European Journal of Obstetrics & Gynecology and Reproductive Biology 177 (2014) 23–28

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Urine peptide patterns for non-invasive diagnosis of endometriosis: a preliminary prospective study

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ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 28 January 2014
Accepted 5 March 2014

Keywords:
Endometriosis
Diagnosis
Urine
Proteomics
MALDI-TOF-MS
LC–MS/MS

ABSTRACT

Objective: To detect endometriosis by urine peptide biomarkers using magnetic beads-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and to identify interesting peptides using liquid chromatography tandem mass spectrometry.
Study design: Prospective case-control study in a university-based gynecological department and central laboratory. A total of 122 patients suffering from dysmenorrhea, pelvic pain and infertility were enrolled in the study. Urine samples were collected before laparoscopy. Urine samples were analyzed by the MALDI-TOF technique to generate peptide profiling and ClinProTools software was used to set up a diagnostic model for endometriosis. Liquid chromatography tandem mass spectrometry (LC–MS/MS) was used to identify interesting peptides.
Results: At laparoscopy 60 patients were diagnosed with endometriosis and 62 patients were disease-free. There were 36 different peptides expressed in endometriosis patients detected by MALDI-TOF compared with controls. We established a genetic algorithm as a diagnostic model with the combination of five peptides (m/z = 1433.9, 1599.4, 2085.6, 6798.0 and 3217.2). The model showed a sensitivity of 90.9% and specificity of 92.9%. Urine from another 26 symptomatic patients before laparoscopy were randomly selected and analyzed accordingly. A genetic algorithm showed a sensitivity of 90.9% and specificity of 92.9% in predicting endometriosis before laparoscopy. We also identified two peptides not belonging to the diagnostic model as collagen precursors.
Conclusions: Patients with endometriosis have a unique cluster of peptides in urine. Peptide proteomic profiling provides a novel method for non-invasive diagnosis of endometriosis.

Introduction

Endometriosis, the presence of endometrial glands and stroma outside the uterus, is a disease associated with chronic pelvic pain, ovarian masses and infertility. Endometriosis affects approximately 15% of women of reproductive age and an even higher percentage of women with pelvic pain symptoms and infertility. However, endometriosis is still an underdiagnosed disease and the interval between the onset of symptoms and the diagnosis of the disease is estimated to be as long as 8 years [1]. Clinical diagnosis of endometriosis is based on direct visualization of endometriosis lesions by laparoscopy, which is invasive and expensive. To date, clinical biomarkers are unsatisfactory [2–4]. A rapid non-invasive technique is urgently required for diagnosis and follow-up of endometriosis.

Recently the proteomic technique has developed rapidly with high throughput and high sensitivity, which makes it a powerful tool for discovery of protein and peptide biomarkers of disease [5–10]. In the present study, we used a magnetic bead-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (ClinProt™, Bruker Daltonics, Germany) to generate peptide profiling for the discovery of potential urine biomarkers of endometriosis.

Materials and methods

This study was designed as a prospective case-control study and was approved by the Human Ethics Committee of Peking Union Medical College Hospital. All participating patients signed an informed consent form before enrollment in this study.

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http://dx.doi.org/10.1016/j.ejogrb.2014.03.011
0301-2115/

Patients and urine sample collection

A total of 122 women who presented to Peking Union Medical College Hospital for diagnosis and treatment of dysmenorrhea, dyspareunia, chronic pelvic pain, ovarian cyst or infertility were selected. None of the patients had estrogen-dependent diseases or received any hormone or medication before being included in the study. All the patients enrolled in the study had a regular cycle of 28 days. Urine samples of the follicular phase were collected on days 6–8 of the cycle, while those of the luteal phase were collected on days 18–22 of the cycle. The patients fasted overnight. All the urine samples were obtained by catheterization into a sterile container before laparoscopy and after anesthesia. After centrifugation, samples were divided into aliquots and kept at −80 °C within 30 min of collection. Repeated freeze/thaw cycles were avoided to protect protein integrity.

Sample pre-treatment with magnetic beads

Urine samples were fractionated with weak cation exchange magnetic beads (MB-WCX) through three steps: binding, washing and elution: 10 μL beads, 10 μL binding solution (Bruker Daltonics) and 5 μL sample were added into a tube and incubated for 5 min. The tube was placed on the magnetic bead separation device and the beads on the tube wall were collected after 1 min. The supernatant was removed and the beads were washed with 5 μL of eluting solution (Bruker Daltonics). The clear supernatant was transferred into a fresh tube and 5 μL of stabilizing solution were added (Bruker Daltonics).

MALDI-TOF-MS analysis

We applied 1 μL of prepared sample to a target spot and let it air-dry. 1 μL of matrix consisting of 3 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 2% trifluoroacetic acid (TFA) was applied four times to each spot and air-dried.

Mass spectra from 1000 Da to 10,000 Da (Daltons) were acquired using a Reflex IV ™MALDI-TOF-MS (Bruker Daltonics) in positive linear mode. Analyses were performed using AutoXecute tool of FlexControl software (Bruker Daltonics) to obtain high quality spectra profiles. Data were accumulated from four different spot positions (100 shots per position). The acquisition of data was carried out at 30% of the maximum laser energy. The data of spots from eight patients were also acquired in reflector mode to get the accurate mass of candidate biomarkers, calibrating the instrument by means of standard peptides.

Data analysis

Data analysis was performed using ClinProTools 2.2 software package13 (Bruker Daltonics, Bremen, Germany). Comparison of multiple spectra and protein pattern identification was achieved through the following workflow: each raw spectrum was normalized to its total ion current, all the spectra were recalibrated using the prominent common m/z values, baseline subtraction, smoothing, and peak detection were performed and peak areas for each spectrum were calculated. The signal-to-noise ratio was set at 5 for peak detection. Peak areas were calculated using end-point level integration type. Spectra were also "top hat" baseline subtracted with the minimum baseline width set to 10%, smoothed and processed in the 1–10 kDa range.

Peak significance between groups was evaluated by Mann–Whitney U-test. The significance level was set to 0.05 and P values less than 0.05 were considered to be statistically significant. Area under the curve (AUC) summaries for peak masses was calculated.

Genetic algorithm (GA), decision tree algorithm (DTA) and quick classifier algorithm (QC) were then used for the selection of peptide peak clusters and set up a diagnostic model using ClinProTools 2.2 software package 13.

Blind test of the diagnostic model

Urine samples from another 26 randomly selected symptomatic patients were collected from Peking Union Medical College Hospital and analyzed accordingly. The patients were classified as either endometriosis or non-endometriosis patients by the selected diagnostic model. The results from the diagnostic model were compared with findings from laparoscopy to generate the diagnostic efficiency of the model.

Biomarker identification

Eluted fractions from three controls and three endometriosis patients were pooled, dried and then dissolved in 25 μL of 0.1% TFA, 2% ACN and water for nanoscale HPLC-ESI MS/MS analysis with an HPLC Ultimate 3000 (Dionex) coupled to the Esquire 3000 Plus™ mass spectrometer (Bruker Daltonics GmbH, Leipzig, Germany). The HPLC was equipped with a Zorbax 300 SB column C18 75 μm × 150 mm, 5 μm (Agilent Technologies) and the flow rate at the end of column was 300 nL/min. Mobile phase A was 0.1% formic acid solution while mobile phase B was 95% acetonitrile with 0.1% of formic acid. Peptides were pre-concentrated into C18 trap column (5 μm, 100 A, 150 μm i.d × 20 mm; Dionex) with a loading flow of 40 μL/min of 0.1% TFA, 2% ACN and water. Then they were eluted from the Zorbax 300 SB column with a multistep gradient from 1.6% to 56% of ACN in 25 min and from 56% to 78.4% in 5 min and after 5 min at the initial conditions directly to the Esquire 3000 plus IT mass spectrometer equipped with a nanoESI ion source. Dry gas was set at 0.5 L/min with a dry temperature of 200 °C.

For LC-ESI MS/MS analysis, 10 μL of samples was injected from autosampler into the column. Helium was used as collision gas (MS/MS Frag Ampl of 0.7 V). Peptides were analyzed in positive ion mode in a range of 200–1500 m/z. The mass spectrometer duty cycle was composed of one MS full scan followed by two consecutive MS/MS scans of the two most intense ions present in each MS scan. MS/MS spectra were limited to three consecutive scans per precursor ion followed by 0.15 min of exclusion. The obtained chromatograms were analyzed with DataAnalysis® (Bruker Daltonics, Bremen, Germany) and the mass lists were used for database search using an in-house Mascot® search on a local server with the MudPIT scoring control. Parameters used to create peak list are: $S/N = 3$; algorithm version 1.0; intensity/area threshold = 0.1; skim ratio = 0.1 and smoothing width = 1. MS/MS spectra were searched against the SwissProt (v. 54.3) restricted to homo sapiens taxonomy without enzyme specificity and missed cleavages. The charge state was defined by 2+ and 3+ settings. Parent ion and fragment mass tolerance were set at ±1.0 Da and ±0.5 Da, respectively. Peptides identification was accepted only for those MS/MS spectra with a Mascot score above the significant threshold of identity ($P < 0.05$).

Results

During laparoscopy, the extent of the endometriosis was staged according to the revised American Fertility Society (rAFS) classification [11]. Sixty patients were diagnosed with endometriosis including two complicated with adenomyosis, and 62 patients were disease-free. In the control group there were 36 patients diagnosed with benign ovarian cyst and 26 with infertility caused by adhesions and inflammation. Detailed information is summarized in Table 1.