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Urinary vitamin D-binding protein is elevated in patients with endometriosis

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BACKGROUND: Recently, proteomic technologies have demonstrated that several proteins are differently expressed in various body fluids of patients with endometriosis compared with those without this condition. The aim of this study was to investigate proteins secreted in urine of patients with endometriosis using proteomic techniques in order to identify potential markers for the clinical diagnosis of endometriosis.

METHODS: Urine samples were collected from women undergoing laparoscopy for different indications including pelvic masses, pelvic pain, suspicious endometriosis, infertility and diagnostic evaluation. Proteomic techniques and mass spectrometry were used to identify proteins secreted in the urine of the patients with and without endometriosis and quantification of identified protein was performed using western blot and specific commercial sandwich enzyme-linked immunosorbent assays (ELISA).

RESULTS: Twenty-two protein spots were differentially expressed in the urine of patients with and without endometriosis, one of which was identified as urinary vitamin D-binding protein (VDBP). ELISA quantification of urinary VDBP corrected for creatinine expression (VDBP-Cr) revealed that urinary VDBP-Cr was significantly greater in patients with endometriosis than in those without (111.96 ± 74.59 versus 69.90 ± 43.76 ng/mg Cr, $P = 0.001$). VDBP-Cr had limited value as a diagnostic marker for endometrioisis (Sensitivity 58%, Specificity 76%). When combined with serum CA-125 levels (the product of serum CA-125 and urinary VDBP-Cr), it did not significantly increase the diagnostic power of serum CA-125 alone.

CONCLUSIONS: Urinary VDBP levels are elevated in patients with endometriosis. They have limited value as a potential diagnostic biomarker for endometriosis but suggest it would be worthwhile to investigate other urinary proteins for this purpose.

Key words: biomarker / endometriosis / proteomics / urine / vitamin D-binding protein

Introduction

Endometriosis is defined as the proliferation of endometrial tissue outside the uterine cavity and is one of the most common benign gynecologic disorders. This disease is present in ~10% of all reproductive-aged women, and its prevalence increases from 20 to 50% in infertile women (Taylor *et al.*, 2002). However, diagnosis of endometriosis is often difficult due to the fact that there is no definite diagnostic biomarker yet available. Imaging techniques, such as ultrasound and magnetic resonance imaging, have been shown to be unreliable in the diagnosis or staging of this disease. Therefore, the direct visualization of lesions and histologic confirmation through surgical procedures are currently essential for the definitive diagnosis of endometriosis. Although laparoscopy is a minimally invasive procedure, it requires general anesthesia, developed surgical skills and procedural costs. Additionally, laparoscopy is associated with the risk of potential complications.

Despite numerous investigative efforts and recent advances in the understanding of the molecular basis of endometriosis, a substantial gap remains in the development of effective non-invasive diagnostic biomarkers for endometriosis diagnosis. Therefore, newly available technologies could play a key role in the identification of novel biological markers in the early stages of endometriosis. Recently, several investigators have advocated the use of proteomic technologies in endometriosis research and have demonstrated that several proteins are differently expressed in various body fluids, including

serum, plasma, peritoneal and endometrial fluids and urine of patients with endometriosis versus those without this condition (Ametzazurra *et al.*, 2009; Ferrero *et al.*, 2007; Liu *et al.*, 2007; Seeber *et al.*, 2010; Tokushige *et al.*, 2011). Proteomic approaches enable the comparison of protein expression in cells, tissues or body fluids under various conditions. The study of protein function and protein–protein interactions may provide more information about the disease than the application of genomics tools because a low correlation exists between the genotype and phenotype in endometriosis (Poliness *et al.*, 2004).

Biomarkers in urine would be ideal because this fluid is easily obtained in relatively large quantities using non-invasive procedures. The clinical importance of urinary angiogenic factors and cytokines has been demonstrated not only in urogenital diseases, but also in other systemic diseases including heart failure and brain tumors (Matsumoto and Kanmatsuse, 2001; Dvorak, 2002; Rivera *et al.*, 2004; Buhimschi *et al.*, 2005; Cho *et al.*, 2007; Smith *et al.*, 2008). Recent studies also indicate that proteomic analysis of urine may lead to new potential biomarkers for various systemic diseases such as ovarian cancer and coronary artery disease (Ye *et al.*, 2006; Zimmerli *et al.*, 2008). We have previously demonstrated the significance of urinary angiogenic factors in patients with endometriosis (Cho *et al.*, 2007). We therefore hypothesized that certain proteins might be differentially excreted in the urine of women with endometriosis compared with those without. The purpose of this study was to conduct comparative proteomic analysis of urine between patients with and without endometriosis and to identify novel potential urinary biomarkers for endometriosis.

Materials and Methods

Study population and sample collection

Ninety-five patients aged 20–47 years participated in this study after giving written informed consent. The study was approved by the Institutional Review Board of Gangnam Severance Hospital. All patients included in the study underwent laparoscopy for various indications including pelvic masses, pelvic pain, suspicious endometriosis, infertility and diagnostic evaluation between January 2008 and October 2010. Pretreatment serum CA-125 levels in all patients were measured using CA-125 II electrochemiluminescence immunoassay with the Roche/Hitachi Modular Analytics E170 system (Roche Diagnostics, Tokyo, Japan). At the time of surgery, all possible endometriotic lesions were excised and sent to pathology for confirmation of diagnosis. Patients were assigned to the endometriosis group only after pathologic confirmation of excised tissue. The extent of endometriosis was determined using the American Society of Reproductive Medicine (ASRM) revised classification (American Society for Reproductive Medicine, 1997). Fifty-seven patients had histologically confirmed endometriosis: 5 patients were classified with minimal-to-mild disease (Stages I and II) and 52 patients with moderate-to-severe disease (Stages III and IV). Thirty-eight patients participated as controls, and this group included 18 cases of dermoid cysts and 20 cases of benign ovarian cysts including serous cystadenoma and paratubal cyst. Exclusion criteria included post-menopausal status; previous hormone or gonadotrophin-releasing hormone (GnRH) agonist use; adenomyosis; endometrial cancer; hyperplasia or endometrial polyps; infectious diseases; chronic or acute inflammatory diseases; malignancy; autoimmune disease and cardiovascular disease. Although no effort was made to control for or assess dietary calcium or vitamin D intake, concurrent medication use, including calcium or vitamin D supplements, was recorded and excluded for final analysis. The menstrual cycle stage was recorded for each patient, including proliferative phase, beginning of menses until 14 days before the next menses, secretory phase and 1–13 days before the next menses.

Urine samples were collected from 57 women with histologically proven endometriosis and 38 women for whom histological or laparoscopic evidence indicated endometriosis was not present. Each urine sample (20 ml) was collected into a sterile plastic tube when the bladder was catheterized after induction of anesthesia and 1X protease inhibitor cocktail was added (Roche, Germany). Urine samples were immediately centrifuged at 1000*g* for 10 min and sediment-free urine samples were obtained. Urine aliquots were frozen at −80°C until used for further analysis. Because previous studies have demonstrated that the collection of 24-h and first-morning urine is less practical for proteomic analysis and may be contaminated with proteins from overgrown bacteria or bladder epithelial cells, no effort was made to obtain samples at a specific time of day (Bottini *et al.*, 2002; Hoorn *et al.*, 2005).

Proteomics

Urine samples from four endometriosis patients and four without endometriosis were used for proteomics analysis. Each urine sample (20 ml) was concentrated to 0.5 ml using Vivaspin (2,000 MW cut-off; Sartorius, Hannover, Germany) and proteins in the concentrated urine were precipitated using a ReadyPrep 2-D cleanup kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The resulting protein pellets were completely dissolved in 2-D buffer (7 M urea, 2 M thiourea, 0.4% [w/v] dithiothreitol and 4% [w/v] CHAPS) and centrifuged at 13 000*g* for 15 min. Protein concentrations in the supernatants were determined by the Bradford method.

For one-dimensional electrophoresis, 450 μg of urine proteins from each sample were rehydrated overnight at room temperature onto linear pH 4–7 (18-cm) Immobiline Dry Strips (GE Healthcare) with 0.8% carrier ampholyte (pH 4–7) and 0.005% (w/v) Bromophenol Blue. The rehydrated Dry Strips were focused a total of 48 kVh using a Multiphor II apparatus (GE Healthcare). The focused Dry Strips were reduced in 5 mM tributyl-phosphine and 15 mM acrylamide for 15 min each at ambient temperature.

The 2-D separations were performed on 8–16% (v/v) linear gradient SDS-polyacrylamide gels. After protein separation, gels were fixed for 1 h in a solution of 40% (v/v) methanol containing 5% (v/v) phosphoric acid and stained with Colloidal Coomassie Blue G-250 solution (ProteomeTech, Seoul, Korea) overnight. The gels were destained in 1% (v/v) acetic acid for 4 h and then imaged using a GS-710 imaging calibrated densitometer (Bio-Rad, Hercules, CA, USA). For the determination of differential protein expression levels, the scanned images of women with and without endometriosis were analysed and compared using ImageMaster V software (GE Healthsciences).

Protein identification by LC-MS/MS

Protein spots of interest were excised from the gels and destained in 1:1 (v/v) acetonitrile/50 mM ammonium bicarbonate (pH 7.8), dried and 10 μl trypsin solution was then added (12 ng/ml, Promega, Madison, WI, USA). After discarding excess trypsin and adding 20 μl of 50 mM ammonium bicarbonate, the gels were incubated overnight at 37°C. The tryptic digest solutions were concentrated and cleaned up using a C18 microcolumn (Millipore, Billerica, MA, USA) as instructed by the manufacturer.

The resulting tryptic peptides were separated and analyzed using a reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) (Bahk *et al.*, 2004). Both a 0.1 × 20-mm trapping and a 0.075 × 130-mm resolving column were packed

with Vydac 218 MS low trifluoroacetic acid C18 beads (5 μm in size, 300 Å in pore size; Vydac, Hesperia, CA, USA) and placed in line. The peptides were then bound to the trapping column for 10 min with 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. The bound peptides were eluted with a 50-min gradient of 5–80% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.2 μl/min. For tandem mass spectrometry, each scan cycle included a full mass scan range mode (m/z 300–2000 Da) and up to three product ion scans with a relative collision energy of 55%. The individual MS/MS spectrum was processed using the Bioworks 3.3 software (Thermo Fisher, San Jose, CA, USA). The generated peak list files were used to query the NCBI human database using the MASCOT program (http://www.matrixscience.com). Search parameters included variable modifications of methionine (oxidation) and cysteine (propionamide), peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 1 Da, allowance of missed cleavage at 1 and charge states (+1, +2, and +3). Matches of peptide(s) with only significant hits as defined by MASCOT probability analysis were accepted.

Western blot

The urine from eight samples (four endometriosis and four control patients), which were distinct from those subjected to 2-DE analyses, were quantified using a BCA assay (Pierce, Rockford, IL, USA). For western blot analyses, equal amounts of urine proteins (70 μg) were subjected to electrophoresis on a 4–20% gradient polyacrylamide gel (Koma Biotech, Seoul, Korea) and transferred to PVDF membranes over 50 min at 15 V using a EZway blotting system (Koma Biotech). Blots were blocked for 1 h at room temperature with 3% (w/v) non-fat dried milk in tris-buffered saline (TBS) solution containing 0.1% Tween-20 (TBS-T). After three washes with TBS-T, the blots were incubated at 4°C overnight with vitamin D-binding protein (VDBP; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-69771) antibody at a dilution of 1:700 in TBS-T. Membranes were washed with TBS-T solution and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) at a 1:2000 dilution at room temperature for 1 h. Immunoreactive proteins were detected by chemiluminescent reaction followed by exposure of the membranes to hyperfilm ECL (Amersham Pharmacia Biotech, Inc., Little Chalfont, UK). The relative density of the protein bands was quantified by Gel Doc XR+ (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of VDBP in urine (100 μl, diluted 1:2) was measured using specific commercial sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's protocols (ALPCO Diagnostics, Salem, NH, USA; enzyme immunoassay no. 30-2314) and expressed in ng/dl. The minimal detectable concentration for urinary VDBP was 1.23 ng/ml. Urine creatinine (Cr) levels were measured with commercial assays at Gangnam Severance Hospital (Hitachi 7600-110, Tokyo, Japan). Urine VDBP values were normalized to urine Cr concentrations and expressed as VDBP-to-Cr ratio (VDBP-Cr; nanograms per miligrams of creatinine).

Statistical analysis

Data were expressed as mean ± SD or median [interquartile (IQR) ranges] where appropriate. The clinical and laboratory characteristics of endometriosis and control group patients, and seasonal variations of urinary VDBP and VDBP-Cr were compared using Student's *t*-test. To analyze variance in VDBP-Cr over the different menstrual cycle stages, the Kruskal–Wallis test was used, followed by the Dunn procedure. To determine if differences in correlations were statistically significant, Pearson's correlation coefficient or Spearman rank correlation coefficient was calculated, where appropriate. The diagnostic performance of urinary VDBP-Cr was assessed using receiver operating characteristic (ROC) curves (Hanley and McNeil, 1982) to plot the test sensitivity versus its false-positive rate and determine the usefulness of a diagnostic test over a range of possible clinical results. The diagnostic utility of the test can be expressed as the area under the ROC curve (AUC), which was calculated as a measure of the ability of each potential biomarker to discriminate between endometriosis and control cases. An AUC of 0.5 indicates classifications assigned by chance. Based on ROC analysis, the best statistical cut-off value of VDBP-Cr was calculated, which corresponds to the point at which the sum of false positives and false negatives is less than any other point. Sensitivity and specificity for selected cut-off points were then assessed. SPSS 16.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis and $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

The clinical characteristics of patients are shown in Table I. The mean age (mean ± SD) of endometriosis patients and controls was 34.22 ± 6.88 and 32.76 ± 10.26 years, respectively. There were no significant differences in mean gravidarum, mean parity or mean body mass index (BMI) between the two groups. In contrast, serum CA-125 levels were significantly higher in patients with endometriosis than in controls (83.09 ± 120.93 versus 20.57 ± 13.25 IU/ml, respectively; $P < 0.001$). No significant differences were observed in mean urine creatinine levels between the two groups.

Proteomics

Two-dimensional gels of urinary proteins revealed an average of 512 spots over a pH range of 4.0–7.0. We identified 22 urinary protein spots with significantly greater expression (% volume) in women with endometriosis compared with control cases (Fig. 1). Of those 22 up-regulated protein spots on the gels in women with endometriosis, 5 spots showed the biggest difference (more than 10-fold increase) compared with women without endometriosis. Five spots were identified as prealbumin [isoelectric point (pI) 5.52, Mr 15.90], enolase-1 (pI 7.01, Mr 47.13), alpha-1 antitrypsin (pI 5.35, Mr 44.25), Chain A solution structure of Bb′ domains of human protein disulfide isomerase (pI 4.84, Mr 25.51) and VDBP (pI 5.40, Mr

Table I Clinical characteristics of patients enrolled in the study.

	Endometriosis ($n = 57$)	Control ($n = 38$)	*P*
Age (years)	34.2 ± 6.88	32.7 ± 10.26	0.443
Gravidarum	1.0 ± 1.31	1.2 ± 1.46	0.448
Parity	0.6 ± 0.86	0.6 ± 0.86	0.923
BMI (m^2/kg)	18.1 ± 7.30	17.7 ± 7.76	0.809
CA-125 (IU/ml)	83.1 ± 120.93	20.6 ± 13.25	<0.001
Urine creatinine (mg/dl)	103.2 ± 52.36	88.4 ± 68.83	0.239

Data are expressed as mean ± SD.

52.93) by LC-MS/MS (Table II). The spot with the biggest difference was VDBP. We decided that VDBP merited further detailed investigation due to its uniform high abundance in all endometriosis samples and its known action as a macrophage activator. The intensity of its expression (% volume) was statistically significantly higher in women with endometriosis, compared with those without endometriosis (0.2005 ± 0.0582 versus 0.0006 ± 0.001, respectively, $P < 0.001$).

Biomarker validation

Western blot analysis of eight urine samples (four samples from patients with endometriosis and four samples from controls) with a VDBP-specific antibody showed that all samples were VDBP positive (Fig. 2A). Quantification of the relative density of the protein bands indicated that the relative density of urinary VDBP was significantly greater in patients with endometriosis than that in controls (2.48 ± 0.11 versus 1.15 ± 0.26; $P < 0.001$; Fig. 2B).

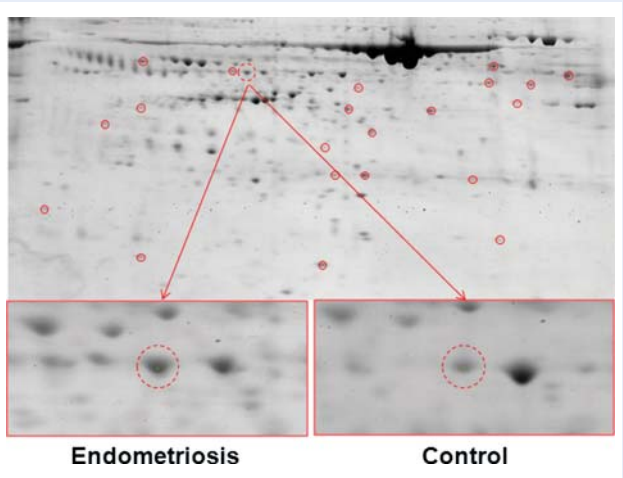

Figure 1 Proteomic analysis of highly expressed proteins in the urine of women with endometriosis. Twenty-two spots that were expressed significantly different (% volume) in urine of women with and without endometriosis were identified. The protein spot circled in dotted red is VDBP.

ELISA quantification of urinary VDBP in 57 patients with endometriosis and 38 patients without endometriosis revealed that urinary VDBP levels were significantly higher in women with endometriosis than those without (106.12 ± 74.55 versus 58.68 ± 60.82 ng/ml, respectively, $P = 0.001$) (Fig. 3). After correcting urinary VDBP levels for creatinine (VDBP-Cr), it was apparent that the levels of VDBP-Cr were also significantly greater in urine of women with endometriosis than of controls (111.96 ± 74.59 versus 69.90 ± 43.76 ng/mg Cr, respectively; $P = 0.001$; Fig. 3). Figure 4 shows the cyclic variation of VDBP-Cr in patients with endometriosis and controls. Data are expressed as median and the IQR. We then evaluated VDBP-Cr according to the menstrual cycle and found no significant difference between the two groups in the median VDBP-Cr during the proliferative phase. The median VDBP-Cr in patients with and without endometriosis during the secretory phase were 91.20 ng/mg Cr (IQR: 53.31–143.32) and 54.13 ng/mg Cr (IQR: 36.02–82.14), respectively. The difference between these values was statistically significant ($P = 0.042$). We also evaluated the correlations between VBDP-Cr and other clinical features. However, no significant correlations existed between VDBP-Cr and disease severity or serum CA-125 levels (data not shown). Date of sampling was used to define seasonality, as fall/winter (September through February) and spring/summer (March through August). The mean values of urinary VDBP and VDBP-Cr were 87.88 ± 72.73 ng/ml and 101.41 ± 75.06 ng/mg Cr for spring/summer season and 86.04 ± 74.09 ng/ml and 85.73 ± 52.40 ng/mg Cr for fall/winter and the differences were statistically insignificant ($P = 0.905$, $P = 0.266$, respectively). Therefore, no seasonal variations were noted in urinary VDBP levels and VDBP-Cr.

ROC curves were used to assess the utility of VDBP-Cr in diagnosing endometriosis (Fig. 5). The AUC of VDBP-Cr was 0.678 (95% CI: 0.569–0.787), and the optimum cut-off value was 87.83 ng/mg Cr with 57.9% sensitivity and 76.3% specificity. We also examined if VDBP-Cr could improve the diagnostic power of CA-125.The AUC for CA-125 alone was 0.857 [95% confidence interval (CI): 0.781–0.933] with sensitivity of 69.6% and specificity of 91.9%, when assessed at a cut-off point of 35 IU/ml, which is the most commonly reported cut-off level in the literature. When CA-125 was combined with VDBP-Cr by multiplication (CA-125 × VDBP-Cr) the AUC for the combined marker was 0.874 (95% CI: 0.802–0.945), and with

Table II Protein spots with significantly different expression (% volume) in urine of women with and without endometriosis.

Protein	pI	Mr (kDa)	Endometriosis	Control	*P* value
Alpha-1 antitrypsin	5.35	44.25	0.082 ± 0.0123	0.002 ± 0.0020	<0.001
Chain A, solution structure of Bb′ domains of human protein disulfide isomerase	4.84	25.51	0.112 ± 0.0383	0.001 ± 0.0020	<0.001
Enolase-1	7.01	47.13	0.184 ± 0.0330	0.001 ± 0.0010	<0.001
Prealbumin	5.52	15.90	0.138 ± 0.0273	0.001 ± 0.0020	<0.001
VDBP	5.40	52.93	0.201 ± 0.0582	0.001 ± 0.0010	<0.001

Data are expressed as mean ± SD.

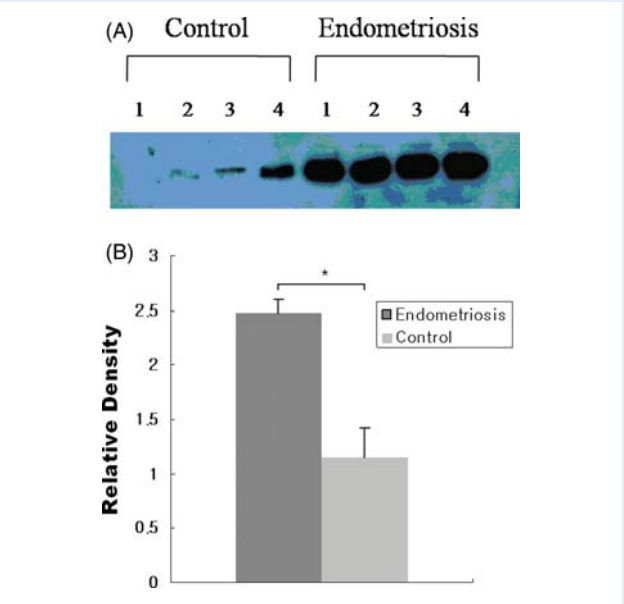

Figure 2 Western blot analysis of eight urine samples probed with VDBP-specific antibody (**A**). The expression of VDBP was significantly greater in urine of patients with endometriosis than that of controls (**B**). Data are expressed as mean ± SD. *$P < 0.001$.

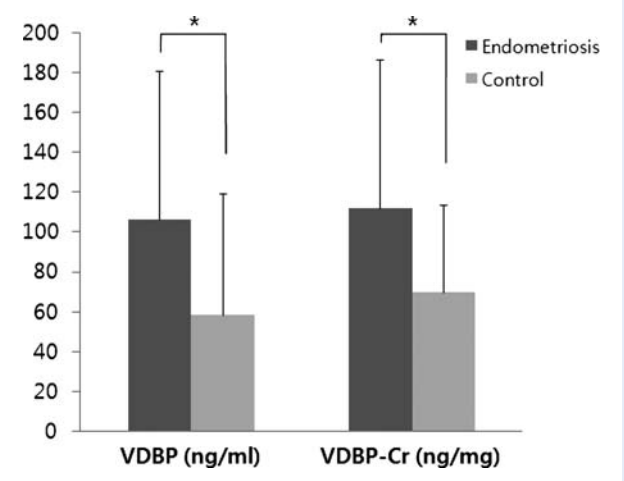

Figure 3 Urinary VDBP levels and VDBP-Cr ratio in the urine of patients with endometriosis and controls. Data are expressed as mean ± SD. *$P = 0.001$.

the optimal cut-off value of 2755 the sensitivity was 73.2% and the specificity 97.3%.

Discussion

A number of recently published studies have used proteomic analysis of urine to identify novel biomarkers for several different diseases. Zimmerli *et al*. (2008) developed the coronary artery disease-specific panel using urinary proteomics techniques that showed sensitivity and specificity of 98% and 83%, respectively, for the prediction of coronary artery disease. Additionally, Ye *et al*. (2006) reported the proteomic-based discovery and characterization of urinary

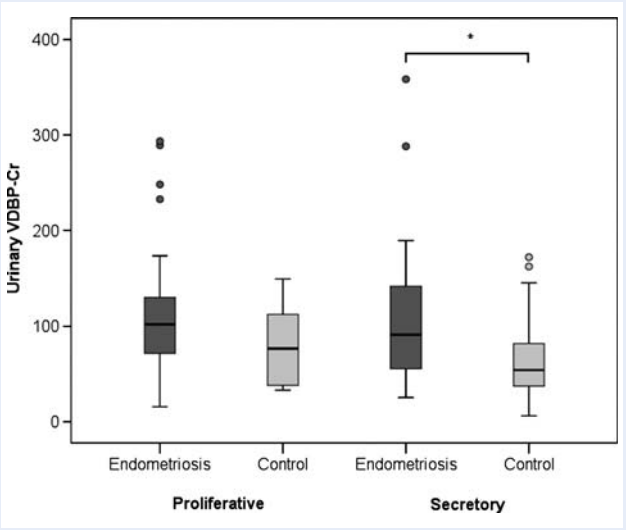

Figure 4 Urinary VDBP-Cr ratio in the urine of patients with endometriosis and controls according to the menstrual cycle. Urinary VDBP-Cr levels were significantly higher in the urine of patients with endometriosis than that of controls during the secretory phase of the menstrual cycle. Data were analyzed by the Kruskal–Wallis test followed by the Dunn procedure. Data are represented by box-and-whisker plots. Boxes indicate the 25th and 75th percentiles, with a solid line within the box showing the median value. Whiskers show the largest and smallest observed value that is not an outlier. Outliers are shown by circles in the plot and are defined as values more than 1.5 box-lengths from the 25th or 75th percentile. *$P = 0.042$.

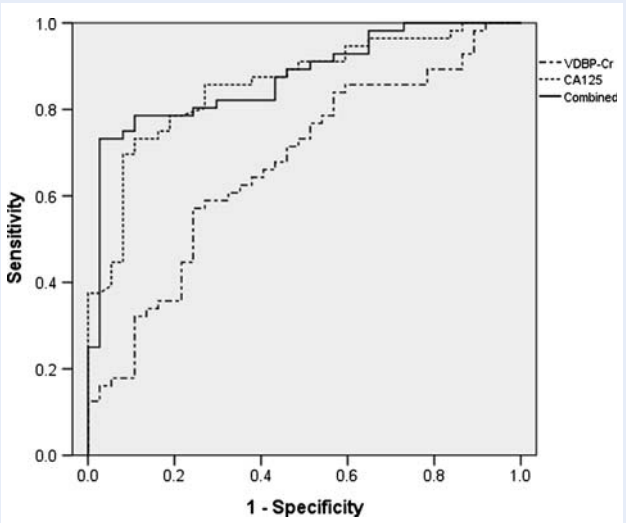

Figure 5 ROC curves of serum CA-125, urinary VDBP-Cr and the combined marker. Serum CA-125 levels were multiplied by VDBP-Cr ratio for the differential diagnosis of patients with endometriosis and controls.

glycosylated eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments for ovarian cancer. More recently, urinary proteomic techniques have been applied in the discovery of biomarkers for endometriosis, and cytokeratin-19, which is known to be a cell structural protein and a member of type I cytokeratin protein genes, has been identified in the urine of patients with endometriosis (Tokushige *et al.*, 2011).

In this study, we found that urinary VDBP is significantly increased in patients with endometriosis. VDBP is a group-specific component of Gc-globulin and is the major plasma carrier protein of vitamin D metabolites, as it is responsible for the transport of cholecalciferol (vitamin D3) to the liver, 25-OH-D to the kidneys and 1,25-OH-D (calcitriol, the active form of vitamin D) to target organs (Daiger *et al.*, 1975). However, <5% of circulating VDBP is complexed with vitamin D metabolites, which leaves a considerable amount of the protein available for other functions that are closely related to the pathogenesis of endometriosis. Previous research has shown that this protein is involved in the transportation of fat and endotoxins, and is known to be an important factor in the actin scavenging system (Berger and Beger, 1987; Williams *et al.*, 1988; Lee and Galbraith, 1992). In addition, there is a growing body of evidence suggesting that VDBP plays an important role in the immune system by acting as a chemotactic factor to recruit neutrophils, monocytes and fibroblasts (Kew and Webster, 1988; Perez *et al.*, 1988; Piquette *et al.*, 1994). Also, at sites of inflammation, sequential contact of VDBP with B and T cells is able to convert VDBP into a potent macrophage-activating factor (Yamamoto and Naraparaju, 1996). Therefore, our results showing increased VDBP expression in urine from patients with endometriosis may be related to the typical systemic subclinical inflammatory process involved with endometriosis (Agic *et al.*, 2006). Adding to the biological plausibility that VDBP is connected with the pathogenesis of endometriosis is the fact that hormones such as estrogen and interleukin-1, which are known to be involved with endometriosis, influence the concentration of Gc-globulin (Guha *et al.*, 1995; Rejnmark *et al.*, 2002).

Despite the important and diverse roles of VDBP, few studies have evaluated its expression in endometriosis. One study showed no significant differences in the amount of serum and peritoneal fluid VDBP between women with and without endometriosis, whereas Agic *et al.* (2007) demonstrated that vitamin D receptor and its hydroxylases were expressed in the endometrium and ovaries of women with and without endometriosis and hypothesized that vitamin D might influence the local activity of immune cells, and cytokines thought to play important pathogenic roles in the development and maintenance of endometriosis (Agic *et al.*, 2007; Borkowski *et al.*, 2008). Another study, using high-resolution two-dimensional gel electrophoresis, reported that one isoform of VDBP was significantly lower in the peritoneal fluid of women with endometriosis than that of controls and that the expression was further decreased following the treatment with GnRH agonist (Ferrero *et al.*, 2005; Ferrero *et al.*, 2009). More recently, proteomic analysis of serum using two-dimensional difference gel electrophoresis showed that the concentration of VDBP is elevated in endometriosis patients and suggested that the inability to sufficiently activate the phagocytic function of macrophages in patients carrying the GC*2 polymorphism may be associated with the implantation of endometriotic tissues in the peritoneal cavity (Faserl *et al.*, 2011).

Evaluation of urinary VDBP has been reported previously, and previous studies indicated that long-term cadmium exposure and type I diabetes are associated with high levels of urinary VDBP (Uchida *et al.*, 2007; Thrailkill *et al.*, 2011). Our results showed that urinary VDBP levels are increased in patients with endometriosis. We also evaluated urinary VDBP based on stages of the menstrual cycle and found that the amount of VDBP was significantly higher in patients with endometriosis than that in controls during the secretory phase of the menstrual cycle. However, we found no significant cyclic variation in urinary VDBP in either the endometriosis group or the control group. There are no previous studies showing the cyclic variations of urinary VBDP, but our findings are consistent with results showing that plasma VDBP expression is not related to menstrual cycle phase (Nielsen *et al.*, 1990; Ferrero *et al.*, 2005).

Another interesting marker shown to be increased in urine of patients with endometriosis in this study was enolase-1. Enolase-1 is a glycolytic enzyme that binds plasminogen at the cell surface, enhancing local plasmin production and migration of monocyte to sites of inflammation (Miles *et al.*, 1991). Recent evidence shows that enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung and anti-α-enolase antoantibody was identified as a potential serum marker for endometriosis (Nabeta *et al.*, 2009; Wygrecka *et al.*, 2009). Therefore, further validation of this marker in urine of patients with endometriosis needs to be considered in the near future.

One potential limitation of this study is related to the patients included in the control group. Healthy women without pelvic pain or disease would have been an ideal comparison group to distinguish between endometriosis and normal patients. However, choosing adequate control groups is a complex and often overlooked problem in endometriosis research. Although we only included patients in the control group surgically proved to be free of endometriosis, the majority of our control group had various other benign diseases, which may have different effects on the levels of urinary VDBP. Another point that needs to be addressed is the discrepancy between the relative expression of urinary VDBP as measured by proteomics and that of which is determined by western blot or ELISA. Such discrepancy has been shown in previous study (Jou *et al.*, 2010). Due to the characteristics of two-dimensional electrophoresis, trailing of protein spots may occur in pI focusing and may decrease the separation power. Furthermore, this effect may lead to blurring of the protein spots and make the images different while image analyzing. Therefore, it is necessary to confirm and validate the data obtained from gel electrophoresis by protein blotting, as we did in this study.

To the best of our knowledge, this study is the first to report the identification and validation of VDBP as a urinary biomarker in detecting endometriosis. Although urinary VDBP levels were significantly increased in patients with endometriosis than those without, they have limited potential as a diagnostic biomarker for endometriosis even alone or after combining with serum CA-125. Addition of VDBP-Cr to CA-125 made no significant difference to the AUC in the ROC curve and some of the slight improvement in sensitivity and specificity may have been because the cut-off for CA-125 was the generally accepted value, whereas that for the combined data was the optimum for this data set. The lack of diagnostic power was disappointing since only a small number of patients included in this study had minimal-to-mild disease. The expression of VDBP has

been reported in the uterus, serum and the peritoneal fluids but it is unclear why or how VDBP is specifically excreted in urine (Cooke *et al.*, 1991; Ferrero *et al.*, 2005; Borkowski *et al.*, 2008; Ferrero *et al.*, 2009). Further research is warranted to investigate the possible role of VDBP and other urinary proteins in the pathogenesis and diagnosis of endometriosis.

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Authors' roles

S.C. contributed substantially to conception and design analysis, interpretation of data, drafted the article and revised and finally approved the version to be published. Su.Y.Y. performed the analysis and interpretation of data, revised the article, finally approved the version to be published. Y.S.C. contributed substantially to conception and design and finally approved the version to be published. H.I.Y., Y.E.J. and K.E.L. contributed to the acquisition of data and finally approved the version to be published. H.Y.K., S.K.S. contributed to analysis and interpretation of data and finally approved the version to be published. B.S.L. contributed substantially to conception and design, reviewed the article and finally approved the version to be published.

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Conflict of interest

None declared.

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