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Serum differential proteomic analysis of endometriosis and adenomyosis by iTRAQ technique

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ABSTRACT

Objective(s): Adenomyosis and endometriosis are two different diseases, although they have similar pathogenesis and characteristic. The serum differential expressed proteins in adenomyosis and endometriosis may result from the different pathogenesis of two diseases. Proteomic technology is a useful method for detecting all the proteins in samples. We try to use isobaric tags for relative and absolute quantitation (iTRAQ) technology to explore the association between the potential pathogenesis of these two diseases and these identified proteins.
Study design: The serum samples from 20 patients with adenomyosis and from 20 patients with endometriosis were analyzed using iTRAQ technology to detect the differential expression of proteins. The validation of the proteins was performed using Western blot.
Results: In the serum of women with adenomyosis and with endometriosis, 14 proteins were found differentially expressed using iTRAQ technology. Nine proteins were high-expression in adenomyosis group and four proteins increased in endometriosis group. And the differential expression proteins were validated by Western blot.
Conclusion(s): The proteins increased in adenomyosis group are related to blood coagulation and complement activation effects, and the proteins high-expression in endometriosis mainly take part in the process of inflammatory response and regulation of apoptosis. The differentially expressed proteins in two groups may due to the different pathogenesis of two diseases.

Introduction

Endometriosis and adenomyosis both are common gynecological diseases characterized by the presence of endometrial glands and stroma outwith their normal locations. Although it has been widely accepted that endometriosis and adenomyosis are two different diseases, the commonness in the pathology and clinical character of endometriosis and adenomyosis are undeniable. In some reports, adenomyosis is considered as a variant of endometriosis or "internal endometriosis" [1]. Till now, the exact relationship between the two is unclear. Previously studies mostly are focus on the expression of one or some particular kind of proteins in endometriosis and adenomyosis, few studies use holistic methodology to study the relationship between factors [2,3].

Proteomics research, as an experimental approach widely used in life science, has facilitated the study of structure and function of protein in integrity. By means of differential proteomic methods, the serum protein expression profile in the different groups was constructed, and the different proteins were detected and confirmed [4]. In this study, we analyzed the serum samples from the patients with adenomyosis and with endometriosis using iTRAQ technology, to find the differential expression proteins in two groups which may contribute to the early diagnosis and correlation mechanism of two diseases.

Materiald and methods

Serum samples were obtained from XuanWu Hospital of Capital Medical University during July 2011 to December 2011 and were divided into two groups: (1) adenomyosis group: 20 women with adenomyosis alone underwent hysterectomy because of dysmenorrhea or menorrhagia, and uteruses were found enlarged more than 12 weeks' gestation by ultrasound and operative exploration; (2) endometriosis group: 20 women with ovarian endometriosis alone underwent laparoscopic cystectomy because of dysmenorrhea or infertility, and at least one side of ovarian were found

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enlarged more than 5 cm in dilameter by ultrasound and operative exploration. All of the diagnoses were confirmed by a pathological examination of the biopsied tissue. In the EMs group, 7 patients were classified as stage I–II, 13 as stage III–IV, and the stage of endometriosis was assessed according to the revised American Fertility Society. All patients studied were of reproductive age. None of the women had received hormonal treatment or undergone surgery within the six months before their inclusion in the study. The study was approved by the Institutional Review Board and all the patients signed an informed consent form.

The serum samples were obtained by venipuncture in the morning before the patients had consumed any food and were centrifuged at 3,000 rpm/min for 5 min at 4 °C. Then they were aliquoted and stored at −80 °C until use. The serum pools were depleted of the most highly abundant serum proteins using the Albumin and IgG Depletion SpinTrap™ reagent (GE Healthcare, London, UK) and were preconditioned using the PD-10 extraction desalting reagent (GE Healthcare, London, UK). Then the eluent of the protein samples was quantitated using the Bradford reagent (Bio-Rad,California, USA) [11,12]. Then 20 μl dissolution buffer, 1 μl denaturant in the kit and 2 μl reducing reagent were added to each tube which contain 100 μg of sample before Incubating at 60 °C for 1 h. 1 μl cysteine blocking reagent and 10 μl of the trypsin solution were added successively to each tube, and then incubate the tubes at 37 °C overnight. Peptide mixtures were labeled using 4-plex iTRAQ labeling kit following the iTRAQ™ Reagents Protocol. Adenomyosis group was labeled with the iTRAQ 114, and the endometriosis group was labeled with iTRAQ 115.

The serum samples were fractionated by two-dimensional (2D) liquid phase chromatography fractionation. The concentrations of buffer salts and organics in iTRAQ labeled peptide solution were reduced by diluting the sample mixture by 10-fold with SCX (strong cation ion exchange chromatography) load-A (5 mM KH_2PO_4 in 20%ACN, pH 3.0). The diluted sample mixture was slowly injected onto the cation exchange cartridge (4.6 × 250 mm, 5 μm, 200 Å) and fractions were eluted with SCX load-B (5 mM KH_2PO_4,350 mM KCL, 20%ACN). And then the fractions were collected at 2-min intervals from the third minute and lyophilized in vacuum concentrator. Some of the fractions were combined according to the chromatogram. The second dimensional chromatography was employed on the Eksigent nanoLC-Ultra™ 2D System. The dried SCX fractions were re-dissolved in 2% acetonitrile, 0.1%formic acid, and loaded on in-house packed trap column (100 μm × 3 cm, C18, 3 μm 150 Å) and washed for 10 min at 4 μL/min. Then, an elution gradient of 5–35% acetonitrile (0.1%formic acid) in 70 min gradient was used on an in-house packed analytical column (75 μm × 15 cm C18- 3 μm 150 Å) with spray tip.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, USA) fitted with a Nanospray III source (AB SCIEX, Concord, USA).

Data was processed with Protein Pilot Software 4.0 (AB SCIEX, Foster City, USA) utilizing the paragon and progroup algorithm. In the software algorithm, all modifications listed in UniMod are searched simultaneous (http://www.unimod.org/). We adopted the fold change of the differentially expressed proteins in the study by Glen et al and the fold change cutoff ratio >1.50 or <0.66 was selected to designate proteins of differential expression ($P < 0.05$). And the PANTHER database was used to elucidate functional classification of identified proteins by iTRAQ. The cellular component (CC) and biological process (BP) classification of the selected proteins were annotated by Gene ontology (GO) database.

The serum samples of 5 patients with adenomyosis and 5 patients with endometriosis were lysed in the 2× loading buffer at 100 °C for 5 min. After centrifugation at 12,000 rpm for 5 min, the proteins were added to the SDS-PAGE. Equal amount of proteins (40 μg) was fractionated using SDS-PAGE and transferred to PVDF membranes. The membranes were blocked at 4 °C for 60 min with 3% BSA, followed by 2 h of incubation with the primary antibodies: fibronection-1 (FN1), CD44, complement factor B (CFB), serum albumin preproprotein (ALB) and fibrinogen alpha (FGA) (Abcam, UK). After washing 5 times with TBST buffer, the membranes were incubated in rabbit polyclonal secondary antibody (Abcam, UK) (1:5000) for 1 h at room temperature. The intensity of target protein was detected with enhanced chemiluminescence detection (ECL) system.

Result

Protein expression profiling

With iTRAQ technology, a total of 151 unique proteins were identified with 95% confidence by the ProteinPilot search algorithm in the serum of two groups. We used 1.3-fold change cutoff for all the iTRAQ ratios to reduce false positives for the selection of differentially expressed proteins. There were 9 proteins significantly over-expressed in adenomyosis group controlling with the endometriosis group (>1.3). And another 5 proteins were significantly decreased (<0.66) in adenomyosis group (Table 1). According to the Gene Ontology (GO) classification, most of these proteins were found to be located in extracellular space. According to the functional categories using the PANTHER classification system, the main molecular functional categories of over-expression proteins in adenomyosis group were blood coagulation, complement activation, immune response and regulation of proteolysis. The top three molecular functional categories of proteins increased in endometriosis group were inflammatory response, regulation of apoptosis and hypoxia.

Western-blot identification

The differential expression levels of the proteins identified by iTRAQ approach were validated using Western blot analysis. In our study, we chose the following five proteins: FN1, CD44, CFB, ALB and FGA for the further validation. As expected, ALB which is one of the differentially expressed proteins identified, was found over-expressed in the serum of endometriosis group. And FGA was found obviously increased in the adenomyosis than in endometriosis group. However, the Western-blot results showed that the expression levels of FN1, CD44 and CFB between two groups were no obvious difference. This result was consistent with the protein expression level obtained in iTRAQ approach (Fig. 1).

Comments

In previous studies, some proteins, such as CD44, VEGF, fibronectin were found to be high-expression in both adenomyosis and endometriosis [5,6], which indicate that adenomyosis and endometriosis have similar mechanism, but not all the changes are identical. In our study, there were significant differences in serum proteome profile between adenomyosis and endometriosis [7]. Fourteen differentially expressed proteins were found in the serum of two groups. This result further proved the theory that adenomyosis and endometriosis are two different diseases.

Significantly increased expression of fibrinogens (fibrinogen-α, fibrinogen-β and fibrinogen-γ) was found in the serum of women with adenoymosis, more than 20-fold the expression in endometriosis. We also found the concentration levels of coagulation factor XIII and antithrombin-IIIin adenomyosis were separately 5.7 times and 1.39 times of them in endometriosis. Fibrinogen, as one of the most important blood coagulation factors, combined with coagulation factor XIII and antithrombin-III synergically take part in the process of blood coagulation. Fibrinogen is an internal acute