

Identification of Biomarkers for Endometriosis Using Clinical Proteomics

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Abstract

Background: We investigated possible biomarkers for endometriosis (EM) using the ClinProt technique and proteomics methods.

Methods: We enrolled 50 patients with EM, 34 with benign ovarian neoplasms and 40 healthy volunteers in this study. Serum proteomic spectra were generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) combined with weak cationic exchange (WCX) magnetic beads. Possible biomarkers were analyzed by a random and repeat pattern model-validation method that we designed, and ClinProt software, results were refined using online liquid chromatography-tandem MS.

Results: We found a cluster of 5 peptides (4210, 5264, 2660, 5635, and 5904 Da), using 3 peptides (4210, 5904, 2660 Da) to discriminate EM patients from healthy volunteers, with 96.67% sensitivity and 100% specificity. We selected 4210 and 5904 m/z, which differed most between patients with EM and controls, and identified them as fragments of ATP1B4, and the fibrinogen alpha (FGA) isoform 1/2 of the FGA chain precursor, respectively.

Conclusions: ClinProt can identify EM biomarkers, which – most notably – distinguish even early-stage or minimal disease. We found 5 stable peaks at 4210, 5264, 2660, 5635, and 5904 Da as potential EM biomarkers, the strongest of which were associated with ATP1B4 (4210 Da) and FGA (5904 Da); this indicates that ATP1B4 and FGA are associated with EM pathogenesis.

Key words: ClinProt, Endometriosis; Liquid Chromatography-tandem Mass Spectrometry, Magnetic Bead; Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry; Proteomics

INTRODUCTION

Endometriosis (EM) is the presence of endometrial-like tissue outside the uterus and is associated with chronic intrapelvic inflammation. It is most common in the ovaries but may appear in the pelvic cavity, peritoneum, other pelvic organs, and even distant organs such as lung, pleura or brain.^[1] It occurs in roughly 5–10% of women during their childbearing years. Although the only way to confirm an EM diagnosis is by laparoscopy or other surgery, the positive rate for laparoscopic diagnosis is only 18–60%.^[2]

Mean delay between symptom onset and the surgical EM diagnosis is an estimated 6.7 years. Each affected woman loses on average 10.8 h of work weekly, mainly from reduced effectiveness while working.^[3] Despite many treatments used to reduce symptoms, EM has a 67% 5-year recurrence rate.^[4] Thus, EM both reduces patients' quality-of-life and generates a huge economic burden.^[5,6] Timely diagnosis is critical.

We previously investigated the applicability of 2-DE gels and peptide mass mapping to identify candidate endometrial proteins by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) protein chip array technology, to detect proteomic patterns in serum of women with EM.^[7,8] However, SELDI technology has very low reproducibility, and cannot detect low-abundance proteins. The ClinProt system facilitates preparation and acquisition of complex proteomic profile patterns and their comparative analyses.^[9] It can optimize searches for biomarkers. In the study, we used ClinProt technology to explore and identify biomarkers from serum of EM patients.

METHODS

Serum samples

In this study, we enrolled 50 patients who had been clinically diagnosed with EM and confirmed postsurgery by 2 senior pathologists. Of these patients, 28 had ovarian EM, 11 had adenomyosis, 8 had adenomyosis complicated with ovarian EM, and 3 had peritoneal and abdominal wall EM. Their average age was 37 ± 8 years (range: 18–51 years). Their

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disease was staged according to the revised American Fertility Society (AFS) classification. As controls, we also enrolled 34 patients with benign ovarian neoplasms, including 19 with mature ovary teratoma, 8 with epithelial neoplasms, 2 with inflammatory masses, and 5 with other benign tumor types; their average age was 34 ± 8 years (range: 19–49 years). Finally, we enrolled 40 healthy women controls who were undergoing health check-ups during the enrollment period, for whom serology, X-ray, and B-ultrasound of their pelvic and abdominal cavities excluded gynecologic diseases and other complications of primary hypertension and renal disorders; the average age of these healthy controls was 38 ± 9 years (range: 23–54 years). Blood samples from all patients were collected preoperatively. Subjects were of reproductive age and had normal menstrual cycles. They had no other diseases on physical examination or by biochemical tests. None had received any hormonal treatment within 3 months before this study. All subjects signed a consent form before participating in this research protocol which was approved by the Institutional Review Board of Peking University People's Hospital.

All blood samples were obtained by venipuncture and centrifuged at 3000 r/min for 5 minutes at room temperature (25°C). The serum was aliquoted and stored at -80°C . Repeated freeze/thaw cycles during further proceedings were avoided to protect protein integrity.

Serum pretreatment with magnetic beads

We used 10 samples from each group to select appropriate magnetic particles; finally WCX was chosen to fractionate samples, following manufacturers' instructions through a standard protocol (ClinProt™, Bruker Daltonics, USA). For each sample, 5 μl of plasma was mixed with 5 μl of beads. Samples were purified through 3 steps – binding, washing, and elution, during which binding incubation took 1 minute. We eluted 5 μl of each sample; the purified plasma samples were further diluted 4-, 8-, and 16-fold.

Data acquisition by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

We prepared diluted solutions for matrix-assisted laser desorption/ionization TOF MS (MALDI-TOF MS) by mixing 1 μl each of diluted samples with 0.5 μl of matrix solution containing 2 g/L α -cyano-4-hydroxycinnamic acid, 10 nmol/L angiotensin II, 10 nmol/L adrenocorticotrophic hormone 18–39 (Bruker Daltonics), and 10 ml/L formic acid in 500 ml/L acetonitrile and allowing each droplet to dry on the MALDI sample plate (Bruker Daltonics). We used an Auto Flex II MALDI-TOF mass spectrometer (Bruker Daltonics), operated in positive ion linear or reflectron mode. Briefly, all spectra were processed by automatic baseline subtraction, peak detection, recalibration, and peak area calculation, according to the predefined settings.

Statistical analysis

When we used ClinProtools Software (Bruker Daltonics) to search for differential proteins and design models,^[10] we found that not only the number, mass of candidate proteins,

but also the potential importance of these proteins; that is, candidate proteins with lower peak intensities appeared to be less meaningful in this context. We, therefore, tried to improve the analytic process.

(A) First, we used ClinProtools to identify the proteins with the greatest differential expression among the three groups. (B) Second, we processed more than 100 stochastic analyses of the EM group versus the healthy controls (total 90 samples – 50 EM and 40 healthy controls), which were then randomly sorted into either the model group or the test group. Of the proteins found by ClinProtools, we identified those with expressions that varied $>30\%$ between the two groups. (C) Third, we used ClinProtools to process Supervised Neural Network (SNN) arithmetic (signal/noise threshold: >5 ; threshold basic peak intensity: 0.1–0.25) and used leave-1-out arithmetic to find the most plausible candidate proteins. (D) Fourth, we combined the 10 initial proteins that showed the highest sensitivity in our first step with other proteins to evaluate the diagnosis veracity in combination. (E) Fifth, we regarded proteins found in the above steps to correlate with differences in disease states to be likely biomarkers; and (F) Sixth, we tried to identify those candidate proteins.

Identification of endometriosis-specific serum peptides

We used online liquid chromatography-tandem MS (LC-MS/MS) (LTQ Orbitrap XL, Thermo Fisher Ltd., USA) to further identify proteins. Our MS analysis used the following source parameters: Nano ion source; spray voltage 2.0 kV; 60 m scanning time; MS mode of data-dependent and dynamic exclusion; mass range: 300–2000 Da; Orbitrap for MS1 at 100,000 resolution; and LTQ for collision-induced dissociation and MS2. The 6 most intense ions were chosen as parent ions. Obtained chromatograms were analyzed with Bioworks Browser 3.3.1 SP1; resulting mass lists were used for database searching using Sequest™ (IPI Human 3.45). Parameters for generating peak lists were as: Parent ion: 50 $\mu\text{g/g}$; fragment mass relative accuracy: 1 Da.

RESULTS

Quality control for the ClinProt System

Stabilization of the mass spectrum using different sample runs was the means of verifying reproducibility of the ClinProt System, so in this study we tested the system stabilization first. We also used commercial standard sample as a reference to compare different chips and experimental runs in which six typical proteins were detected; the results show that the mean coefficient of variation (CV) was $<10\%$, which indicates good system reproducibility [Figure 1] and indicates that our results are credible.

The preliminary results of biomarker exploring

First, we constructed models from ClinProtools analyses of 30 EM patients and 20 controls, and then we used another 20 patients and 20 controls to test the model. We found many differentially expressed proteins [Figure 2], which scored high in the cross test, which indicates a very stable

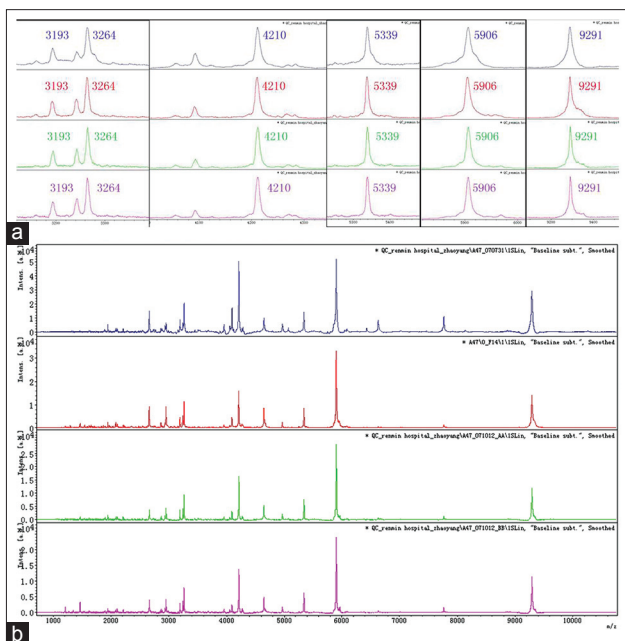


Figure 1: Protein standard fingerprints in different experimental runs. (a) Six proteins in different experimental runs; (b) Entire standard fingerprint in different experimental runs. Blue: 1st run; red: 2nd run; green: 3rd run; purple: 4th run.

model [Table 1]. From the four table analysis, sensitivity and specificity were 92.0% and 97.5%, respectively. Positive and negative prediction rates were 97.8% and 90.70%, respectively. However, as we found no differentially expressed proteins when we used 30 EM patients and 20 benign controls to construct models [Table 2], we tried to establish whether patients with early-stage EM and healthy controls showed differentially expressed proteins.

The AFS system classifies EM into 4 stages, in which patients with Stage I–II EM normally have only pelvic disease and no ovarian neoplasms; we considered them to have early-stage disease for this study. Notably, we found these early-stage patients to have two groups of differential proteins with average cross-validation of 86.25%. These proteins merit wider attention; our next work will focus on them.

Exploring peaks of differentially expressed proteins

The ClinProt system analyzes proteins by their protein fingerprint, and constructs an optimized model, in which numbers and mass of candidate proteins may vary when target samples changed, and its calculations might not seem biologically plausible for all the protein peaks. We, therefore, tried to find the most stable model and appropriate differential proteins. We independently constructed a reiterative model test method and used several statistical paths, after many random testing runs and associative steps to get the most stable and accurate proteins.

Differential proteins between endometriosis and control groups

We then randomly distributed the EM and healthy control samples into the modeling and testing groups, on which we carried out 100 random analyses of these candidate proteins,

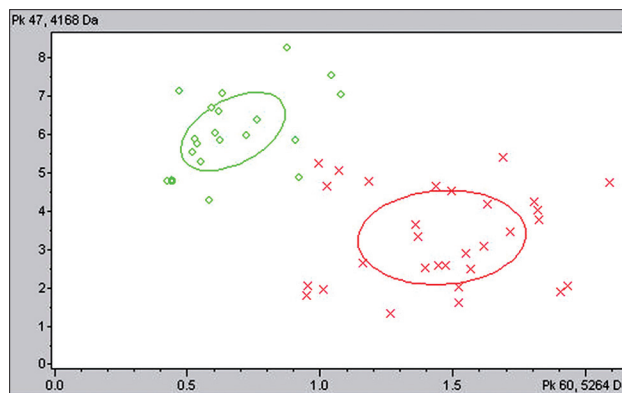


Figure 2: Distribution of endometriosis (EM) and control. X: EM samples; O: Control samples.

Table 1: Model and test results for patients with EM and healthy controls

Groups	Serum sample	n	Correct	False	Accuracy (%)	Cross-validation (%)
Model	Control	20	20	0	100.00	100
	EM patients	30	29	1	96.67	90
Test	Control	20	19	1	95.00	
	EM patients	20	17	3	85.00	

EM: Endometriosis.

Table 2: Model and test results for patients with EM and those with benign gynecological tumors (controls)

Groups	Serum sample	n	Correct	False	Accuracy (%)	Cross-validation (%)
Model	Benign controls	30	2	28	6.67	26.67
	EM patients	20	20	0	100.00	100.00
Test	Benign controls	20	0	20	0	-
	EM patients	14	14	0	100.00	-

EM: Endometriosis.

which showed a 99.75% mean recognition accuracy rate, and mean validation accuracy rates of 85% for EM and 99% for healthy controls. Table 3 shows proteins with > 30% occurrence frequency.

Stable differential proteins among groups

Combining the sensitivity and specificity of each protein with reiterative random tests, we used ClinProtocols SNN (signal-to-noise: >5; correlation threshold base peak: 0.1–0.25), and the leave-1-out method to cross test, which gave the differential proteins combination for each group [Table 4].

Confirmation of stable differential proteins

After testing with the random repeat-validation self-determination system, the stable differential protein peaks were for 4210, 5264, 2660, 5635, and 5904 Da [Figure 3]. The 5635 Da peak distinguished patients with Stage I–II EM from healthy controls very well. Peak intensity of some proteins was transformed with greater disease stage: 2660 Da, 5264 Da, 5635 Da, 5904 Da gradually increased

Table 3: Proteins with expression that varies by $\geq 30\%$ between healthy controls and patients with EM

Mass (Da)	Frequency (%)	Change in EM
4209.64	100	Down
5263.87	89	Up
4247.51	87	Down
4963.78	84	Up
2659.26	69	Up
4168.75	63	Down
8106.89	60	Down
5805.74	59	Up
8142.45	53	Down
3240.52	52	Up
5634.22	40	Up
5864.94	39	Up
7009.98	37	Down
7398.71	37	Down
4266.52	32	Down
5754.13	31	Up

Da: Daltons; EM: Endometriosis.

Table 4: Differential proteins selected by ClinProtocols software

Protein combinations (Da)	Sensitivity (%)	Specificity (%)	Average cross verification (%)
EM patients vs. healthy controls			
4209.26	96.67	100	97.77
5904.97			
2658.79			
Other benign tumors vs. healthy controls			
5264.28	100.00	100	100.00
Stage I–II EM vs. healthy controls			
5905.37	100.00	100	97.73
4209.68			
Stage III–IV EM vs. healthy controls			
2659.98	100.00	100	96.30
4210.09			
3191.88			

Da: Daltons; EM: Endometriosis.

and 4210Da decreased compared with normal. This pattern implies that those proteins affect disease development.

Identification of endometriosis-specific serum peptides by liquid chromatography-tandem mass spectrometry

We collected data for nine samples using an Auto Flex II MALDI-TOF/TOF operated in linear mode for 400 shots to draw peptide mass fingerprints for of 4210, 5904, 5264, 5635, and 2600 m/z [Figure 4]. Of these five peaks, 4210 and 5904 m/z had high discrepancy peaks without interference peaks, which facilitate follow-up peptide identification; whereas peak intensities of 5263, 5635, and 2600 m/z were too low or had intensive interference peaks, which

impede follow-up identification. The two selected peaks (4210 m/z, 5904 m/z) also showed the biggest differences between EM patients and controls [Figure 5]. LC-MS/MS showed the amino acid sequence of the 4210 m/z peptide as IMSEYLWDPERRMFLARTGQSWSLILLIYFFFY, a fragment of ATP1B4; and the 5904 m/z peptide sequence as SSSYSKQFTSSTS YNRGDSTFESKSYKMADEAGSE ADHEGTHSTKRGHAKSRPV, which is a fragment of the fibrinogen alpha (FGA) isoform 1/2 of the FGA chain precursor.

DISCUSSION

Although many EM patients develop dysmenorrhea, infertility or chronic pelvic pain long before forming detectable ovarian neoplasms, it is difficult to diagnose in its early stages, even in women who develop EM in puberty.^[11]

For the last decade, researchers have used SELDI technology to explore biomarkers for EM, and have found differential peaks at 8141, 6096, 5894, 3269, 3956.83, 11710.70, 6986.45, 4974, 5813, and 4290 Da.^[12,13] These results did not accord well, partly because of variations in samples, and possibly because their data were simply imported to the analysis software with little further statistical analysis for in-depth validation. Some investigators got different proteins using the same type of sample in different sites or times, which made identifying these proteins much harder.

Matrix-assisted laser desorption/ionization TOF MS is a sensitive and effective approach for identifying potential biomarkers of health and disease.^[14] In our previous study, WCX was a superior means of finding differential proteins and establishing steady models.^[15] Here, we enriched low-abundant serum peptides/proteins by WCX magnetic beads and performed serum protein profiling through MALDI-TOF-MS, which offers a wide selection. It is also noninvasive and might allow dispensing with tissue biopsies.

In this study, we used the ClinProt System to explore serum biomarkers of EM, and found many differential proteins between EM patients and healthy controls from which we constructed a model with high sensitivity and specificity. Although we also used serum from patients with other benign ovarian neoplasms as a control group, we found no proteins that significantly differed between them and the EM patients. Further studies showed differences in protein fingerprints between patients with minimal and early-stage EM and healthy controls, and also significant differential proteins that can stably distinguish these two groups, which may aid in early diagnosis of EM.

Other technologies use different serum EM markers, possibly because different systems focus on different mass area of target proteins. The advantage of our system is that it facilitates identification of those proteins which has been otherwise so time-consuming.

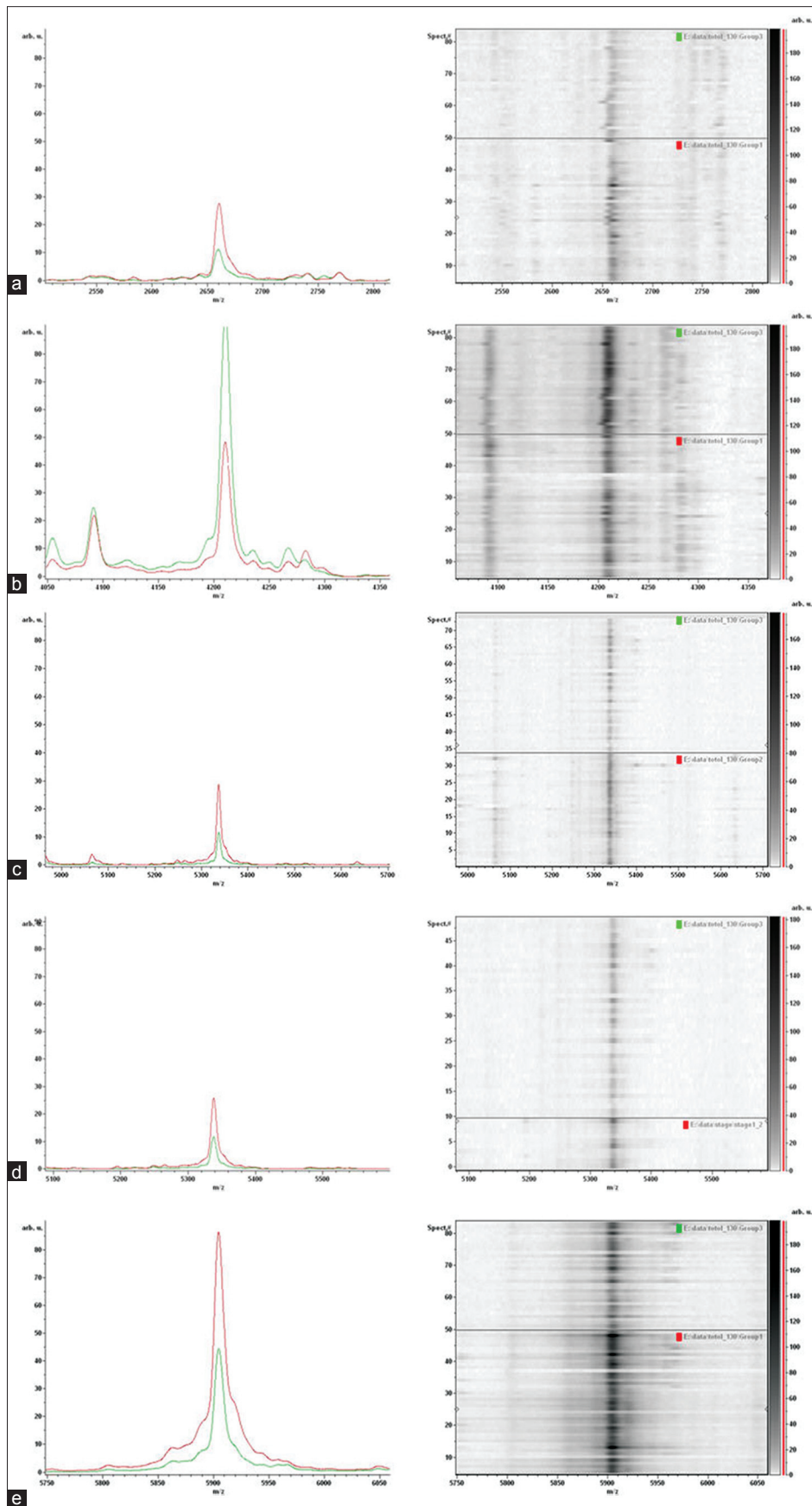


Figure 3: Differential protein peaks in typical samples, and in electrophoresis gel bands. Left: Differential protein peaks; red: Patient samples; green: Healthy controls; Right: Electrophoresis gel bands; Upper: Healthy controls; lower: Patients. (a) 2660 Da; (b) 4210 Da; (c) 5264 Da; (d) 5635 Da; and (e) 5904 Da.

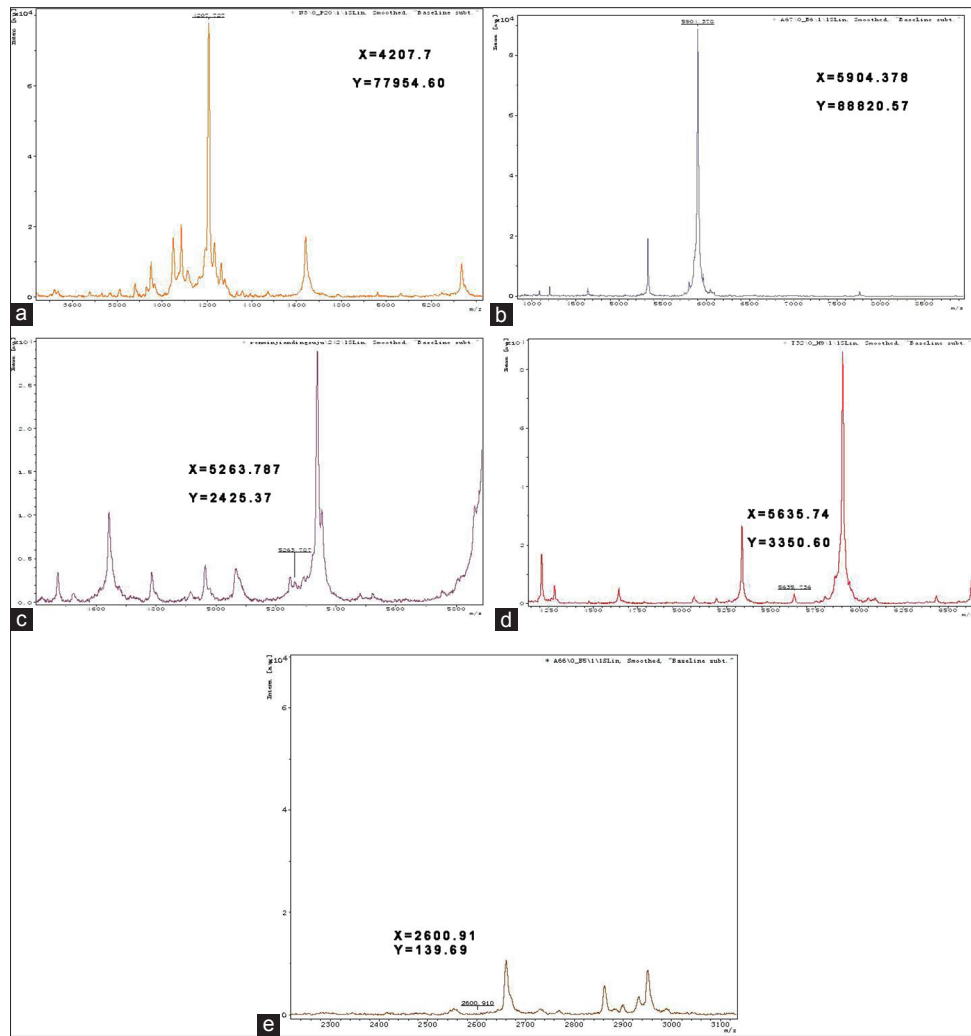


Figure 4: Peptide mass fingerprinting of (a) 4210 m/z; (b) 5904 m/z; (c) 5264 m/z; (d) 5635 m/z; and (e) 2600 m/z.

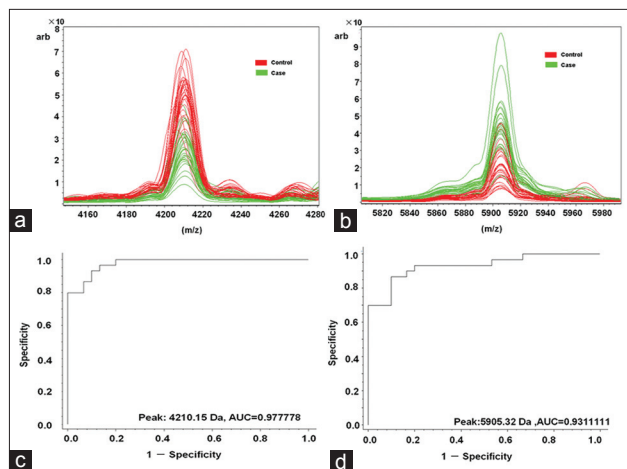


Figure 5: Distribution and performance of serum peptide biomarker for (a) 4210 m/z and (b) 5904 m/z. Receiver operator characteristic of (c) 4210 m/z and (d) 5905 m/z distinguishes patients with endometriosis (EM) from healthy controls. Red: Controls; green: Patients with EM.

As the weakest aspect of SELDI is reproducibility, we focused on this point. First, we designed a strictly

standardized experimental operation: Sample collection and storage, transport, magnetic beads selection, equilibrium for matrix formula, data collection. We then used the CV value for several typical peaks in different samples to evaluate the stability of the system. We randomly chose a commercial sample as the standard serum; all CV values of these selected peaks were <10%, which indicates high stability. Inter- and intra-group consistency was also satisfactory. To assess stability of differential proteins in different groups, we first found cross-validation results (average diagnosis values from different samples) to construct model and validation groups; in our study all results were $\geq 85\%$, which indicated a stable model. We then used randomly selected samples to construct our models, and found the differential proteins remained when groups changed. Thereafter, the biggest challenge was to confirm the differential proteins.

We also found results to change when using a different analysis system, which we thought was because of variations in bioinformatics. Because complicated algorithms are based on provided sampling data, and may modify parameters to

make analyses more suitable for the data at hand, they may change with different sample sets. If the protein fingerprint differed dramatically between two groups, random combinations for each 2-protein set could show strong sensitivity and specificity for the model, but would change with the variables.

After employing these methods, we found differential proteins at 4210, 5264, 2660, 5635, 5904 Da to distinguish between patients with EM and healthy controls. These proteins have high diagnostic accuracy, either singly or combined. We also found that the 5635 Da peak is associated with early-stage or minimal EM and could help monitor early disease. LC-MS/MS sequencing showed the 4210 Da peptide to correspond uniquely to ATP1B4, and the 5904 Da protein to FGA isoform 1/2 subunit.

ATP1B4 is crucial in forming functionally active X, K-ATPases.^[16] In a previous study, we discovered that the ATP synthase β subunit is expressed differently in patients with EM than in normal controls. We considered that there is a close relationship between ATP function and EM generation and development. EM results from a menstrual bleeding countercurrent, the formation of which depends on functions that use ATP enzymes (ATP synthesis, signal transduction, metabolic regulation, etc.).^[17,17]

Fibrinogen alpha fibrinogen is a glycoprotein secreted by hepatocytes,^[18] which mainly affects blood coagulation and hemostasis.^[19] Fibrinogen is considered to be an acute-phase reactant protein; it increases during tumor progression, apparently as an ongoing inflammatory response to tumor. Some studies suggest that tumor cells could promote coagulation by interacting with endothelial cells and platelets, and then releasing active biologics that activate platelets, thus increasing the fibrinogen in the blood of cancer patients. FGA, isoform 1 of FGA chain precursor, has also been found (using ClinProt) to significantly differ between adult patients with acute lymphocytic leukemia and healthy controls, and is a potential biomarkers for forecasting relapse, and monitoring residual disease and therapeutic response in these patients.^[20] Although EM is not cancer, it displays malignant behavior. Nothnick *et al.* utilized a novel mouse model in which eutopic endometrial fragments used to induce EM were deficient in miR-451 expression, which resulted in fewer ectopic lesions *in vivo* and was associated with differential expression of alpha polypeptide isoform 2 precursor.^[21] As this polypeptide contained arginyl-glycyl-aspartic acid (RGD) cell adhesion motifs that could modify early-stage lesion development, RGD peptides might help prevent recurrent EM development.

Our results using ClinProt to identify biomarkers for EM screening indicate that it is effective even for early-stage or minimal EM. We found five stable peaks with molecular weights of 4210, 5264, 2660, 5635, and 5904 Da as potential

EM biomarkers. Furthermore, our results indicate that ATP1B4 and FGA are associated with EM pathogenesis; these associations merit further study.

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