems). The mass/charge spectra were generated by Ciphergen Proteinchip software with automatic denoising algorithm, and the accuracy of mass spectra was calibrated using the all-in-one peptide mass standard (Ciphergen) as the external standard at the day of experiment. Data were collected with laser intensity at 225, detection sensitivity at 9.

2.4. Data analysis

The MS spectra of pITP, sITP, and control groups were analyzed with Biomarker Wizard 3.1 software. Protein peaks were selected for further analysis with frequency threshold at 10% and signal to noise ratio (S/N) set at >10. Relative intensities were calculated from the percentage of single peak intensity to total peak intensity. Variance analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA) to screen for the marker proteins with significantly different expression levels between pITP, sITP, and control healthy subjects. The differences were assessed by the receiver operating curve (ROC), and the area under the curve (AUC) value was used to screen for marker proteins. Data from training group samples were used to build a diagnosis model with ANN software using a back-propagation algorithm. The discrimination ability of the established model was tested with the test group samples using SPSS software.

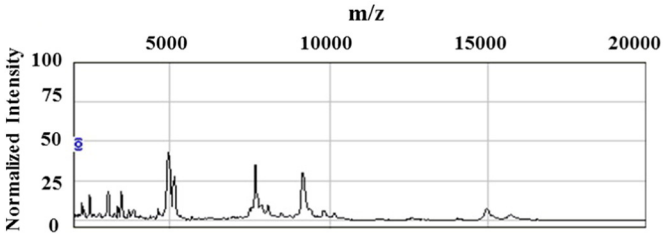

Fig. 1. Typical SELDI-TOF MS spectra of the platelet lysate of a pITP patient.