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Serum Biomarker for Diagnosis of Endometriosis

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Endometriosis is estimated to affect 10% of women during the reproductive years. The lack of a non-invasive diagnostic test significantly contributes to the long delay between onset of the symptoms and definitive diagnosis of endometriosis. This case–control study was conducted to identify specific endometriosis antigens using 2D gel analysis in women with endometriosis (n = 5) and without endometriosis (n = 5). Differentially expresses spots were analyzed using matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry (nanoLC-ESI-MS/MS) with MASCOT analysis, in order to identify the corresponding proteins. ELISAs were performed on a different cohort of endometriosis (n = 120) and healthy patients (n = 20) in order to confirm the differential expression of the identified proteins. ROC analysis of ELISA results confirmed the statistical significance of the differential expression for one of these proteins: Zn-alpha2-glycoprotein ($P = 0.019$). We propose the analysis of the expression level of this protein in the serum as a new non-invasive diagnostic test for endometriosis.

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Endometriosis is defined as the presence of tissue similar to endometrium but residing outside of the uterine cavity. It is one of the most common gynecological diseases affecting up to 10% of all reproductive-aged women (Bulun, 2009). Nevertheless, the prevalence rises to 30–50% in women with chronic pelvic pain and infertility (Baldi et al., 2008). The localization of the endometrial-like tissue is primarily on the pelvic peritoneum and ovaries, but it can be found under the peritoneal surface, the so-called deep-penetrating endometriosis, that is associated with pelvic pain symptoms (Signorile et al., 2009b). Implants can result in substantial morbidity, and infertility, often requiring extensive medical and surgical treatments with associated costs and risks (Fuldeore et al., 2011). Hence, this disease has significant socio-economic implications. The pathogenesis of the disease is still unknown, being retrograde menstruation and coelomic metaplasia the most recognized hypotheses (Giudice and Kao, 2004; Benagiano and Brosens, 2006). Recent data form our research group have underlined the possibility that endometriosis can be caused by fine-tuning alterations of the female genital system development during the fetal life (Signorile et al., 2009a, 2010a,b, 2012; Crispi et al., 2013). However, this demonstration is still debating in the scientific community.

Unfortunately, the symptoms are mostly non-specific, mimicking those associated with other chronic pain disorders and in the great majority of the cases, the definitive diagnosis can be reached only by invasive surgical procedures (Bulun, 2009). Indeed, endometriosis remains a significantly under-diagnosed and under-treated disease, with an estimated time-interval between insurgence of the disease and definitive diagnosis of 8–12 years (Hadfield et al., 1996). So far, non-invasive approach such as blood tests, ultrasound and magnetic resonance imaging have not yielded sufficient power for the definitive diagnosis of endometriosis (Ballard et al., 2006). Therefore, development of a non-invasive diagnostic test for endometriosis would be of great value in clinical practice, because it could allow to identify among women with sub-fertility with or without pains, those with endometriosis that could benefit from laparoscopic surgery that has been reported to decrease pain and increase fertility (D'Hooghe et al., 2006). In particular, concerning peripheral biomarkers, still there are not reliable blood tests, as recently reviewed (May et al., 2010). Nevertheless, several studies evaluating biomarkers for the diagnosis of endometriosis have been proposed so far, but none of them have been recommended in routine clinical care (Mihalyi et al., 2010; Fassbender et al., 2012; Gajbhiye et al., 2012).

In this study, we performed a proteomic approach, based on 2D-gel analysis, for the identification of proteins differentially expressed in a cohort of endometriosis patients respect to a group of healthy women used as control. From this analysis we identified several potential biomarkers for early diagnosis of endometriosis. The diagnostic value of one of these biomarker was finally shown.

Materials and Methods

Study population

The study participants were recruited by the Centro Italiano Endometriosi, Roma. Two independent cohorts were created: one for biomarker discovery through a proteomic approach, and another for subsequent biomarker validation. The study was approved by the ethical committee of the Fondazione Italiana Endometriosi. Written informed consent was obtained from all the subjects before the collection of the blood samples.

For the biomarker discovery study, 5 endometriosis patients were recruited, with the diagnosis confirmed by laparoscopy and histology and 5 control healthy women. For biomarker validation, 120 additional endometriosis patients and 20 additional control healthy women were recruited. In all patients, the entity of endometriosis was classified according to the Revised American Society for Reproductive Medicine classification of endometriosis (1997). The control group included women who were regularly cycling and spontaneously ovulating. Patients using hormonal medication and patients operated within 6 months before the time of sample collections were excluded. Serum level values for CA125 and CA19.9, two serum markers for endometriosis, were

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristics; 2DE, two-dimensional gel electrophoresis.

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TABLE 1. Clinical characteristics of the 120 endometriosis patients

Median age	Stage of the disease	Cycle phase	CA125 U/ml	CA19.9 U/ml
31	86 pts stage IV 18 pts stage III 12 pts stage II 4 pts stage I	66 pts secretory 28 pts proliferative 26 pts Menstrual	59 pts > 35 U/ml	25 pts > 35 U/ml

Pts, patients.

evaluated for all the patients enrolled. Table 1 depicts the characteristics of all the patients enrolled in the study.

Sample collection and serum preparation

Plasma samples were collected from a total of 125 women undergoing laparoscopic surgery and from 25 control healthy women. All the peripheral blood samples (10 ml) were collected by venipuncture, put in ice and moved to the laboratory. Sera were centrifuged at 3,000 rpm for 10 min at −4°C, separated in a tube, labeled, and aliquoted at −80°C. The hemolyzed samples were discarded from the study protocol.

Depletion of high-abundant proteins from human serum

For depletion, the Agilent Multiple Affinity Removal System was used. This system removes the six high abundant proteins: albumin, IgG, IgA, haptoglobin, transferrin, and antitrypsin via polyclonal antibodies packed into an HPLC column. Samples processing were performed according to the manufactures protocol. The serum was diluted with four volumes of buffer A (Agilent Multiple Affinity Removal Buffer A, Agilent, Waldbronn, Germany) and spun through a 0.22-μm spin tube at 16,000*g* at room temperature for 2 min. 100 μl sample was injected onto a 4.6 × 50 mm^2 affinity column in buffer A at a low rate of 0.25 ml/min for p minutes. The bound fraction was eluted with buffer B at a flow rate of 1.0 ml (min for 3.5 min). Then, the column was re-equibrated with buffer A for 10 min. Flow-through was collected at 1.5–4.5 min automatically into Deep Well Plates. After HPLC the collected samples were concentrated via a 10-kDa spin tube and the protein concentration was determined with Bradford assay.

Sample preparation for 2DE electrophoresis

Sample preparation was performed according to Proteome Factory's (Berlin, Germany) 2DE sample preparation protocol for liquid samples. Urea, ampholytes, and DTT were added to a final concentration of 9 M urea, 2% ampholytes, and 70 mM DTT. After incubation for 30 min and centrifugation for 45 min at 15,000*g*, the supernatant was frozen in new tubes at −80°C.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2DE) was performed according to Proteome Factory's 2D electrophoresis technique. Protein (70 μg) was applied to vertical rod gels (9 M urea, 4% acrylamide, 0.3% PDA, 5% glycerol, 0.06% TEMED, and 2% carrier ampholytes [pH 2–11], 0.02% APS) for isoelectric focusing at 8,820 Vh in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mM triphosphate (pH 6.8), 40% glycerol, 65 mM DTT, and 3% SDS for 10 min; subsequently, second dimension SDS–PAGE (20 cm × 30 cm × 0.1 cm) were prepared containing 375 mM.

Tris HCL buffer (pH 8.8), 12% acrylamide, 0.2% bisacrylamide, 0.1% SDS, and 0.03% TEMED. After thawing, the Proteome Factor equilibrated IEF gels were immediately applied to SDS–PAGE gels. Electrophoresis was performed using 140 V for 5 h 15 min until the front reached the end of the gel. After 2DE separation the gels were stained with FireSilver (Proteome Factory, PS-2001).

Image analysis

The 2DE gels used for comparison analysis were digitized at a resolution of 150 dpi using a PowerLook 2100XL scanner with transparency adapter. Two-dimensional image analysis was performed using the Proteomweaver software (Definiens AG, Munich, Germany).

Trypsin-in-gel-digestion/nanoLC-ESI-MS/MS

Protein identification using nanoLC-ESI-MS/MS was performed by Proteome Factory. The MS system consisted of an Agilent 1100 nanoLC system (Agilent), NanoMate 100 (Advion, Ithaca, NY) and a Finnigan LTQ-FT mass spectrometer (Promega, Mannheim, Germany) and applied to nanoLC-ESI-MS/MS. Peptides were trapped and desalted on the enrichment column (Zorbax SB C18, 0.3 mm × 5mm, Agilent) for 5 min using 1% acetonitrile/0.5% formic acid as eluent, then peptides were separated on a Zorbax 300 SB C18, 75 μm × 150 mm column (Agilent) using an acetonitril/0.1% formic acid gradient from 5% to 40% acetonitrile within 40 min. MS spectra were automatically recorded by the mass spectrometer according to manufacturer's instrument setting for nanoLC-ESI-MS/MS analyses. Proteins were identified using MS/MS ion search of the MASCOT search engine (Matrix Science, London, England) and nr protein database (National Center for Biotechnology Information, Bethesda, MD). Ion charge in search parameters for ion form ESI-MS/MS data acquisition were set to "1+, 2+, or 3+" according to the instrument's and method's common charge state distribution.

Enzyme-linked immunosorbent assay (ELISA)

Elisa tests were performed using the dedicated Abnova kit for Zn-alpha2-glycoprotein, following the manufacturer's protocols.

Statistical analysis

Statistical analysis was performed with the SPSS package (version 13.05, SPSS, Inc., Chicago, IL). Receiver operating characteristics (ROC) curve analysis was performed to determine the best predictor of endometriosis between the tested variables.

Results

2DE analysis

2DE gel were performed as described in the Material and Methods section. In every gel were detected 1,000–1,200 spots. The comparison between the first group (control) and the second group (endometriosis) showed 19 differentially expressed protein spots. These differentially expressed proteins spots were selected according to two parameters: (a) a minimal significant factor which was evaluated as follow with a replicate quality test: based on the 500 highest intensity spot-pairs, an average replicate deviation of 159.29% was found. The standard deviation of the average intensities for a group with five gels is 35.16%. The regulation factor between two such groups has a standard deviation of 53.13%. The selected confidence level (0.05) results in a trust factor of 1.96. Exponentiating the standard deviation of the regulation factors

TABLE 2. Regulated spots group control (G1) versus group endometriosis (G2) with *t*-test and Mann–Whitney test

Spot ID	Annotation	Average G1 vs. G2	Average G2 vs. G1	Factor	*t*-Test	Mann–Whitney test
774	u t-	0.042	0.093	22.071	0.8056	0.2222
295	u	0.360	0.835	23.187	0.0137	0.0159
351	u	0.129	0.514	39.691	0.0245	0.0952
564	u	0.161	1.435	88.890	0.0008	0.0000
483	u	0.091	0.296	32.525	0.0104	0.0317
1240	u	0.088	0.280	31.866	0.0114	0.0952
1245	u	0.077	0.572	74.388	0.0055	0.0159
263	d t-	0.116	0.036	0.3118	0.7813	0.1429
1261	d	0.699	0.178	0.2544	0.0090	0.0317
431	d	0.069	0.025	0.3598	0.0084	0.0159
1513	d	0.051	0.021	0.4188	0.0167	0.0635
542	d	0.264	0.108	0.4105	0.0118	0.0317
1543	d	0.138	0.042	0.3018	0.0159	0.0317
930	d	0.151	0.063	0.4186	0.0126	0.0159
471	d	0.155	0.055	0.3575	0.0008	0.0000
1129	d	0.082	0.037	0.4506	0.0156	0.0635
212	d	0.071	0.019	0.2717	0.0118	0.0317
36	d	0.078	0.033	0.4254	0.0132	0.0635
275	d	0.072	0.031	0.4381	0.0213	0.1111

D, down-regulated spots in comparison to group 1; u, up-regulated spots in comparison to group 1; f-, factor not fulfilled; t-, t-test not fulfilled; NA, not annotated.

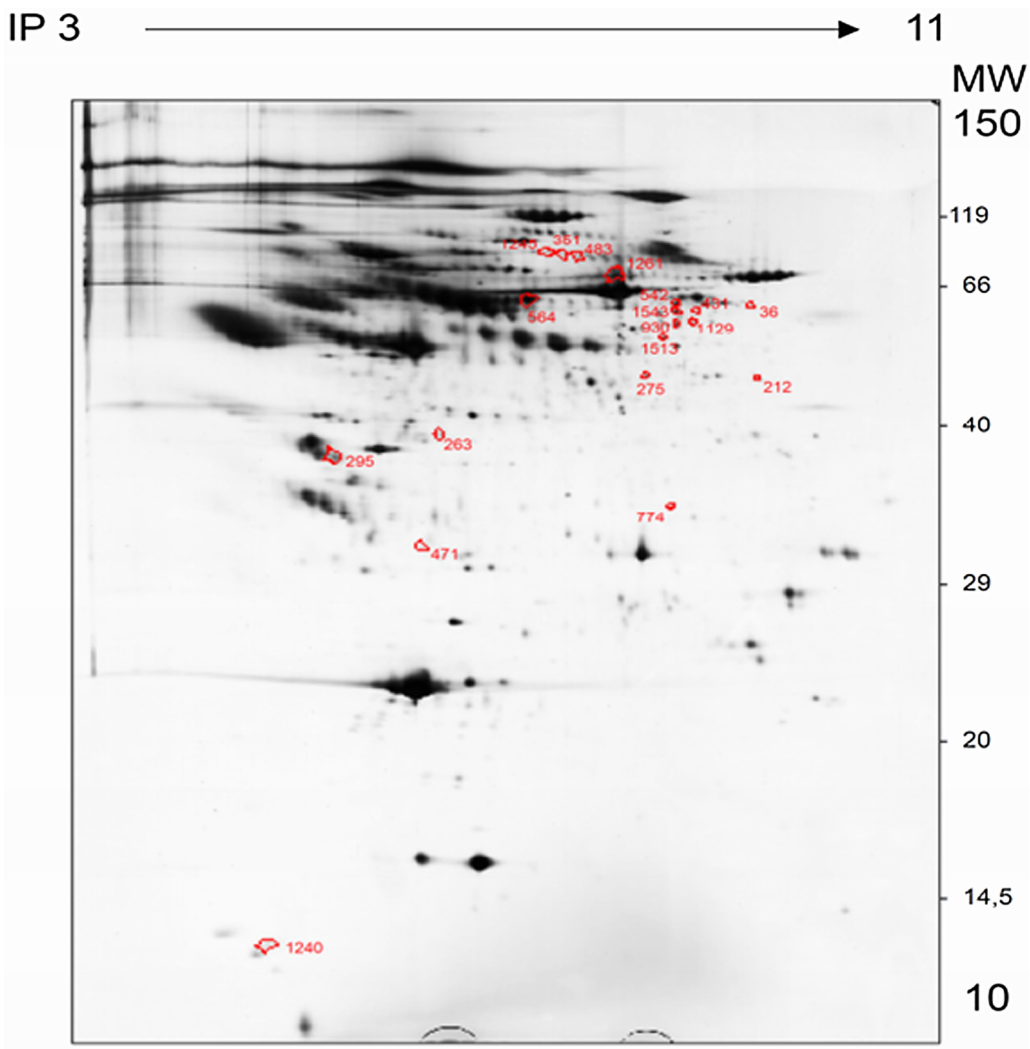

Fig. 1. Silver-stained 2DE gel. Highlighted are analyzed spots.

with the trust factor results in a minimal significant regulation factor of: 2.3. A minimal significant factor of 2.0 was therefore applied for the selection of differentially expressed protein spots. (b) *t*-Test: $P = 0.05$.

However, a few spots were considered as modified although the present criteria were not fulfilled. These spots are marked with f- (factor not fulfilled), t- (*t*-test not fulfilled). The different expressed spots are listed in Table 2 and in Figure 1. All differentially expressed proteins spots were picked ad identified by mass spectrometry.

Identification of regulated spots

The 19 regulated spots are being analyzed and identified via nanoLC-ESI_MS/MS and using MASCOT search engine (Matrix Science, London, England) and nr protein database (National Center for Biotechnology Information). Identified proteins to date are listed in Table 3.

Biomarker validation

ELISA tests were performed on a different cohort of endometriosis (n = 120) and healthy patients (n = 20) in order to confirm the differential expression of two among the identified proteins. ROC analysis of ELISA results confirmed the statistical significance of the differential expression for one of these proteins, when they were taken one at a time: Zn-alpha2-glycoprotein ($P = 0.019$). In Figure 2, the ROC curve for this protein is depicted, together with specificity (100%) and sensitivity (69.4%). This result was clearly significant, when compared to the sensitivity achieved in the some cohort of patients by CA125 and CA19.9, respectively of 33% and 13% (Fig. 3).

Discussion

One of the utmost task in gynecology is to diagnose patients with endometriosis without recurring to surgery. This would allow to identify endometriosis patients in a short window of time after commencement of the symptoms, thus avoiding a substantial number of usefulness diagnostic procedures. In fact, it is well known that a definitive diagnosis of endometriosis in these patients generally is made after several years form the start of the symptoms (Bulun, 2009). The data of our study show that it could be possible to diagnose endometriosis using plasma level analysis of some biomarkers by means of routinely used biochemical techniques, with a high sensitivity and specificity. Indeed, several studies have found some promising potential markers in the peritoneal fluid and serum, but none of those has demonstrated suitable properties to permit their use alone for diagnosis (Seeber et al., 2008). The relevance of our observation in clinical practice is straightforward, especially for women of reproductive age. In fact, early non-invasive diagnosis of minimal-mild endometriosis in women who have the desire to conceive, should allow to select these patients for laparoscopic excision of endometriosis which improves fertility (Kennedy et al., 2005) and may avoid progression of the disease. The most important goal of a non-invasive diagnostic test is that no women with endometriosis are neglected. Therefore, the test should have high sensitivity with a low number of false negative results and high specificity, that implies a low number of false positive results. Taking into account this

TABLE 3. Identified proteins by mass spectrometry

Number	Sup.Spt.ID	Identification
1	1261	Complement C3 precursor
2	1245	To be defined
3	564	Chain A, human serum albumin
4	0483	To be defined
5	471	Apolipoprotein E mutant E3K
6	431	To be defined
7	351	To be defined
8	295	Zn-alpha2-glycoprotein
9	212	To be defined
10	36	Complement component C3
11	263	To be defined
12	275	Complement component C3
13	542	Chain A, complement C3b in complex with factor H domains 1–4
14	774	To be defined
15	930	Complement component C3
16	1129	To be defined
17	1240	To be defined
18	1513	Complement C3 precursor
19	1543	Chain A, complement C3b in complex with factor H domains 1–4

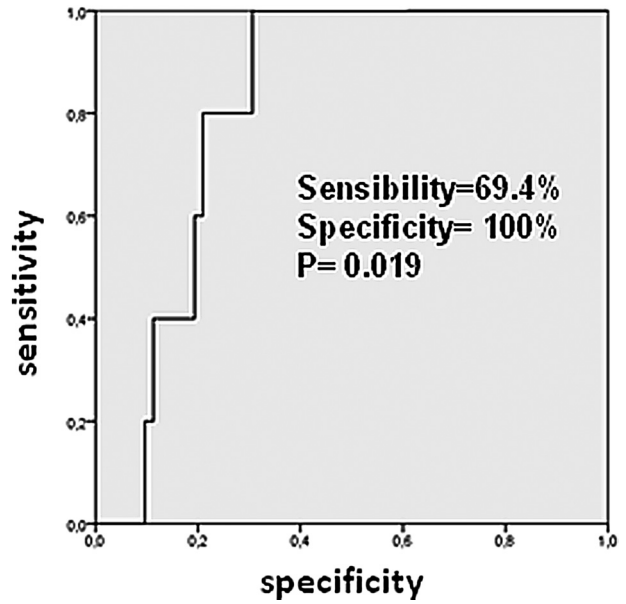

Fig. 2. ROC curve for Zn-alpha2-glycoprotein.

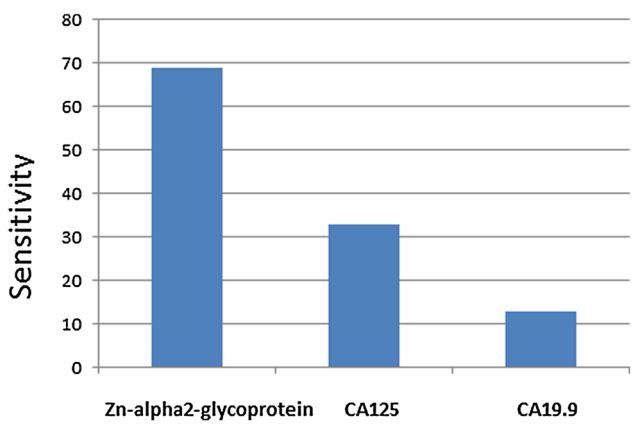

Fig. 3. Histogram depicting the different sensitivity values achieved by Zn-alpha2-glycoprotein, CA125, and CA19.9 in diagnosing endometriosis in the cohort of patients analyzed.

clinical perspective, the data presented in this study (sensitivity 100%; specificity 69.4%) come close to this ideal. Moreover, it should be considered that the patient population has been enrolled independently from the cycle phase and independently from endometriosis stage, thus avoiding any influence from the hormonal input and from the effects of the disease on the normal physiology of the body.

Major limitation of the study is the small number of control patients enrolled. However, the statistical results obtained are very promising; moreover, it should be noted that the median values of serum concentration obtained by ELISA test for the marker analyzed in the control population (30 mg/L for Zn-alpha2-glycoprotein) are very close to the normal values registered in the general population for that markers (Stejskal et al., 2008). Therefore, even if small, the cohort of control can be considered valid. Further studies are ongoing to confirm the pattern of expression found in a larger patients population and to further characterize the proteins identified with the proteomic approach.

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