Proteomic analysis of eutopic and ectopic endometriotic tissues based on isobaric peptide tags for relative and absolute quantification (iTRAQ) method

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Abstract

OBJECTIVE: The present study aimed at performing proteomic analysis of matched eutopic endometrium and ovarian endometrioid cysts from women with endometriosis in order to discover any abnormal protein expression related to the disease.

DESIGN AND SETTING: The study included 8 women with stage III/IV endometriosis according to revised American Fertility Society (rAFS) classification and one woman with no signs of the disease as a reference. Proteomic analysis was performed using a novel isobaric tag-based methodology for relative and absolute peptide quantification (iTRAQ) coupled with multidimensional liquid chromatography and tandem mass spectrometry.

RESULTS: The selection of 419 proteins was found in all endometriosis specimens. Using normal eutopic endometrium from woman without endometriosis as a reference, some proteins expressions were significantly increased in all endometriosis samples. They included collagen α1(XIV), calmodulin, collagen α(VI), plexin, integrin αVβ3, transgelin, desmin, and vimentin. The comparison of these proteins' expression in paired eutopic and ovarian endometriosis samples has revealed that only vimentin was significantly increased in ovarian endometrioma.

CONCLUSIONS: It was confirmed that endometriosis is associated with different expression of proteins in endometriotic samples. Nevertheless, further studies seem to be necessary as they may reveal possible markers that would be useful in clinical diagnosis of the disease.

ORIGINAL ARTICLE

INTRODUCTION

Endometriosis is a common gynecologic disorder affecting around 10% of women of reproductive age. The disease manifests by the presence of endometrial tissue outside the uterus, most commonly in the peritoneal cavity and the ovary, rarely found in other locations i.e. urinary tract or intestines (Ropacka-Lesiak *et al.* 2013). Endometriosis is a chronic inflammatory condition that may be accompanied by chronic pelvic pain, dysmenorrhea, dyspareunia, as well as subfertility or infertility (Giudice *et al.* 2004). Due to the above the disease constitutes an important clinical and social problem.

Endometriosis appears to be an enigmatic multifactorial trait depending on a variety of genetic and environmental factors (Giudice *et al.* 2004, Zondervan *et al.* 2001). It has been postulated that ectopic endometrioid tissue may originate from retrograde menstruation or metaplasia (Nisolle *et al.* 1997), however, it still remains unclear what facilitates survival, heterotopic implantation and growth of endometrial epithelial and stromal cells. This may be related to abrogated elimination of endometrial cells by local immune mechanisms and/or their increased survival owing to some intrinsic or environmental factors (Gazvani *et al.* 2002, Matarese *et al.* 2003, Harada *et al.* 2004, Kajihara *et al.* 2011). Elucidation of these mechanisms and identification of factors responsible for development and persistence of endometriosis will be a milestone in understanding the etiopathogenesis of the disease and will possibly provide a basis for development of novel and more effective therapeutic approaches.

It is plausible that endometriosis is related to some, yet poorly defined alterations of some protein expression in both eutopic and ectopic endometrial tissue. Such alterations may be revealed by proteomic analyses and, indeed, a number of studies showing differential protein expression analyses of various samples from patients with endometriosis have been reported so far (Upadhyay *et al.* 2013, Ferrero *et al.* 2008, Siristatidis 2009, Meehan *et al.* 2010). However, the obtained results were either conflicting or not satisfactory. Therefore, the present study aimed at performing proteomic analysis of matched eutopic endometrium and ovarian endometrioid cysts from women with endometriosis using a novel isobaric tag-based methodology for relative and absolute peptide quantification (iTRAQ) coupled with multidimensional liquid chromatography and tandem mass spectrometry (Wiese *et al.* 2007).

MATERIALS AND METHODS

Patients and clinical material collection

The study was approved by the Local Bioethical Committee of the Medical University of Warsaw and a written informed consent was obtained from all participating patients. The study included 8 women with stage III/IV endometriosis according to revised American Fertility Society (rAFS) classification (American Fertility Society 1985) and one woman in whom no signs of the disease could be found (mean age 32.7±3.1 years). The diagnosis was based on laparoscopic and histopathological examinations. The patients had regular menstrual cycles of 28–32 days. They did not suffer from any other chronic disease and were not a subject of any hormonal treatment at least 3 months before the onset of the study.

All tissue specimens were collected in the follicular phase of the menstrual cycle. Eutopic endometrium samples were obtained by Pipelle® endometrial suction curette and ovarian endometrioma tissues were collected during a routine laparoscopic procedure. The samples were bisected; one part was subjected to histopathological examination and the second one was snap frozen on dry ice and stored at –80 °C until used for total protein extraction.

Protein extraction

The tissue samples were washed in ice-cold sterile phosphate-buffered saline (PBS) in order to remove contaminating blood, then cut into 1 mm^3 pieces using tissue scissors, placed in mortars, and ground into powders in liquid nitrogen. The resulting tissue powders were then homogenized in an ice-cold lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 2% pH 3–10 ampholyte, all from Bio-Rad) containing protease inhibitor cocktails (Sigma). The DNase (200 µg/ml) and RNase (50 µg/ml) were added and the sample mixtures were incubated for 30 min at 37 °C in order to remove DNA and RNA. The homogenates were then centrifuged at 14,000 rpm for 1 hour at 4 °C. The supernatants were afterwards collected and precipitated with cold acetone at –20 °C for 1 hour. The precipitated proteins were again collected by centrifugation and then dissolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 2% pH 3–10 ampholyte, Bio-Rad). The protein concentrations were determined using the DC protein assay kit (Bio-Rad) and the individual sample concentrations were adjusted using the same rehydration buffer. The protein samples were immediately subjected to isoelectric focusing (IEF) or stored in aliquots at –80 °C for future assays.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and computer-assisted image analysis

Two-dimensional electrophoresis was performed by combining isoelectric focusing (IEF) in the first dimension and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The IEF was carried out using the PROTEAN IEF system (Bio-Rad), which includes the PROTEAN IEF cell, and ReadyStrip immobilized pH gradient (IPG) strips. Protein samples were applied to IPG strips (11 cm, pH 3–10) with a ReadyPrep 2-D Starter Kit (Bio-Rad) using a passive rehydration method. After

IEF, the strips were equilibrated in the equilibration buffers provided in the kit. The second dimension (SDS-PAGE) was performed using an 8–16% linear gradient Ready Gels on a Criterion Precast Gell System (Bio-Rad). Following SDS-PAGE, the gels were stained using the MS Compatible Silver Stain Plus Kit (Bio-Rad and USA) and scanned with the Molecular Imager GS-800 Calibrated Densitometer (Bio-Rad). The 2-D maps were then analyzed by PDQuest 2-D Analysis Software Version 8.0 (Bio-Rad), which allowed automatic detection and quantification of protein spots as well as the resizing, alignment, and matching between different gel images. Finally, a database of all protein spots from the digital images was created and the integrated intensity of each spot was normalized based on spot area and spot optical density. Only spots, which were up-regulated or down-regulated more than two-folds, were selected for mass spectrometric analysis.

Mass spectrometry (MS) and protein identification

The protein spots of interest were excised from the silver stained gels manually. The excised spots were then processed for in-gel digestion with MS grade Trypsin Gold (Promega) according to the manufacturer's suggestions. Briefly, the gel spots were unstained, dehydrated, dried, and incubated in 0.01 mg/ml of trypsin solution overnight at 37°C. Next the digested peptides were extracted and dried and processed for isobaric tag for relative and absolute quantitation (iTRAQ) labeling coupled with multidimensional liquid chromatography-tandem mass spectrometry. The mass spectral data were processed into peak lists and searched against the Swiss-Prot Database using the Mascot search algorithm. The searching parameters were then set up as follows: Database, Swiss-Prot; taxonomy, Homo sapiens; enzyme, trypsin; the number of missed cleavage, up to 1. Carbamidomethyl (C) and oxidation (M) were set as variable modifications. A high level of confidence was assigned to the protein identities with multiple matched peptides from each protein. Peptides with a Mascot score of more than 30 were considered significant hits.

Statistical analysis

Statistical analysis was performed in order to identify the proteins which were consequently present or expressed in endometriotic samples. The Kolmogorov-Smirnov test was used to verify the normality of the distribution and Levene's test was used to assess the equality of variances in the different samples. Comparisons between the independent groups were performed with the use of Mann Whitney U test.

RESULTS

The example of a representative gel is shown in Figure 1. Proteomic analysis of eutopic endometrium and ovarian endometrioma paired samples from all patients allowed the identification of the selection of 419 proteins which were present in all analyzed specimens. Using normal eutopic endometrium from a woman without endometriosis as a reference, we have identified some proteins expression which was significantly increased in all endometriosis samples. These proteins included collagen α1(XIV), calmodulin, collagen α(VI), plexin, integrin αVβ3, transgelin, desmin, and vimentin. The comparison of expression of these proteins in paired eutopic and ovarian endometriosis samples has revealed that only vimentin was significantly increased in ovarian endometrioma (Figure 2).

DISCUSSION

In the present study, using iTRAQ, a novel quantitative proteomic approach, the protein expression patterns in eutopic and ectopic endometrium from women with endometriosis was analyzed. It was found that, comparing to normal eutopic endometrium as a standard, endometriosis is associated with overexpression of several endometrial proteins, such as some extracel-

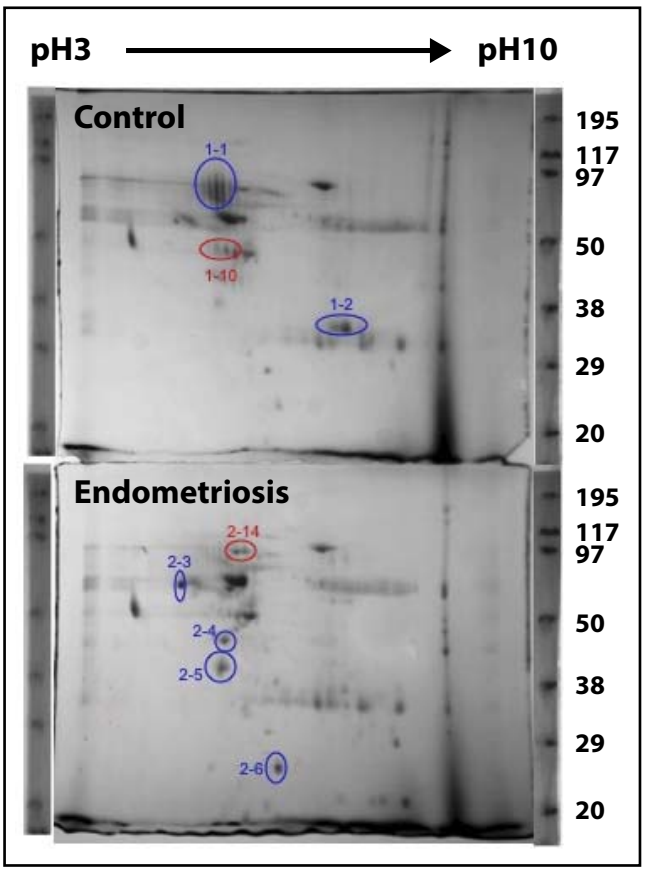

Fig. 1. 2D PAGE gel of representative samples from normal eutopic endometrium (control) and ovarian endometrioid cyst (endometriosis). The material was prepared as described in material and methods and 200 µg samples were separated in 12% polyacrylamide gel and stained with Coomassie Blue R250. Blue circles indicate significantly differentially expressed proteins, and red circles indicate likely differentially expressed proteins.

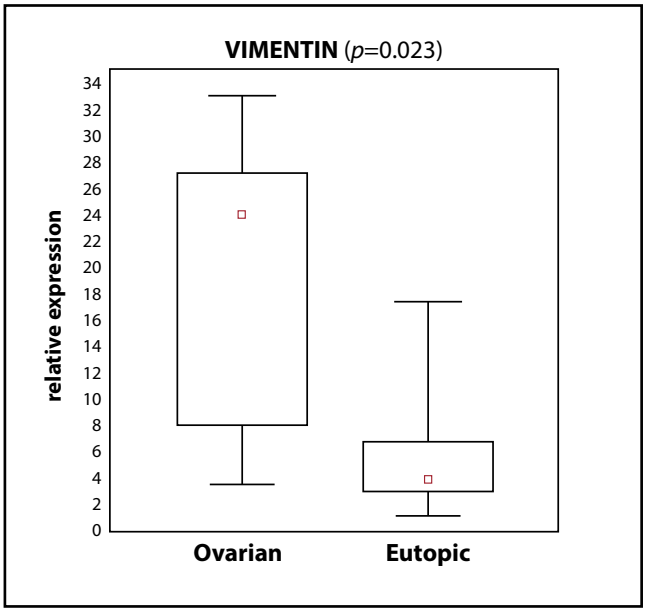

Fig. 2. Relative expression of vimentin in paired samples of eutopic endometrium and ovarian endometrioma from women with endometriosis. The data are shown as medians and interquartile range from 8 patients. Statistical significance was calculated by Mann-Whitney U test.

lular matrix components (collagen α1(XIV) and α(VI) chains), adhesion molecules (integrin αVβ3), signaling molecules (calmodulin, plexin) and structural proteins (transgelin, desmin and vimentin). Only vimentin was found to be significantly increased in ovarian endometrioid cyst when compared to eutopic endometrium from women with endometriosis.

To our knowledge only few papers concerning proteomic analysis of the endometrioid tissue have been published so far (Kyama *et al.* 2006; Fowler *et al.* 2007; Zhang *et al.* 2006; Assinder *et al.* 2009). The results of these studies showed that endometriosis may be related to aberrant expression of different proteins, however, the reproducibility of the results seems to be rather poor. In the presented analysis we have revealed that endometriosis may be associated with increased expression of transgelin and vimentin. Transgelin is a recently discovered actin-binding protein being a marker of smooth cells differentiation (Assinder *et al.* 2009; Robin *et al.* 2012) whereas vimentin is a type III intermediate filament ubiquitously expressed in mesenchymal cells (Fuchs *et al.* 1994). These proteins have previously also been reported to be aberrantly expressed in eutopic endometrium of patients with endometriosis by 2D-PAGE approaches (Kyama *et al.* 2006; Fowler *et al.* 2007). However, a putative role of these particular proteins in the development and etiopathogenesis of endometriosis remains obscure.

It is difficult to ascertain what the reason for inconsistencies in the results of proteomic analyses of endometrioid tissues is. It must be taken into consideration that these studies were performed using different methods. It is also plausible, that some differences may arise from different time of menstrual cycle at which clinical material was collected. The histological composition of the specimens may also have an impact on the results of total protein analysis as the proportion between stromal and glandular tissue may vary considerably. Therefore, further proteomic analyses of endometriosis-derived tissue samples will require more restrictive criteria for material collection and should allow for possible differences in histological pattern of evaluated samples.

It is noteworthy that studies in endometriosis also included proteomic analyses of the patients' plasma or sera and peritoneal fluid (Ferrero *et al.* 2008; Meehan *et al.* 2010; Ferrero *et al.* 2009; Liu *et al.* 2007; Wölfler *et al.* 2011). These studies also seem to be inconclusive and need replications. Nevertheless, further studies seem to be necessary as they may reveal possible markers that would be useful in clinical diagnosis of the disease.

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