Biomarkers of peritoneal fluid in endometriosis identified by surface-enhanced laser desorption/ionization time-of-flight

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Summary

Objectives: This work aims to detect the peritoneal fluid proteomic patterns in endometriosis patients, build diagnostic models, and evaluate its clinical significance. *Study Design:* The authors used SELDI-TOF-MS protein chip array technology to detect biomarkers of peritoneal fluid in endometriosis patients. Fourteen endometriosis patients and 16 persons without endometriosis as control group were tested. *Results:* Four potential biomarkers (4428m/z, 6891m/z, 13766m/z, and 6427m/z) were found. *Conclusions:* This method showed great potential in screening better biomarkers for endometriosis.

Key words: Endometriosis; Peritoneal fluid; Surface-enhanced laser desorption/ionization time-of-flight; Biomarkers; adenomyosis.

Introduction

Endometriosis, a gynecological disorder primarily of reproductive-aged women, is characterized by the presence of endometrial tissue (glandular and stromal epithelium) in sites outside of the uterus [1, 2]. Early diagnosis of endometriosis will greatly reduce its occurrence and reduce infertility [3]. Peritoneal fluid originates mainly from ovarian surface tissues exudation secondary to increased vascular permeability. Transudation from blood plasma as well as transudation/ exudation from kidneys, liver, pancreas, intestine, and intra-abdominal fat may contribute to the overall peritoneal fluid volume, which is greatest at mid-cycle and in the early luteal phase (mean 8.7 ml; range, 1 - 21 ml) [4, 5]. Results of research into the flow of peritoneal fluid support the hypothesis that peritoneal fluid represents a specific microenvironment that could play a role in the pathogenesis of endometriosis [6-8]. CA125 is the most widely used biomarker of endometriosis and progressive elevation of CA125 has been observed in peritoneal fluid of endometriosis patients [9]. Recent studies showed that peritoneal fluid CA-125 levels were 100- to 1000-fold higher than serum levels in women with pelvic endometriosis [10]. However, many women with endometriosis have normal CA125 values whereas high CA125 values can be seen in women with other gynecologic diseases such as ovarian cancer [11, 12], and thus CA125 does not have an adequate predictive value. How to find another sensitive marker in peritoneal fluid is very important for early diagnosis of endometriosis.

The authors have used SELDI-TOF-MS protein chip array technology to detect the proteomic patterns of serum and eutopic endometrium in endometriosis patients [13, 14]. The purpose of our present study was to find the biomarkers of peritoneal fluid in endometriosis patients by using SELDI-TOF-MS protein chip array technology.

Materials and Methods

A total of 30 women who had normal menstrual cycles volunteered for this study. All of them had not received any hormonal treatment for at least six months before operation. At the time of surgery, pelvic organs were carefully examined for the presence and extent of endometriosis. Fourteen patients with ovarian endometriotic cyst or adenomyosis were identified as endometriosis group. Among them, nine had one side of ovarian endometriotic cyst, three had two sides of ovarian endometriotic cysts, and two had adenomyosis. The median age of the endometriosis patients was 35 years (range 23 – 44). Diagnosis was pathologically confirmed and specimens were obtained before treatment. The distribution of endometriosis according to the revised American Fertility Society classification [15] was as followed: Stage I (n = 2), Stage II (n = 3), Stage III (n = 5), and Stage IV (n = 4). The control group (16 cases), with a median age of 37 years (range 20 – 47), underwent surgery for tubal ligation (three cases) or hysterectomy for benign indications (13 cases). They had no visible evidence of endometriosis. All of the peritoneal fluid samples were obtained at the beginning of surgery and centrifuged at 1,000 rpm for two minutes and then the peritoneal fluid samples were stored at -80°C until use. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Zhejiang University School of Medicine. Written informed consent was also obtained from all participants.

Peritoneal fluid samples in ice were thawed and centrifuged at 3,000 rpms for five minutes at 4°C, and supernatants were retained. Ninety µl of five g/l 3–((3–cholamidopropyl)dimethylammonio)–1–propanesulfonic acid (CHAPS) (pH 7.4) was added into phosphate buffered saline (PBS) to make up ten µl of each peritoneal fluid sample, and vortex-mixed. The diluted samples were added to 100 µl Cibacron Blue 3GA (previously equilibrated thrice with five g/l CHAPS) in 96-well cell culture plate and agitated on a plat-

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form shaker at 4°C for 60 minutes. After centrifugation at 1000 rpm, 50 µl supernatants were sampled and further diluted by 150 µl 20 mmol/l N–2–hydroxyethylpiperazine–N'–2–ethanesulfonic acid (HEPES) (pH 7.4) and applied to each well of a bioprocessor containing hydrophobic surface (H4) chips previously activated with 20 mmol/l HEPES. The bioprocessor was then sealed and agitated on a platform shaker for 60 minutes at 4°C. The excess peritoneal fluid mixtures were discarded, and the chips were washed three times by gently shaking on a platform shaker at a speed of 700 rpm for five minutes with 200 µl of 20 mmol/l HEPES (pH 7.4), were air-dried, and were crystallized by the addition of α-cyano-4-hydroxycinnamic acid.

Chips were detected on the Protein Biological System II (PBS-II) plus mass pectrometer reader. Data were collected by averaging 65 laser shots with an intensity of 190, a detector sensitivity of 7, a highest mass of 30000 Da and an optimized range of 2000-20000 Da. Mass accuracy was calibrated to less than 0.1% using the All-in-1 peptide molecular mass standard. The spectra intensities of all samples were normalized to the total ion current of mass to charge ratios (*m/z*) between 2,000 Da and 30,000 Da. Noise was filtered from the spectra and peaks were detected with an automatic peak detection pass. Peak clusters were completed with second-pass peak selection (signal-to-noise ratio > 2, within a 0.3% mass window), and estimated peaks were added.

All these were performed using ProteinChip Software 3.1. SELDI-TOF-MS can produce thousands of peaks that mostly represent the peritoneal fluid proteins and peptides but also contain the signals generated from the CHCA, the in-sample and sample-to-sample variations. To remove these signals, the authors excluded all the signals with m/z values below 2000. The collected protein mass-dependent velocities (m/z) peaks were analyzed using an artificial neural networks (ANN) [16-18]. ANN are advanced computer algorithms, able to recognize complex patterns in measured input variables which are not apparent to other forms of analyses. After processing of the incoming data by several transformation steps, the ANN produced an output, indicating a specific category within a given classification [19]. ANN has been successfully applied to a broad range of biomedical problems. All of the calculations were made with the STATISTICA 6.0 software package.

Results

After filtrating noise by Ciphergen ProteinChip Software 3.1, there were 267 peaks detected for discriminating endometriosis patients from healthy individuals. The peaks were 2 kDa to 30 kDa. Peaks with *m*/*z* < two kDa were mainly ion noise from the matrix and therefore excluded. The 267 qualified peaks detected from the two groups were ranked by receiver operating characteristic (ROC). The top four peaks with higher area under curve values were finally selected as potential biomarkers by using the stepwise approach. The *m/z* of the four candidate biomarkers were 4,428, 6,891, 13,766 and 6,427. The peaks of 4,428 m/z and 6,427 m/z were highly expressed in endometriosis patients but weakly expressed in control group, but the peaks of 13,766 m/z and 6,891 m/z seemed to be expressed in a contrary way, as shown in Figure 1. In the two groups, the *p* values of *t*-tests and the area under the ROC curve showed the statistical significance of all the four peaks, as shown in Table 1.

Table 1. — *The statistics of the candidate biomarkers; mean and standard deviation (SD).*

m/z	*p* value	Healthy (mean ± *SD)*	endometriosis (mean ± *SD)*
4428	0.0092446	2469.231 ± 1,772.43	4,752.407 ± 4,180.2754
6427	0.043213	1,360.464 ± 675.22	2,613.776 ± 4,002.54
6891	0.043213	3,173.666 ± 6,243.92	2,361.758 ± 1,270.45
13766	0.065073	3,794.985 ± 1,903.15	2,850.713 ± 1,462.49

Discussion

This time the present study indicated that changes in the peritoneal fluid composition may contribute to the pathogenesis of endometriosis. However, the analysis of peritoneal fluid protein fingerprinting by means of SELDI-TOF-MS can give only indirect insights into the process of endometriotic protein. Thus, this approach has to be considered as a form of screening assay, which allows for the identification of important marker that might be relevant in the pathogenesis of endometriosis. Further studies will be needed to determine the relationship between the peaks and protein in peritoneal fluid of endometriosis. In summary, in the present study the authors used SELDI-TOF-MS protein chip array technology to find the biomarkers of peritoneal fluid in endometriosis patients. Four potential biomarkers were found and the peritoneal fluid diagnostic system of endometriosis was built. Further studies should provide evidence that fundamental abnormal changes may occur within the peritoneal fluid of women with endometriosis compared to that of women without endometriosis.

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